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THEME

Effect of storage time on the quality of olive oil mill wastewaters obtained from the cold extraction of olive oil in the region of Khenchela and their biological properties

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DEDICATION

WITH GREAT LOVE AND RESPECT,

I DEDICATE THIS WORK,

TO THE MEMORY OF MY DEAR FATHER MOSTAPHA, WHO SACRIFICED FOR ME AND SUPPORTED ME THROUGHOUT HIS LIFE.

TO MY DEAR MOTHER ZINEB, MY PARADISE, MY TRUE SOURCE OF STRENGTH AND ENERGY, WHO LIGHTS UP MY PATH AND MY LIFE.

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TO ALL THOSE WHO LOVE ME, AND THOSE WHOM I LOVE.

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ABSTRACTS

Effect of storage time on the quality of olive oil mill wastewaters obtained from the cold extraction of olive oil in the region of Khenchela and their biological properties

Abstract

The objective of this work is to evaluate the effect of storage time on the quality of olive oil mill wastewater (OMW) obtained from the cold extraction of olive oil in the region of Khenchela, Eastern Algeria, and their biological properties. The physicochemical quality of OMW from two types of separated and mixed showed that the OMW storing could help decrease the pollution produced by this waste over time. During one year of storage, the reduction rate of chemical oxygen demand, biological oxygen demand, total oxidizable matter, and biodegradability index is correspondingly (29.4%), (54.8%), (39.16%), and (54.2%); however, C/N, BOD_5/COD, and pH continue to decrease. The highest phenolic concentration (961.11 ± 65.95 µg GAE/mL) was recorded in OMW of Abani variety after 12 months of storage. The highest concentration of flavonoids (27.96 ± 3.71 µg QE/mL) was recorded in OMW of Zlitni, just after olive oil extraction. The highest concentration of total tannin (90.47 ± 21.24 µg CAE/mL) was recorded in Zlitni after 1 month of storage. For condensed tannin, the highest concentration (8.66 ± 3.22 µg TAE/mL) was recorded in Abani after 6 months of storage. Qualitative analysis by LC-MS revealed the presence of 28 constituents, of which kampherol was the major constituent and rosmarinic acid was detected for the first time in OMW. The analysis of the antioxidant activity showed a very highly significant difference for DPPH, ABTS, FRAP, and H_2O_2. The best results were obtained in OMW of Abani after 12 months of storage for the four tests; DPPH (IC_{50} = 151.12 ± 0.22 µg/mL), ABTS (IC_{50} = 129.32 ± 26.09 µg/mL), FRAP (IC_{50} = 72.42 ± 8.59 µg/mL) and H_2O_2 (IC_{50} = 75.52 ± 0.85 µg/mL). The anti-inflammatory activity of the phenolic compounds showed a very highly significant difference between the two tests, IPD and MSP. The best value obtained for the IPD test (IC_{50} = 25.47 ± 1.50 µg/mL) was recorded after 1 month of storage of OMW from the Abani variety. The best value obtained for the MSP test (IC_{50} = 16.11 ± 0.10 µg/mL) was recorded in OMW from Abani after 12 months of storage. For the anticoagulant activity, the finding showed, for the time effect, a very highly significant difference, as regards APTT and PT. The best values obtained for APTT (80.07 ± 0.15 s) and PT (37.13 ± 0.31 s) were recorded in Zlitni OMW just after olive oil extraction. It concluded that the storage time affects the physicochemical and biological properties of OMW. In addition, OMWs are a source of molecules with biological activity, and their extraction can constitute one of the strategies for recovering this waste.

Keywords: Storage time, olive oil mill wastewater, cold extraction, Khenchela, biological properties, olive oil.

Effet du temps de stockage sur la qualité des margines issues de l'extraction à froid de l'huile d'olive dans la région de Khenchela et leurs propriétés biologiques

Résumé

L'objectif de ce travail est d'évaluer l'effet du temps de stockage sur la qualité des margines issues de l'extraction à froid de l'huile d'olive dans la région de Khenchela, à l'Est de l'Algérie, et leurs propriétés biologiques. La qualité physico-chimique des margines issues de deux types séparés et mélangés a montré que le stockage peut contribuer à diminuer la pollution produite avec le temps. Pendant un an de stockage, le taux de réduction de la demande chimique en oxygène, de la demande biologique en oxygène, de la matière oxydable totale et de l'indice de biodégradabilité est de (29.4%), (54.8%) (39.16%) et (54.2%), cependant, C/N, DBO_5/DCO et le pH continuent de diminuer. La concentration phénolique la plus élevée (961.11 ± 65.95 µg EAG/mL) a été enregistrée dans les margines de l'Abani, après 12 mois de stockage. La concentration la plus élevée de flavonoïdes (27.96 ± 3.71 µg EQ/mL) a été enregistrée dans les margines de Zlitni, juste après l'extraction de l'huile d'olive. La concentration la plus élevée de tanin total (90.47 ± 21.24 µg EAC/mL) a été enregistrée à Zlitni après 1 mois de stockage. Pour le tanin condensé, la concentration la plus élevée (8.66 ± 3.22 µg EAT/mL) a été enregistrée pour Abani après 6 mois de stockage. L'analyse qualitative par LC-MS a révélé la présence de 28 constituants, dont le kamphérol était le constituant majeur et l'acide rosmarinique est détecté pour la première fois dans les margines. L'analyse de l'activité antioxydante a montré qu'il y avait une différence très hautement significative pour le DPPH, l'ABTS, le FRAP et le H_2O_2. Les meilleurs résultats ont été obtenus dans les margines d'Abani après 12 mois de stockage pour les quatre tests ; DPPH (IC_{50} = 151.12 ± 0.22 µg/mL), ABTS (IC_{50} = 129.32 ± 26.09 µg/mL), FRAP (IC_{50} = 72.42 ± 8.59 µg/mL) et H_2O_2 (IC_{50} = 75.52 ± 0.85 µg/mL). Les résultats de l'activité anti-inflammatoire montrent qu'il y avait une différence très hautement significative entre les deux tests IPD et MSP. La meilleure valeur obtenue pour le test IPD (IC_{50} = 25.47 ± 1.50 µg/mL) a été enregistrée après 1 mois de stockage des margines de la variété Abani et aussi pour le test MSP (IC_{50} = 16.11 ± 0.10 µg/mL) après 12 mois de stockage. Pour l'activité anticoagulante, la constatation a montré, pour l'effet temps, une différence très hautement significative, en ce qui concerne l'APTT et le PT. Les meilleures valeurs obtenues pour APTT (80.07 ± 0.15 s) et PT (37.13 ± 0.31 s) ont été enregistrées chez la variété Zlitni juste après l'extraction de l'huile d'olive. D'après les résultats trouvés, il a été conclu que les propriétés physico-chimiques et biologiques des margines sont affectées par la durée de stockage. De plus, les margines présentent une source de molécules à activité biologique et leur extraction peut constituer une des stratégies de valorisation de ces déchets.

Mots clés : Durée de stockage, margine, extraction à froid, Khenchela, propriétés biologiques, huile d'olive.

تأثير مدة التخزين على جودة المياه النباتية الناتجة عن الاستخلاص البارد لزيت الزيتون في منطقة خنشلة وخصائصها البيولوجية

الملخص

الهدف من هذا العمل هو تقييم تأثير وقت التخزين على جودة المرجين المتحصل عليه من الاستخلاص البارد لزيت الزيتون في منطقة خنشلة، شرق الجزائر، وخصائصها البيولوجية. أظهرت الجودة الفيزيائية والكيميائية للمرجين من نوعين منفصلين ومختلطين أن تخزين المرجين يمكن أن يساعد في تقليل التلوث الناتج عن هذه النفايات بمرور الوقت. خلال سنة واحدة من التخزين، كان معدل خفض الطلب على الأكسجين الكيميائي، والطلب على الأكسجين البيولوجي، وإجمالي المواد المؤكسدة، ومؤشر التحلل البيولوجي المقابل (29.4٪)، (54.8٪)، (39.16٪)، و (54.2٪)؛ ومع ذلك، يستمر C/N وBOD_5/COD ، و pH في الانخفاض. تم تسجيل أعلى تركيز فينولي (961.11 ± 65.95 ميكروغرام مكافئ حمض الغاليك / مل) في المرجين من صنف عباني بعد 12 شهرًا من التخزين. تم تسجيل أعلى تركيز للفلافونويد (27.96 ± 3.71 ميكروغرام مكافئ الكارستين / مل) في المرجين من صنف زليتني بعد استخلاص زيت الزيتون مباشرة. تم تسجيل أعلى تركيز من إجمالي التانين (90.47 ± 21.24 ميكروغرام مكافئ حمض الكاتشيك / مل) في زليتني بعد شهر من التخزين. بالنسبة للتانين المكثف، تم تسجيل أعلى تركيز (8.66 ± 3.22 ميكروغرام مكافئ حمض التانيك /مل) في صنف عباني بعد 6 أشهر من التخزين. كشف التحليل النوعي بواسطة الكروماتوغرافيا (مطياف الكتلة اللوني السائل عالي الأداء LC-MS) عن وجود 28 مكونًا ، كان الكومفيرول هو المكون الرئيسي وحمض الروزمارينيك تم اكتشافه لأول مرة في المرجين. أظهر تحليل نشاط مضادات الأكسدة أن هناك فرقًا مهمًا للغاية في DPPH و ABTS و FRAP و H_2O_2 .تم الحصول على أفضل النتائج في صنف أباني بعد 12 شهرًا من التخزين بالنسبة للاختبارات الأربعة ؛ DPPH (IC_{50} = 0.22 ± 151.12 ميكروغرام / مل)، ABTS (IC_{50} = 26.09 ± 129.32 ميكروغرام / مل) ، FRAP (IC_{50} = 72.42 ± 8.59 ميكروغرام / مل) و H_2O_2 (IC_{50} = 75.52 ± 0.85 ميكروغرام / مل). أظهرت نتائج النشاط المضاد للالتهابات للمركبات الفينولية وجود فرق كبير للغاية بين الاختبارين IPD و MSP للتأثير. تم تسجيل أفضل قيمة تم الحصول عليها لاختبار IPD (IC_{50} = 25.47 ± 1.50 ميكروغرام / مل) بعد شهر واحد من تخزين المرجين من صنف عباني تم تسجيل أفضل قيمة تم الحصول عليها لاختبار MSP (IC_{50} = 16.11± 0.10 ميكروغرام / مل) في صنف عباني بعد 12 شهرًا من التخزين. بالنسبة للنشاط المضاد للتخثر، أظهرت النتائج، بالنسبة للتأثير الزمني، فرقًا مهمًا للغاية فيما يتعلق بكل منAPTT و PT. تم تسجيل أفضل القيم التي تم الحصول عليها لـ APTT (80.07 ± 0.15 ثانية) و PT (37.13 ± 0.31 ثانية) في صنف زليتني بعد استخلاص زيت الزيتون مباشرة. وخلصت هذه الدراسة إلى أن الخصائص الفيزيائية والكيميائية والبيولوجية للمرجين تتأثر بوقت التخزين. بالإضافة إلى ذلك، يعد المرجين مصدرًا للجزيئات ذات النشاط البيولوجي ويمكن أن يشكل استخلاصها إحدى الاستراتيجيات لإعادة تدوير واسترداد هذه النفايات.

الكلمات المفتاحية: مدة التخزين، المرجين، الاستخلاص البارد، خنشلة، الخصائص البيولوجية، زيت الزيتون.

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A: Abani

ABTS: Ammonium salt of 2,2 '-azinobis (3-ethylbenzothiazothiazoline) -6-sulfonic acid

ACD: Acid citrate dextrose

AIS: Steroidal anti-inflammatory drugs

$AlCl_3$: Aluminum chloride

AMPc: Adenosine monophosphate cyclique

ANOVA: Analysis of variance

AP-1: Activator protein-1

APTT: Activated partial thromboplastin time

As Ac: Ascorbic acid

ATP: Adenosine triphosphate

BHT: Butylated hydroxytoluene

BI: Biodegradability index

BK: Bradykinin

BOD: Biological oxygen demand

BOD_5: BOD measured after 5 days

BSA: Bovine serum albumin

C: Carbon

C-: Negative control

C+: Positive control

CAE: Catechuic acid equivalent

COD: Chemical oxygen demand

COX: Cyclooxygenase

CTC: Condensed tannin content

DM: Dry matter

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl

EC: Electrical conductivity

EC_{50}: Half-maximal effective concentration

FCR: Folin Ciocalteu Reagent

Fe: Iron

$FeCl_3$: Iron chloride

FR: Free radicals

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalent

GC: Glucocorticoids

Glc: Glucose

GPX: Glutathione peroxidase

GRE: Glucocorticoid response element

GSH: Glutathione

GSSG: Glutathione disulfide

H_2O_2: Hydrogen peroxide

HK: High molecular weight kininogen

HOCL: Hypochlorous acid

HPLC-MS: High-performance liquid chromatography-mass spectrometry

HSP: Heat shock proteins

HSV-2: Herpes simplex virus type 2

IC_{50}: Half-maximal inhibitory concentration

IL: Interleukin

IOC: International Olive Council

IPD: Inhibition of protein denaturation

KC: Kaolin cephalin

KCT: Kaolin clotting time

KDa: Kilodalton

LDL: Low Density Lipoprotein

LMWH: Low molecular weight heparin

LOO: Lipid peroxyl radical

LOOH: Lipid hydroperoxides

LOX: Lipoxygenase

LSD: Least Significant Difference

M: Mixture

MAPK: Mitogen-activated protein kinases

MHz: Megahertz

MM: Mineral matter

MPO: Myeloperoxidase

mS/cm: Millisiemens per centimeter

MSP: Membrane stabilizing potential

N.D: Not detected

N: Nitrogen

NK: Natural killer

NSAIDs: Non-steroidal anti-inflammatory drugs

OH: Hydroxyl group

OM: Organic matter

OMW: Olive oil mill wastewater

P: Para

PAF: Platelet activating factor

PBS: Phosphate-Buffered Saline

PCA: Principal Component Analysis

PK: Prekallikrein

PKC: Protein kinase C

PLA2: Phospholipases A2

ppm: Parts per million

PT: Prothrombin time

PUFAs: Polyunsaturated fatty acids

QE: Quercetin equivalent

QT: Quick prothrombin time

RNA: Ribonucleic Acid

ROS: Reactive oxygen species

rpm: Rotation per minute

RT: Retention time

S: Seconds

SD: Standard deviation

SOD: Superoxide dismutase

T: Time

t0: Time immediately following sample collection

TAE: Tannic acid equivalent

TF: Tissue factor

TFC: Total flavonoid content

TKN: Total kjeldahl nitrogen

tM1: Time after one month of storage

tM12: Time after twelve months of storage

tM2: Time after two months of storage

tM6: Time after six months of storage

TNFα: Tumor necrosis factor α

TOC: Total organic carbon

TOM: Total oxidizable matter

TPC: Total phenolic content

TSS: Total suspended solids

TTC: Total tannin content

UFH: Unfractionated heparin

UV: Ultraviolet

V: Variety

VM: Volatile matter

VTE: Venous thromboembolism

V×T: Variety time interaction

WHO: World Health Organization

XO: Xanthine oxidase

Z: Zlitni

GENERAL INTRODUCTION

General Introduction

Mediterranean countries have become the largest consumers, producers, and exporters of olives and olive oil globally. The olive oil production of the countries of the Mediterranean basin represents approximately 98% of the world's production. The production is concentrated in Spain, followed by Italy, Greece, Turkey, Tunisia, Morocco, Portugal, and Algeria **(Manzanares et al., 2020).**

Nowadays, with the promotion of the beneficial virtues of olive oil on human health, Olive oil production has risen in recent decades as a great source of antioxidants and vital fatty acids in the human diet, and it is now one of the world's most potent diet trends **(Souilem et al., 2017).** The demand for olive oil does not cease increasing, and consequently, the production increases. In addition to oil as the main product, the olive industry generates large amounts of solid by-products called olive mill pomace (OMP) and the other liquid called vegetable water or olive oil mill wastewater (OMW). The world production of olive oil mill wastewater represents a volume of more than 30 million m^3 / year **(Nunes et al., 2018).**

For the production of olive oil, the fruits are harvested from the tree from November, crushed, and then kneaded to increase the yield of released oil. The extraction of the oil from the olive paste can be carried out according to different processes: (i) batch press, (ii) three-phase continuous, and (iii) two-phase continuous **(Klen and Vodopivec, 2012).** Three different products and by-products are generated for the first two processes (olive oil, pomace and vegetable water) and only two (olive oil and wet pomace) with the two-phase system. Even if they are less environmentally friendly and generate large quantities of olive oil mill wastewater, traditional pressing and three-phase centrifugation are still used in Algerian oil mills **(Yakhlef, 2019).**

Olive oil mill wastewaters or vegetable waters are water from vegetation generated during the extraction of virgin olive oil. These are effluents rich in organic matter (phenolic compounds, lipids, sugars, proteins, etc.) and mineral salts (potassium, sodium, magnesium, etc.). These vegetable waters are often spread uncontrollably on agricultural soils or stored in evaporation ponds near oil mills, thus exposing water-soil-plant systems to inevitable pollution. The physicochemical and biological treatments of olive oil mill wastewaters, which reduce their impact on water resources, are still insufficient and costly **(La Scalia et al., 2017).**

Algeria, a country with approximately 6.2×10^6 olive trees spread over 471 657 ha according to provisional figures from the Directorate for the Regulation of Agricultural

Production, is among the countries of the Mediterranean basin where the Olivier finds its area of extension. It is the ninth olive oil producer country in the world, with a production of 80 000 tons in 2017/2018 of Mediterranean production. Thus, like all Mediterranean olive oil-producing countries, Algeria is faced with the problem of eliminating OMW with a production of 200 000 tons of OMW per year. In order to reduce the costs of the various treatments applied to OMW and rationalize the management of their waste, research is focused on their recovery in various fields: composting, agriculture, cosmetics, and even in the pharmaceutical industry **(Senani-Oularbi, 2018).**

There has always been considerable interest in molecules of natural origin, which continues to increase in the face of growing consumer mistrust of synthetic products, which sometimes have side effects that are sometimes very serious for human health. In this context, the valuation of by-products from the olive sector can prove to be attractive from an environmental and even economic point of view. The purpose of this is to use these products either in their raw form with some inexpensive treatment or extract bioactive molecules from them. Phenols in OMW are group components with a broad range of chemical structures, ranging from low molecular weight chemicals to complicated high molecular weight compounds **(Da Rosa et al., 2019).**

Phenolic compounds are among the most interesting and widespread bioactive molecules that may be produced at a minimal cost from various plant sources, agricultural wastes, and industrial by-products **(Nollet and Gutierrez-Uribe, 2018).** They are currently among the most widely studied molecules for their multiple biological and functional properties. Their presence at significant levels in olive oil by-products, particularly vegetable water, justifies their choice of recovery **(Gullón et al., 2020).**

This thesis aims to evaluate the effect of storage time on the physicochemical characterizations, phenolic profile, and biological properties of mono-varietal and combined olive oil mill wastewaters obtained from the cold extraction of olive oil from two varieties, Zlitni and Abani. The study of the biological properties of the phenolic extracts of olive oil mill wastewater is concerned with three activities *in vitro*: antioxidant, anti-inflammatory, and anticoagulant.

This thesis is subdivided into two parts: the bibliographic synthesis and the experimental study. The bibliographic part has three chapters: The first chapter deals with general information about the olive tree and OMW; the second is devoted to studying phenolic

compounds; the third deals with biological activities. The experimental part is subdivided into three chapters. The first chapter concerns studying the effect of storage time on the physicochemical properties of olive oil mill wastewaters. The second is devoted to the quantitative (content in polyphenols, flavonoids, total tannins, and condensed tannins) and qualitative (LC-MS analysis) characterization of the phenolic extracts of olive oil mill wastewaters. The third is devoted to evaluating *in vitro* the antioxidant, anti-inflammatory, and anticoagulant activities of phenolic extracts.

Part 1: Bibliographic Synthesis

Chapter 1: General information about the olive tree and olive oil mill wastewater (OMW)

1. General information about the olive tree and OMW

1.1. The olive

The olive is the fruit of the olive tree and is considered one of the oldest cultivated trees in the world. The olive is a food and a source of edible oil. The oil is obtained by grinding the fruit (the pericarp and not the seeds) in a specific oil mill. It is an evergreen tree grown in the Mediterranean region, with more favorable climatic conditions for cultivation of oil and table olive. It is grown in abundance all over the Mediterranean because of its high drought resistance, its tolerance to salt, its long life span, and the supply of high-quality fruit and oil **(Gigon and Le Jeune, 2010; Purcaro et al., 2014).** It is a smooth, fleshy drupe with a rigid bony core that sometimes contains two seeds. Its ovoid shape is typical. Its color, initially green, turns purple and black at full maturity through October-November in the Northern Hemisphere **(Gigon and Le Jeune, 2010).** Its lipid and oil content varies in olive components depending on the terroir, local agronomic practices, variety (or cultivar), and ripeness at harvest **(Purcaro et al., 2014).** The characteristics of olive oil are as follows: density 0. 92 (1 liter of olive oil weighs about 920 g); calorie intake: 9 kcal/g. This oil has a low iodine number and has a longer shelf life if kept cool and protected from light. Therefore, it is best to consume it within two years of making it.

Olea europaea L is one of the rare plant species to synthesize polyols (mannitol) and oligosaccharides (raffinose and stachyose), metabolites of photosynthesis, in its leaves. These carbohydrates, along with the sucrose, are transported from the leaf to the fruit, the olive, to participate as precursors in synthesizing the oil. Developing olives contain chloroplasts that can fix CO_2 and thus contribute to the carbon economy of the fruit. The overall quality of olive oil is directly dependent on this stage of maturation of the olive **(Gigon and Le Jeune, 2010).**

The Algerian national olive grove represents about 33% of tree production. The favorable climate and traditional olive growing traditions provide competitive advantages for the development of the olive oil sector and contribute to self-sufficiency in vegetable oils **(Senani-Oularbi, 2018).**

1.2. Olive Oil

Olive oil is a crucial element of the famous Mediterranean diet, recognized as a protective factor against cardiovascular diseases, cancer, and neurodegenerative diseases. The health benefits of olive oil are even more significant when consumed early in life, before puberty. However, it has been reported that the usual dose of olive oil by healthy volunteers has

already protected a meal by rapidly altering the expression of specific genes **(Lombardo et al., 2018).**

1.3. Olive oil production

1.3.1. Olive cultivation in the Mediterranean basin

In the Mediterranean basin, the olive tree (*Olea europaea* L.) is an important fruit species, both in terms of the number of species grown and its cultural and environmental role **(Gomes et al., 2012).** It indicates the existence of more than 805 million olive trees worldwide, of which 98% are concentrated around the Mediterranean. The worldwide olive genetic heritage is very rich in variety. It consists of more than 2600 different species **(Muzzalupo et al., 2014).** The Mediterranean basin is the productive and commercial heart of olive oil. About 98% of the world's olive trees are concentrated in 3 million farms. Olive oil has been rooted in Mediterranean culture for centuries. The Mediterranean remains the leading producer and consumer of olive oil, whose production is concentrated in Spain, followed by Italy, Greece, Turkey, Tunisia, Morocco, Portugal, and Algeria **(Manzanares et al., 2020).**

According to **IOC (2021),** world production for the 2020/21 campaign should reach 3 034 000 t, a drop of 6.9% compared to the previous campaign, and consumption would be around 3 211 000 t (- 0.2%). Imports would reach 1 074 000 t (- 9.3%), while exports would fall by 8.8% compared to the previous season, to 1 132 000 t. In pole position in production in the 2020/21 campaign are the member countries of the IOC International Agreement on Olive Oil and Table Olives 2015, which alone would produce 2 834 000 t or 93.4% of the world total. The production of the group of European countries is estimated at 2 057 000 t, up 7% from the previous season. The European group is led by Spain, which would register an increase of 24.4%, with an estimated production of 1 400 000 t; followed by Italy, with 270 000 t (- 26.2%); Greece, with 270 000 t (- 1.8%); and Portugal, with 100 000 t (- 28.8%). The production of the other IOC member countries is expected to reach 777 000 t (- 32.4%). In Tunisia, production would be around 140 000 t (- 68.2%), in Turkey 210 000 t (- 8.7%), in Morocco 160 000 t (+ 10.3%) and in Algeria 90 000 t (- 28.7%).

1.3.2. Algerian olive cultivation

Algerian olive cultivation is mainly located in the northern part of the country, where most orchards (80%) are located in mountainous areas. Olive oil production in Algeria increased from the previous year, reaching around 80 000 tons. The expected yield of oily olives established by the agricultural departments of potential Wilaya varies between 7 and 25 q/ha.

The latter registers an interesting threshold, especially in the Wilaya of Skikda, Tizi Ouazou, and Jijel with 25, 22, and 21 q/ha, respectively. Wilaya of Bejaia alone occupies an area of 51 874 hectares with an olive oil production of 123 316 hectares. The olive tree also helps fight erosion, improve agricultural land and settle the population in mountainous areas **(IOC, 2018).**

1.4. Extraction processes of olive oil

The processing of olives for oil extraction can be done mechanically (by pressure or by centrifugation). Various extraction systems are used to extract olive oil **(Baccioni and Peri, 2014).**

1.4.1. Basic process or press system (most common system)

The press system **(figure 1)** is the most common and well-known traditional olive oil extraction system in ancient times. Milling takes place with granite stone stones, converted into a tank whose floor is also made of stone. The grinders used for grinding are then slightly unbalanced concerning the axis of rotation. So, they slide a little on the surface when it turns, allowing it to knead the dough. The dough is obtained in about 30 minutes. After that, the kneaded dough is placed in a thin 2 cm thick layer on a nylon fiberboard called scourt. They are stacked on top of each other around a center pin mounted on a small cart. The set is placed on a hydraulic grass piston that allows a dough pressure of 400 kg/cm^2. The liquid phase flows into the receiving tank. This operation takes 45 minutes. Finally, centrifuges allow the separation of olive oil from vegetable waters. The solid and liquid phases are separated by simple pressure, while the oil is separated from the plant water by natural decantation or centrifugation in vertical centrifuges. The productivity of the extract is 86-90% compared to the oil in the fruit **(El-Abbassi et al., 2012; Ben Hassine et al., 2013).**

1.4.2. Continuous process (centrifugation system)

This modern extraction design replaces the traditional pressing. Instead, it uses horizontal centrifuges called decanters, which improve the yield and productivity of the oil mills. There are two types: the two-phase system and the three-phase system.

1.4.2.1. Three-phase centrifugation system

This system **(Figure 1)** allows the separation of three substances: olive oil, press residues, and vegetable water **(Zbakh and El Abbassi, 2012).** It requires hot water to the olive paste from the kneading before entering the centrifugal edge. So the different steps are separated according to their density utilizing high centrifugal forces in machines called horizontal centrifuges to separate solid-liquid phases or vertical centrifuges to separate liquid-liquid phases that rotate at high speeds of about 3500 rpm **(El-Hajjouji, 2007; Zbakh and El Abbassi, 2012).**

1.4.2.2. Two-phase centrifugal system

This two-phase system (oil and pomace), also called the ecological system **(Figure 1),** allows the production of olive oil without the need to add water to the decanter. So that the latter separates the oil and pomace-water mixture from the vegetation into a single phase of pasty consistency called wet pomace or two-phase pomace limiting the production of vegetable water **(Rahmanian et al., 2014).**

1.4.3. Refining

There is also another industrial process, which is refining. Refining is a relatively recent technology; it is the set of operations that transform crude oil into an industrial product by removing impurities that make it unfit for consumption. As a results, the oil contains desirable and valuable elements (vitamins, unsaponifiable, etc.) and other undesirable elements such as (waxes, peroxide, free fatty acids, etc.). The purpose of refining is to maintain or improve organoleptic characteristics: (taste, neutral odor, clarity, light, youthful color), nutritional characteristics, and the stability of fatty substances in general. To do this, it implements several steps to eliminate, for example, waxes, free fatty acids, pigments, metallic traces, etc.), which are compounds that are harmful to the quality **(Peri, 2014).**

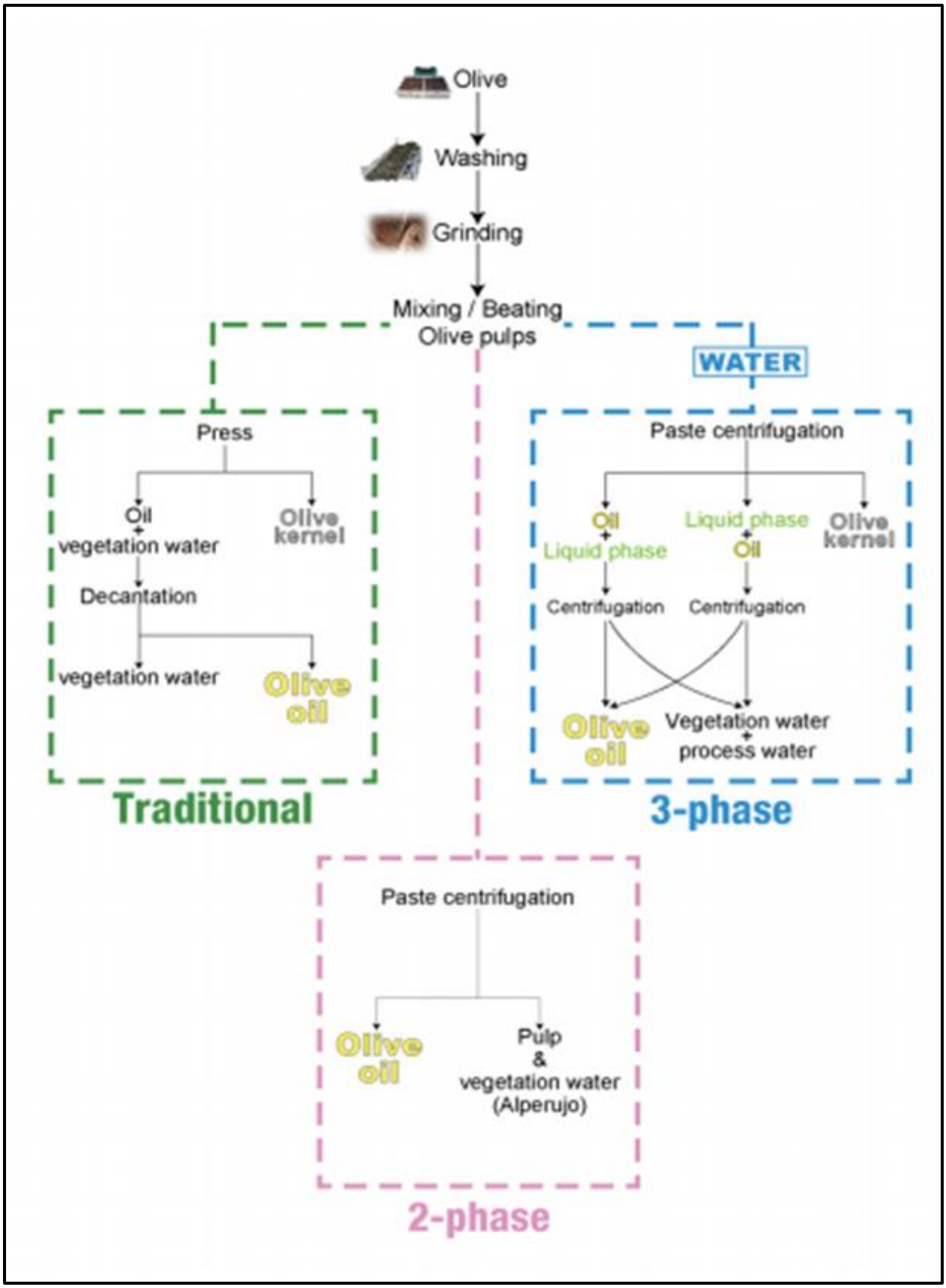

Figure 1: Schematic diagram of traditional, two-phase, and three-phase olive oil extraction methodologies **(Roselló-Soto et al., 2015).**

1.5. By-products of olive oil production

In recent years, the development of agro-industrial activity has generated a large production of waste, particularly from the processing of agricultural raw materials. Like all food industries, the olive industry, in addition to its main product, which is oil (virgin oil and press oil), the extraction process requires large amounts of water, which generates large amounts of solid waste (olive press) and liquid residues (vegetable water) **(Figure 2)** is estimated at 3 million m^3/year **(Dermeche et al., 2013; Roselló-Soto et al., 2015).**

1.5.1. Olive mill pomace

Olive mill pomace or olive cake is a by-product of the extraction process of olive oil from the first pressing or centrifugation. It is a solid residue made up of skins, remnants of the pulp, and fragments of the pips. It consists of one lignin-rich fraction of the nuclear fragments, and the other contains mainly carbohydrates such as cellulose and hemicellulose, and to a lesser extent, proteins and oil, which depend on the extraction technique **(Roselló-Soto et al., 2015).** It is divided into three types, according to the step extraction process: raw cake, depleted cake, and partially stoned pomace.

- **The raw cake**

It is the residue from the extraction of whole olive oil. It is relatively humid water contents and in oil favor its rapid deterioration when it is left in the open air **(Dermeche et al., 2013; Roselló-Soto et al., 2015).**

- **The depleted cake**

It is the residue obtained after oily removal of the raw cake with a solvent, generally hexane **(Roselló-Soto et al., 2015).**

- **The partially stoned pomace**

It results from the partial separation of core debris from the pulp by sieving or ventilation. It says "fatty" if its oil is not extracted by solvent and "degreases" or "exhausted" if the oil is extracted by solvent **(Roselló-Soto et al., 2015).**

1.5.2. Olive oil mill wastewater OMW

1.5.2.1. Definition of olive oil mill wastewater

Vegetable wastewater is the principal waste generated during the extraction of olive oil in highly contaminated acidic liquid form. It is characterized by an intense purple-brown or reddish-brown to a black color and an olive oil odor. Its composition is variable and complex, consisting mainly of sugars, lipids, polyalcohols, proteins, organic acids, and polyphenols **(Daassi et al., 2014).**

Olive oil mill wastewaters (OMW) or vegetable waters, called (Aqua Reflu in Italy, Alpechin in Spain, Katsigaros in Greece, Zebar in Arab countries), are the main liquid effluents that arise during the production of olive oil. In general, 40 to 50% of this water comes from the fruit, and the rest of the water is added during crushing **(Rahmanian et al., 2014; Roselló-Soto et al., 2015).**

Originally, olive oil mill wastewater appears as a residual aqueous liquid, reddish-brown in color, transforming into black vegetable water depending on the decomposition state of its chemical substances, especially phenolic compounds. It is known that the concentration of large amounts of polyphenols comes from the fruit of the olive tree. These biomolecules can be used to develop new food products, food additives, and foodstuffs. In addition, OMW contains high amounts of other valuable compounds, dietary fiber with promising gelling properties, and other valuable organics, such as nitrogen compounds (mainly proteins) and sugar **(Dermeche et al., 2013; Zirehpour et al., 2014).** They have a cloudy appearance, a specific oil odor and are characterized by an acidic pH (4.5 - 5.5) and a very high electrical conductivity **(La Scalia et al., 2017).**

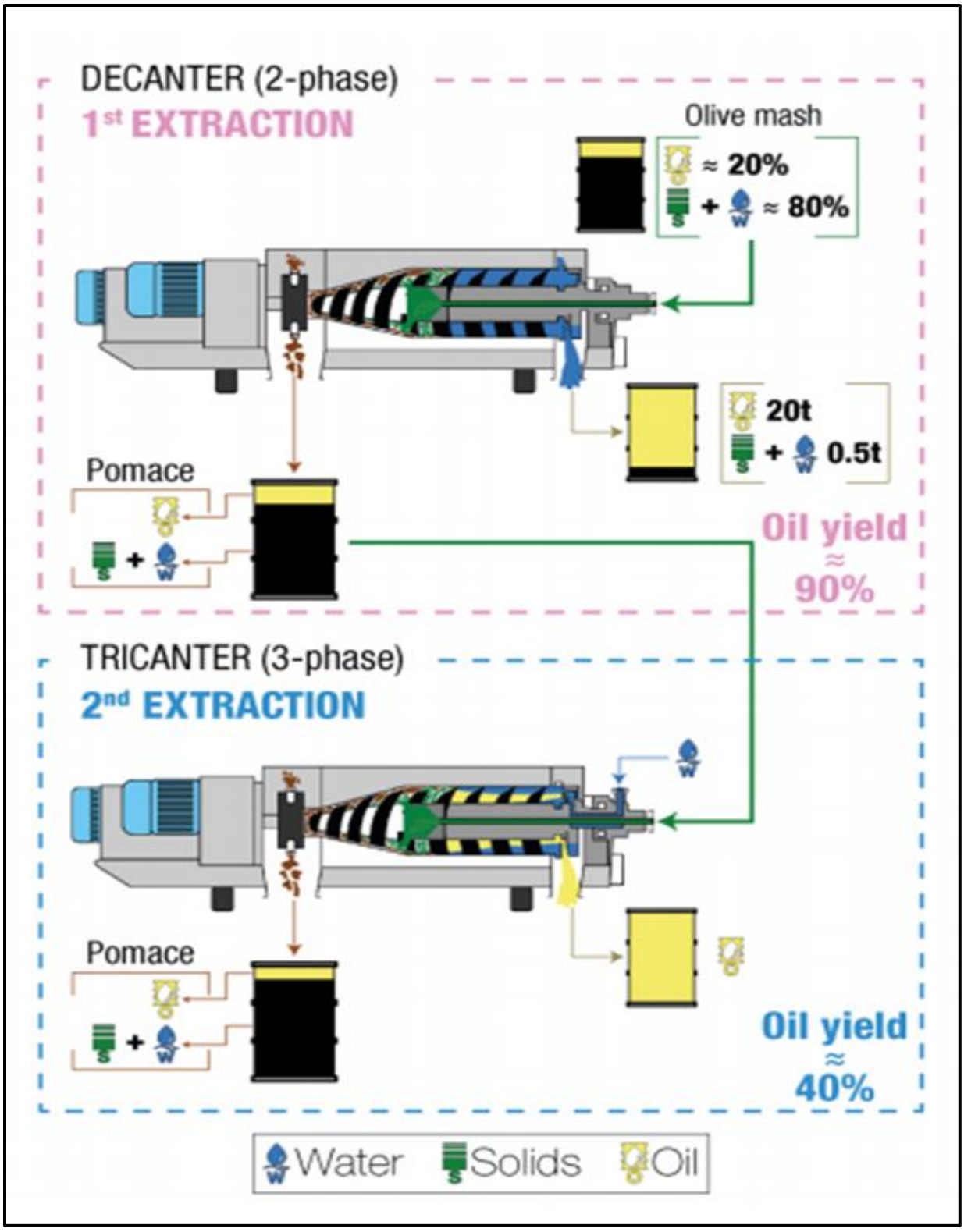

Figure 2: Wastes and by-products generated during olive oil production process (Three-phase extraction method) **(Roselló-Soto et al., 2015).**

1.5.2.2. Types, composition, and management of olive mill wastes

Dealing with a large amount of solid and liquid waste from olive oil production is a challenge for olive growers and producers from an economic and ecological point of view. However, solid waste from the olive oil industry represents a promising biomass resource because its thermochemical properties allow potential use for energy production. For example, lignocellulose can produce bioethanol and therefore offers a solution to management problems **(Khdair and Abu-Rumman et al., 2020).**

The oil yield is similar in the two main types of processing (two- and three-stage centrifugation), but there is a significant difference in the amount and composition of the remaining parts of the solid liquids and liquids produced **(El Hanandeh, 2015).**

Mediterranean countries produce more than 30 million m^3 of olive mill waste annually. These wastes can be in a liquid (OMW) or semi-solid (OMSW) state. The amount and composition of the waste varies according to oil extraction techniques. Due to its high organic content, OMW is extremely harmful to the environment and has high values for biological oxygen demand (50-100 g/L) and chemical oxygen demand (80-200 g/L) **(Khdair and Abu-Rumman et al., 2020).**

1.5.2.3. Characteristics of OMW

1.5.2.3.1. Physicochemical characteristics

OMW has a very complex and heterogeneous chemical composition. It contain various organic and mineral compounds in very different types and concentrations. This variation is mainly due to the oil extraction process of olives, which is the essential element, the stage of olive ripening, the olive tree variety, climatic conditions, the duration of the olive storage before crushing, the cultivation system and geographical location, storage time of olive oil mill wastewater after oil extraction, method of preservation of olives, techniques and storage location **(Zaier et al., 2017; Gueboudji et al., 2021b).**

OMW is characterized by a pH value between 4.2 and 5.9 and a high salt content, expressed in electrical conductivity (18 to 50 mS/cm), mainly due to potassium chloride, ions of calcium, and magnesium **(Zaier et al., 2017; Gueboudji et al., 2021b).**

1.5.2.3.2. Microbiological characteristics

In OMW, only a few microorganisms grow. They are mainly yeasts and molds. Therefore, there are no pathogenic microorganisms in the most cases, and they do not cause

problems from a health point of view **(Ntougias et al., 2013).** The antimicrobial activity of OMW is mainly related to the action of monomeric phenols and catechol-melanin brown pigments. This effluent affects the bacteria, distorts cellular proteins, and alters the membranes **(Zair et al., 2017).**

1.5.2.3.3. Chemical composition

OMW's main compounds are water (83. 2%), organic matter (15%), and minerals (1.8%). The chemical composition of vegetable water varies according to the ripening stage of the olives, the extraction process, the conditions, and the variety of olives **(Dermeche et al., 2013; Gueboudji et al., 2021b).**

- **Mineral fractions**

Vegetable water contains a very diverse mineral content. They are very rich in potassium, sodium, calcium, and phosphorus. In some cases, these effluents may contain traces of heavy metals such as nickel, cadmium, and cobalt. Minerals are composed of 0.06 to 2 kg of nitrogen, 0.1 to 0.5 kg of phosphorus, 1.2 to 3.6 kg of potassium, and 0.05 to 0.2 kg of magnesium **(Dermeche et al., 2013).**

- **Organic fraction**

OMWs have two organic fractions: an insoluble fraction consisting mainly of olive pulp, representing the suspended and colloidal substance. A fraction soluble in the water phase contains sugars, lipids, organic acids, pectin, phenolic compounds, vitamins, and traces of pesticides **(Jail et al., 2010).**

Carbohydrates are mainly represented by the components of the wall, particularly cellulose and pectins, the latter playing an important role in the texture of olives, representing about 0.6% by weight of the fresh pulp. The sugars represent between 4.1 and 4.8% of the total weight of vegetable water, which can be divided as follows: arabinose (62-71%), galactose (17-25%), rhamnose (2-3%), xylose (12%), and glucose (1%) **(Dermeche et al., 2013).**

- **Nitrogen compounds**

Proteins mainly represent the nitrogen fraction with a concentration ranging between 1.2 and 2.4% (w/v). All amino acids in OMW have been identified. The most common are aspartic acid, glutamic acid, proline, and glycine **(Jail et al., 2010).**

- **Vitamins**

Several vitamins have been identified. The most common are vitamin E and vitamin PP, with a concentration of 124 mg/kg content that can be exploited on an industrial scale **(Aissam, 2003).**

- **Organic acids**

The proportion of organic acids in olive oil mill wastewater varies between 0.5 and 1.5% (w/v). The main organic acids found are fumaric, glyceric, lactic, malic, and malonic **(Dermeche et al., 2013).**

- **Oil**

The residual oil concentration in vegetable water varies greatly depending on the extraction process used. It varies between 0.02 and 1% (v/v). Oleic acid is the most abundant fatty acid with a percentage of 65% of the total oil **(Aissam, 2003).**

- **Phenolic compounds**

The phenolic compounds in OMW are very different, and their structure is very varied. More than 50 different phenolic compounds have been identified in olive oil mill wastewaters **(Dermeche et al., 2013).** They are derived from enzymatic hydrolysis of carbohydrates and esters of olive mass during the extraction process. However, their solubilization in oil is much lower than in olive oil mill wastewater, which explains their high concentration detected in vegetable water. The organoleptic properties of virgin olive oil are due to phenolic compounds and volatiles.

The content of phenolic compounds in vegetable water varies between 3 and 5 g/L and may even exceed 9 g/L **(Aissam, 2002; Değirmenbaşı and Takaç, 2018).**

1.5.2.4. Impact of OMW on the environment and processing

The impact of OMW on the environment is characterized by the influence on the different compartments of the ecosystems **(Souilem et al., 2017).** Because they are frequently disposed of in evaporation ponds or other environmental receptors, resulting in high odor nuisance, soil pollution, plant growing limitation, natural stream pollution, and severe consequences on aquatic fauna and ecological balance **(Komnitsas et al., 2016).**

- **Soil pollution**

The direct spread of olive mill wastewater on the soil is the source of various irritations. Their acidity, high salinity, and abundant phenolic compounds destroy the soil microflora and cause toxic effects on vegetable crops; this leads to the sterility of the soil and an imbalance in the symbiosis between the soil microflora and plants **(Belaqziz et al., 2016; Souilem et al., 2017).**

- **Air pollution**

The outflow of vegetable water in open evaporation ponds, on land, or in natural water generates fermentation processes and releases several gases, predominantly methane, carbon dioxide, and hydrogen sulfide; this causes the release of an unpleasant odor **(Ruiz-Mendez et al., 2013; Souilem et al., 2017).**

- **Water pollution**

Metallic and organic waste, biological and chemical oxygen demand are a source of water pollution transferred to groundwater and surface water on earth. Discharges of these drain into rivers and drains without prior treatment cause severe problems for the water system. The harmful effect is primarily derived from the content of phenolic compounds, which can inhibit the growth of microorganisms, mainly bacteria, which reduces natural biodegradation **(Pavlidou et al., 2014; Souilem et al., 2017).**

1.5.2.5. Treatment and recycling of OMW

Due to the impact of olive mill wastewater on the environment and for economic reasons, several researchers have chosen to treat and recycle OMW to limit pollution. These applications are mainly dependent on these effluents' physicochemical and microbiological characteristics **(Zenjari et al., 2006; El-Abbassi et al., 2017).**

To date, the treatment of OMW has a complex problem given the quality and quantity of the substances it contains. Therefore, applying simple treatment is unsatisfactory **(El-Abbassi et al., 2017)**. Thus, different processes also apply such as biological treatment, physicochemical treatments (coagulation/flocculation, membrane filtration, etc.) **(Ouabou et al., 2014; Sbai and Loukli, 2015),** heat treatment (combustion, evaporation), and treatment with evaporation ponds, which is still the most widely used technology today due to its simplicity **(Khdair and Abu-Rumman et al., 2020) (Annex 4).**

Recovery and recycling of OMW **(Figure 3)** carried out is mainly based on the valuation of olive mill wastewater biologicaly (aerobic, anaerobic). Work has also been done to convert phenolic compounds in olive mill wastewater from microorganisms into molecules of interest, such as hydroxytyrosol **(Zenjari et al., 2006; El-Abbassi et al., 2017; Khdair and Abu-Rumman et al., 2020).**

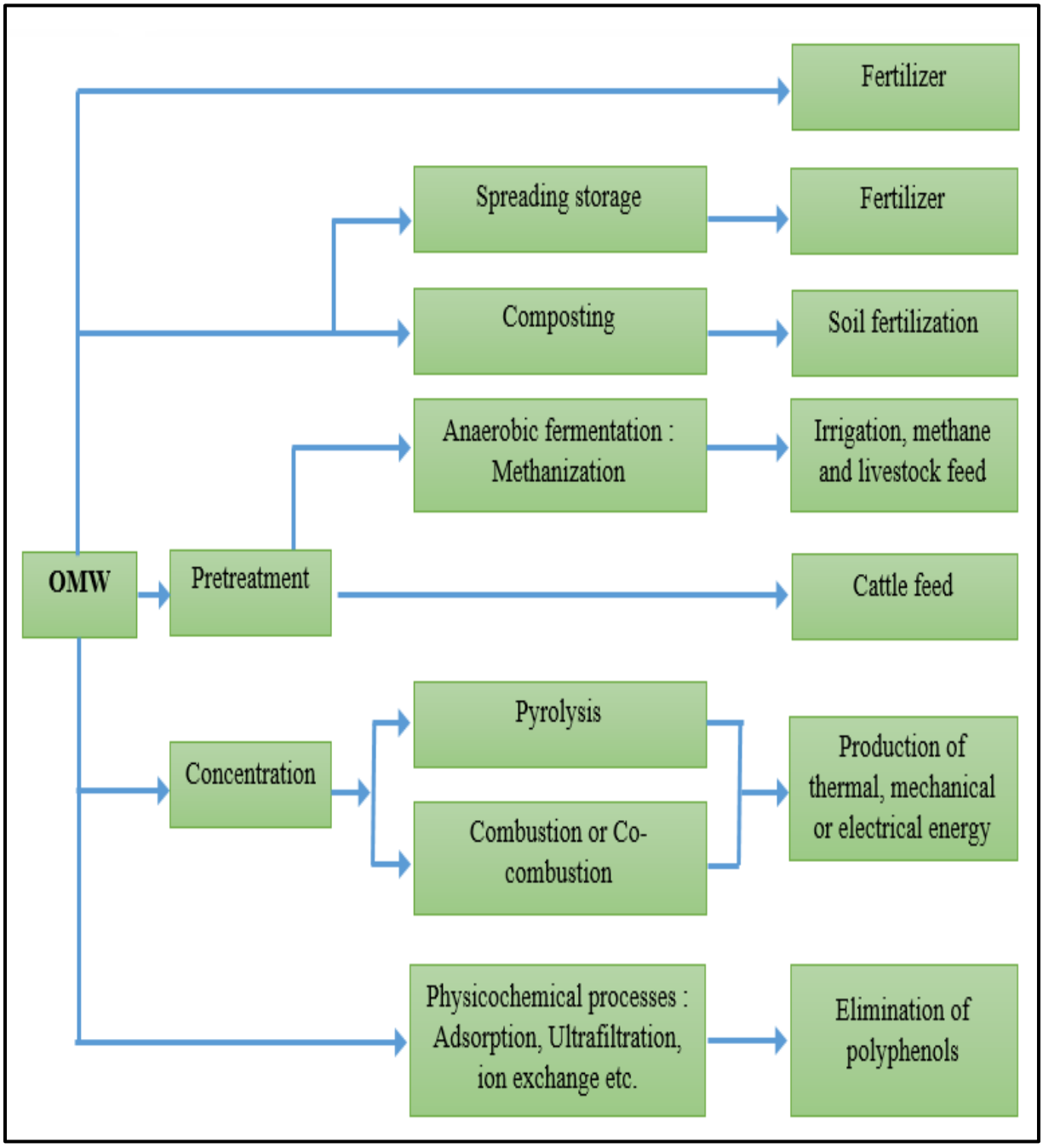

Figure 3: Different OMW recovery channels **(El-Abbassi et al., 2017)**

Chapter 2:
Phenolic Compounds

2. Phenolic compounds

2.1.Chemical structure and diversity

A class of chemical substances extensively prevalent in a plant kingdom is phenolic compounds or polyphenols. It is present on plants from roots to fruits; thus, it is part of our nutrition. They are secondary plant metabolites that interact with other plants and animals. They do not have a direct role at the level of the organism's essential processes, such as growth or breeding **(Lattanzio, 2013).**

The word phenolic is used to designate compounds with an aromatic ring replaced by at least one hydroxyl group (OH). The chemical compound phenol is the simplest name. Consequently, natural polyphenols can be simple molecules like phenolic acids, and highly polymerized compounds like tannins. So far, there have been many thousands of phenolic compounds in the Kingdom of Plants. Nearly 8 000 chemicals are available. Although they are highly varied, they all feature one or more benzene rings with one or more hydroxyl functionalities in common. Phenolic compounds can be classified into many groups **(Figure 4)**, first by the complexity of the basic backbone (from a simple C6 to a highly polymerized one), then by the degree to which this backbone is modified (degree of oxidation, hydroxylation, methylation, etc.). Then probable links between these fundamental molecules and others (carbohydrates, lipids, proteins, other secondary metabolites may or may not be phenolic compounds, etc.) **(Lattanzio, 2013; Kumar et al., 2019).**

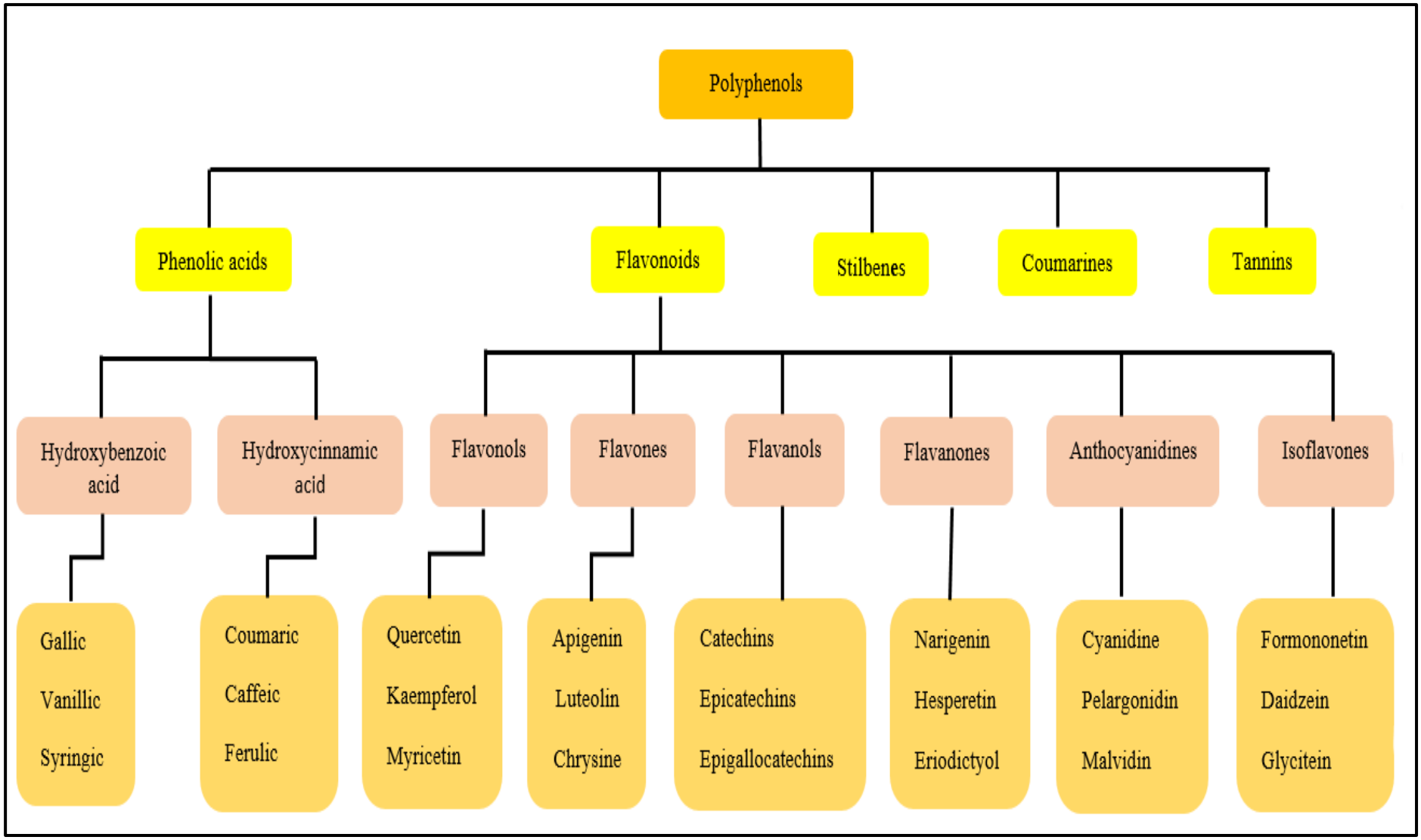

Figure 4: Classification of polyphenols **(Kumar et al., 2019)**

2.2. Biological properties of interest for phenolic compounds

Phenolic compounds have a wide range of biological characteristics that are used in a variety of industries. Without a doubt, antioxidant activity is what distinguishes the best and most frequently occurring polyphenols. Indeed, several studies have attributed to them the function of efficient scavengers of reactive species directly derived from oxygen ($O2^{-\bullet}$, $HO^{\bullet}$, $NO^{\bullet}$, H_2O_2, $1O_2$, HOCl, $RO^{\bullet}$, and $ROO^{\bullet}$) from biomolecules such as lipoproteins, proteins, and oligo-nucleic acids (DNA and RNA). This well-studied and well-recognized faculty are usually highlighted as a key to the prevention and/or reduction of oxidative stress, which is directly connected to chronic illnesses such as cardiovascular disease, carcinogenesis, and neurological disorders. Free radicals are also thought to have a role in the aging process **(Quideau et al., 2011).**

Phenolic compounds defend plants against microbial invasions and have various modes of action against fungus, bacteria, and viruses. These antifungal and antiviral characteristics have several uses in human health. For example, grapes (*Vitis vinifera*) have been found to have major pharmacological characteristics, particularly antibacterial activity, due to the presence of numerous polyphenols, including gallic acid, hydroxycinnamic acid, flavanols, flavonols, and tannins. The most typical of these effects are cinnamic and caffeic acids, found in thyme and tarragon, and are especially efficient against numerous bacterial, fungal, and viral types **(Haminiuk et al., 2012).**

For a long time, flavonoids have been used to treat various ailments. Hippocrates recommended propolis balm for wounds and sores. Antimicrobial activities of propolis have been linked to chemicals classified as flavonols and flavanones centuries later. Indeed, because they prevent the development of pathogenic plant spores, they are good candidates for fighting pathogenic fungus in humans. Flavonols derived from propolis fractions have also effectively fought sexually transmitted herpes simplex virus type 2 (HSV-2), which is a major risk factor for HIV transmission. Tannins' ability to form complexes with proteins via hydrogen bonds, hydrophobic bonds, or covalent bonds allows them to deactivate microbial adhesions, enzymatic and enclose cells harboring microbe proteins. Over 50 years ago, the impact of flavonoids in lemon extract on membrane permeability was the first identified pharmacological effect of these chemicals. Although inflammation is natural self-defense in the body against damage, it may become uncontrolled in autoimmune disorders (rheumatoid arthritis) or when related to allergy reactions (asthma). In the stilbenes family, resveratrol has shown anti-

inflammatory properties *in vivo* and *in vitro* **(Haminiuk et al., 2012; Soto-Hernández et al., 2017).**

2.3. Industrial applications of polyphenols

Such characteristics have been harnessed and find uses in a wide range of industries, including food, cosmetics, and pharmaceuticals. For example, because of the antibacterial characteristics of some polyphenols, such as flavan-3-ols, flavanols, and tannins, it is now feasible to create food preservatives and novel therapeutics for various infectious illnesses taking microbial resistance to specific antibiotic treatments into account **(Daglia, 2012).** In addition, the antioxidant activity of phenolic compounds is employed in food to combat lipid peroxidation and therefore improve food stability. They are also advised to enhance the stability of colored juice pigments (such as beet juice) and food flavorings and include them into the formulations of therapeutic goods for oral use and cosmetics for local applications **(Garg et al., 2019; de Araújo et al., 2021).** Finally, the effect of certain flavonoids in human medicine is being studied more and more in the treatment of certain diseases, particularly in the control of the immunodeficiency virus, the main cause of acquired immunodeficiency syndrome (AIDS), which is reducing the use of synthetic drugs **(Garg et al., 2019).**

2.4. Phenolic compounds in OMW

OMW's phenolic compounds (or biophenols) are extremely varied, with a wide range of structures. Biophenols are transported from olive paste to oil and other by-products, notably OMW, throughout the olive oil manufacturing process. Phenolic compounds have a hydrophilic propensity; nevertheless, their solubilization in oil is significantly lower than in the aqueous phase. In reality, just 2% of the phenolic chemicals found in olives are transported to oil, 53% to OMW, and 45% to olive pomace. Nonetheless, some of these components are being synthesized for the first time because of numerous enzymatic biotransformation routes **(Klen and Vodopivec, 2012; Gueboudji et al., 2021c).**

OMW phenols include molecules with a wide range of chemical structures, ranging from low molecular weight chemicals to complicated high molecular weight compounds **(Da Rosa et al., 2019).** The most important biophenol classes are phenolic acids, phenolic alcohols, flavonoids, and secoiridoids. For example, 3,4-dihydroxyphenyl ethanol (hydroxytyrosol) and p-hydroxyphenyl ethanol are the phenolic alcohols of OMW (tyrosol). The anthocyanins cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside, and luteolein 7-O-glucoside, rutin, and

apigenin 7-O-glucoside, are the most often reported vegetable flavonoids. The primary secoiridoids in OMW are oleuropein and ligustroside, derived from the olive pulp.

Several oleuropein derivatives have also been identified, including dimethyloleuropeine, oleuropein aglycone, and elenolic acid. The primary hydroxycinnamic acid derivative in vegetable water is verbascoside **(Castro-Muñoz et al., 2019).** Structures of the main phenolic compounds found in OMW are illustrated in **(Figure 5).**

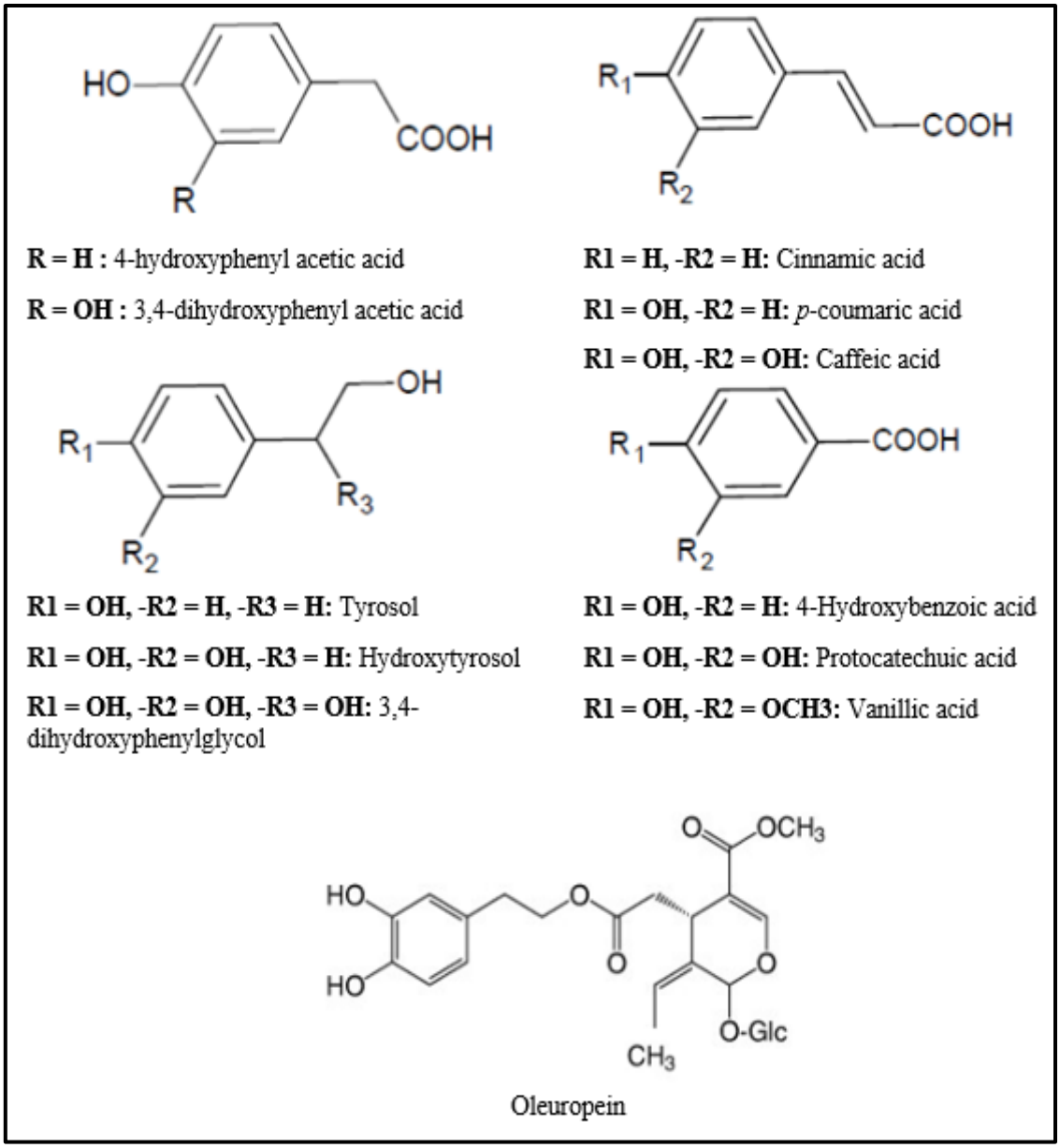

Figure 5: Structures of the main phenolic compounds found in OMW. **Glc:** Glucose **(Dermeche et al., 2013)**

2.5. Biological interest of polyphenols of OMW

There is mounting evidence that free radicals have a role in various human illnesses by attacking biomolecules such as lipids, proteins, DNA, and bio-membranes, and playing a significant part in the oxidative destruction of foods and cosmetic goods. As a result, the extraction of physiologically active phenolic components (biophenols) from vegetable fluids represents a feasible alternative for upgrading this hazardous effluent **(Zbakh and El-Abbassi, 2012; Castro-Muñoz et al., 2019).** Indeed, the phenolic fraction possesses interesting biological properties. Several *in vitro* and *in vivo* investigations have demonstrated that biophenols of OMW had several bioactivities **(Zbakh and El-Abbassi, 2012).** First, hydroxytyrosol had an antioxidant effect. It activates the biogenesis of mitochondria and inhibits lipid oxidation during food storage. In addition, it has cardioprotective and antiatherogenic effects because it traps and reduces the production of the superoxide anion in the culture of human promonocytes, antimicrobial and antiviral effects against human pathogens. In agriculture, against phytopathogenic bacteria against the influenza virus, moreover, anti-inflammatory effect to Inhibits the production of leukotrienes B4 leukocytes. Second, oleuropein had an antioxidant effect against anti-free radical activity (DPPH test). Antiatherogenic and cardioprotective prevents oxidative damage to the myocardium induced by ischemia. Vasodilator and anti-platelet aggregation effects. Hypoglycemic in diabetic rabbits. Antihypertensive, effective in patients with stage 1 hypertension. Antimicrobial and antiviral antibacterial antimycoplasma against pathogenic bacteria and fungi. Anti-inflammatory, identified in all cell cultures human blood. Third, oleuropein aglycone had a more excellent antioxidant activity than oleuropein, protecting against LDL oxidation. Neuroprotective inhibits aggregation rate neurodegenerative. Cytoprotective prevents cytotoxic amyloid aggregation of human amylin. Reduces DNA oxidation using in high concentrations. Anti-inflammatory effect inhibits carrageenan-induced pleurisy in mice. Finally, Tyrosol:

Antioxidant against lipid peroxidation in intestinal Caco-2 cells. Anti-inflammatory in humans. Cardioprotective, modulate superoxide anion production and MMP-9 expression in PMA-stimulated THP-1 cells, and neuroprotective, the protective effect of transient cerebral ischemia in rats **(Zbakh and El-Abbassi, 2012; Aggoun, 2016).**

Chapter 3: Biological Activities

3. Biological activities

3.1. Antioxidant activity

3.1.1. Oxidative stress

Oxygen is normally converted into water molecules in the mitochondrial respiratory chain in biological systems. This reaction is crucial because it provides the cell with most of the energy it needs (in adenosine triphosphate ATP) to perform its many functions. However, oxidative stress imbalances the balance between antioxidant defense systems and reactive oxygen species (ROS) production. Thus, this leads to biochemical damage to the body's cells due to its molecular consequences, such as changes in proteins, the occurrence of DNA breaks, or damage to the integrity of the cell membrane by induction of lipid peroxidation **(Sies et al., 2017; Shilpa et al., 2017).**

Oxidative stress is involved in many pathologies **(Figure 6);** including obesity, high blood pressure, diabetes, cardiovascular and neurodegenerative diseases, skin aging, and renal failure **(Shilpa et al., 2017; Tan et al., 2018a).**

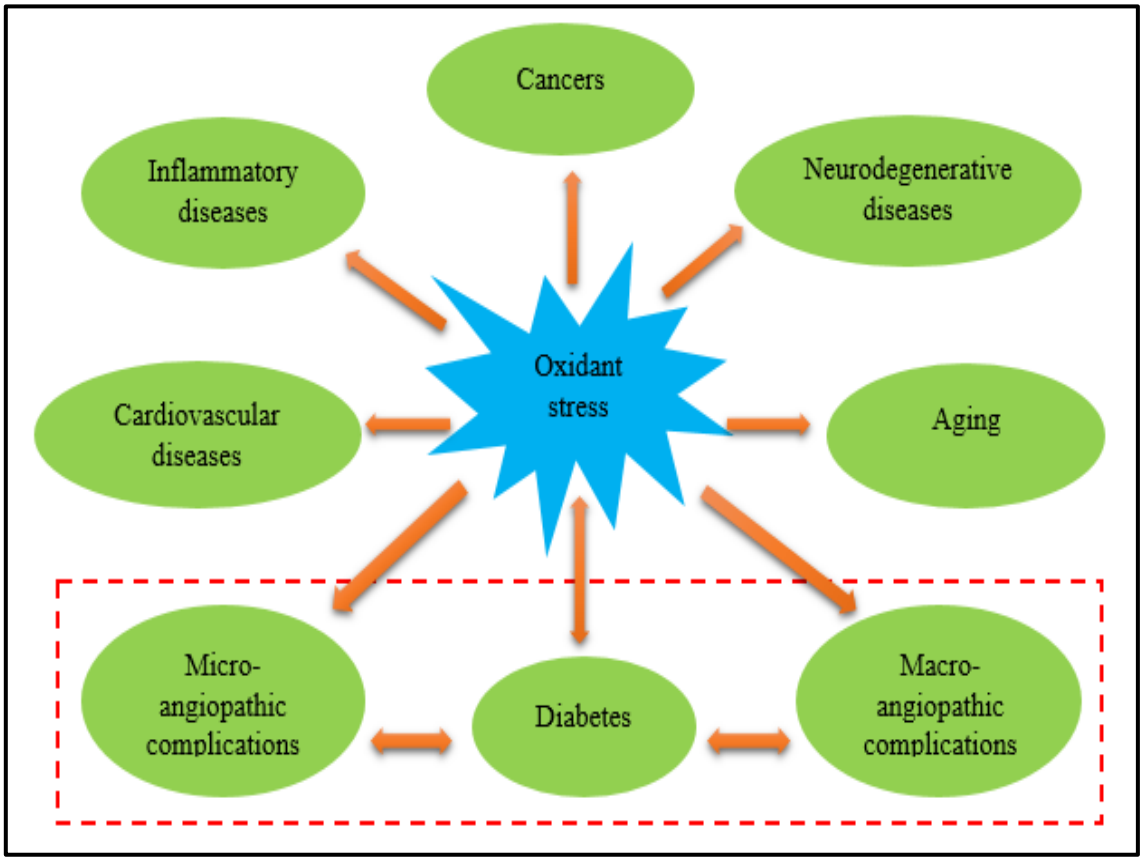

Figure 6: Consequences of oxidative stress **(Tan et al., 2018a)**

3.1.2. Biological consequences of oxidative stress and pathological implications

Free radicals present a paradox in their biological function. These are very hazardous species that, during oxidative stress, are susceptible to oxidative damage to various biological molecules, especially lipids, proteins, and DNA **(Tan et al., 2018b).**

- **The oxidation of proteins**

Free radicals are exerted on proteins, especially on the side chains of some amino acids such as the thiol group of cysteines and methionine, and cause the formation of sulfur bridges and thus the aggregation of proteins. The other functional protein groups also targets free radicals (FR) attacks. Thus, OH ° attacks valine methyl groups, the phenyl group of the amino acids tyrosine and the methyl group of alanine, and the amino group of arginine and lysine. FR can also cut peptide bonds and form protein fragments. These oxide modifications of proteins generally cause changes in the spatial conformation and consequently the change in the biological function of this protein (enzyme, receptor, transcription factor, etc.) **(Sies et al., 2017).**

- **DNA oxidation**

Whether nuclear or mitochondrial, DNA is a major target of reactive oxidized and nitrogenous species, leading to various damages such as base modification, base formation, strand breakage, and DNA bridging protein. Oxidation cell membranes in polyunsaturated fatty acids (PUFAs) (oleic, linoleic, and arachidonic acid) are susceptible to FR-induced oxidation by triggering a chain reaction called cell lipoperoxidation. Altering the fluidity, permeability, and excitability of membranes by creating abnormal biological molecules and overexpression of specific genes is associated with oxidative stress in several diseases like cancer, cataracts, and accelerated aging. It is also a potentiating factor in developing multifactorial diseases such as diabetes, neurodegenerative, pulmonary, and cardiovascular diseases **(Shilpa et al., 2017; Tan et al., 2018b).**

3.1.3. Free Radicals

Free radicals are unstable molecules or atoms with one or more odd electrons, making them highly reactive. This free radical always seeks to fill its orbit to stabilize; oxidizing the second compound, therefore, reduces it. Then speak of the major radical species of the reactive oxygen species (ROS) concentrated in **Figure 7** during this electron atom of oxygen **(Losada-Barreiro et al., 2017).**

It is advisable to distinguish a limited number of radical compounds, which have a special role in physiology, called primary free radicals. They are most dangerous because they come directly from oxygen, such as the superoxide radical $O^{-.2}$, the hydroxyl radical $OH^{\bullet}$, hydrogen peroxide H_2O_2, or even singlet oxygen $O^{.}{}^{2}$ **(Maurent, 2017; Diallo, 2019).** The main sources of free radicals are either endogenous or exogenous.

- **Endogenous sources**

- The mitochondria are the main source of ROS (Reactive Oxygen Species) through its respiratory chain. It is thought to produce 90% of cellular ROS **(Losada-Barreiro et al., 2017).**

- Enzymes such as; Catalase, Xanthine oxidase (XO), NADPH oxidase (NADPHO) **(Losada-Barreiro et al., 2017).**

- Metal ions: like iron and copper in their reduced form **(Losada-Barreiro et al., 2017).**

- **Exogenous sources**

Several environmental factors, such as ultraviolet and ionizing radiation, can stimulate the endogenous production of free radicals after interaction with tissues or cells **(Losada-Barreiro et al., 2017).** In addition, various other exogenous sources contribute directly or indirectly to the total oxidant charge. These include the effects of air pollution and natural toxic gases, like chemicals and toxins. In addition, foreign microorganisms induce the formation of secondary oxidants and their release in the host, in addition to their sometimes-direct oxidative capacities **(Losada-Barreiro et al., 2017; Maurent, 2017).**

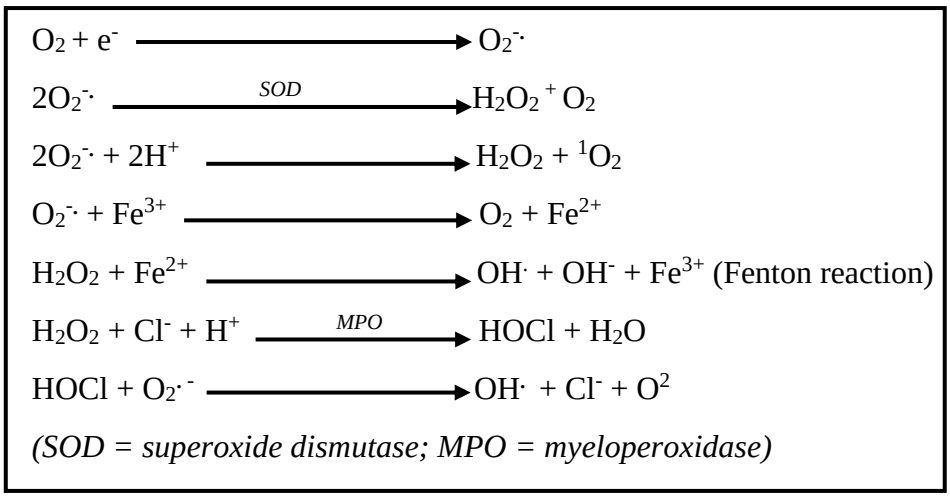

Figure 7: Main reactive oxygen species present in the organism **(Maurent, 2017)**

3.1.4. Antioxidants

An antioxidant is any substance at a lower concentration than the oxidizing substrate that can delay or prevent the oxygen content of this substrate **(Jeeva et al., 2015).**

They are produced in the body (endogenously) or supplied by food (externally), according to their mechanism of action; a distinction is made between enzyme and non-enzyme antioxidants **(Gulcin, 2020) (Figure 8).**

3.1.4.1. Enzymatic antioxidants

Enzymatic antioxidants are produced only in the human body and further subdivided into primary and secondary antioxidants.

- Superoxide dismutase (SOD) catalyzes the disproportionation of the superoxide anion to H_2O_2.

- Catalase is an enzyme capable of converting hydrogen peroxide into water and molecular oxygen.

- Glutathione peroxidase (GPx) acts in synergy with SOD since its role is to accelerate the disproportionation of H_2O_2 into H_2O and O_2. During this reaction, two molecules of reduced glutathione (GSH) are oxidized to glutathione disulfide (GSSG) **(Jeeva et al., 2015).**

3.1.4.2. Non-enzymatic Antioxidants

External antioxidants are molecules not produced by the body but are provided by food or therapies. Trace elements (Mg, Zn, Se and, Mn), vitamin E, vitamin C, carotenoids, and polyphenols represent this class of antioxidants.

- Vitamin E binds to the cell membrane and prevents the chain of lipid peroxide reactions by capturing peroxyl lipid radical (LOO^-) **(Maurent, 2017).**

- Vitamin C (ascorbic acid) is a water-soluble vitamin found in the cytosol and extracellular fluid. It can take directly O_2^- and OH^-. It can also reduce the α-tocopherol group and thus allow for the better activity of vitamin E **(Maurent, 2017).**

- Carotenoids are very numerous and represent the main origin of retinol. In addition to the activity of pro-vitamin A, carotenoids can inactivate one oxygen and free radicals by neutralizing the unpaired electrons and thus converting them into stable molecules or ions **(Maurent, 2017).**

- Polyphenols are secondary metabolites that can inhibit the accumulation of oxygen in biomolecules. These compounds can act in a variety of ways in the oxidative stress management process: by directly capturing reactive oxygen species, by clinging to transition metals such as iron (thus preventing the Fenton reaction), or by blocking the action of certain enzymes responsible for the production of ROS **(San Miguel-Chávez, 2017).**

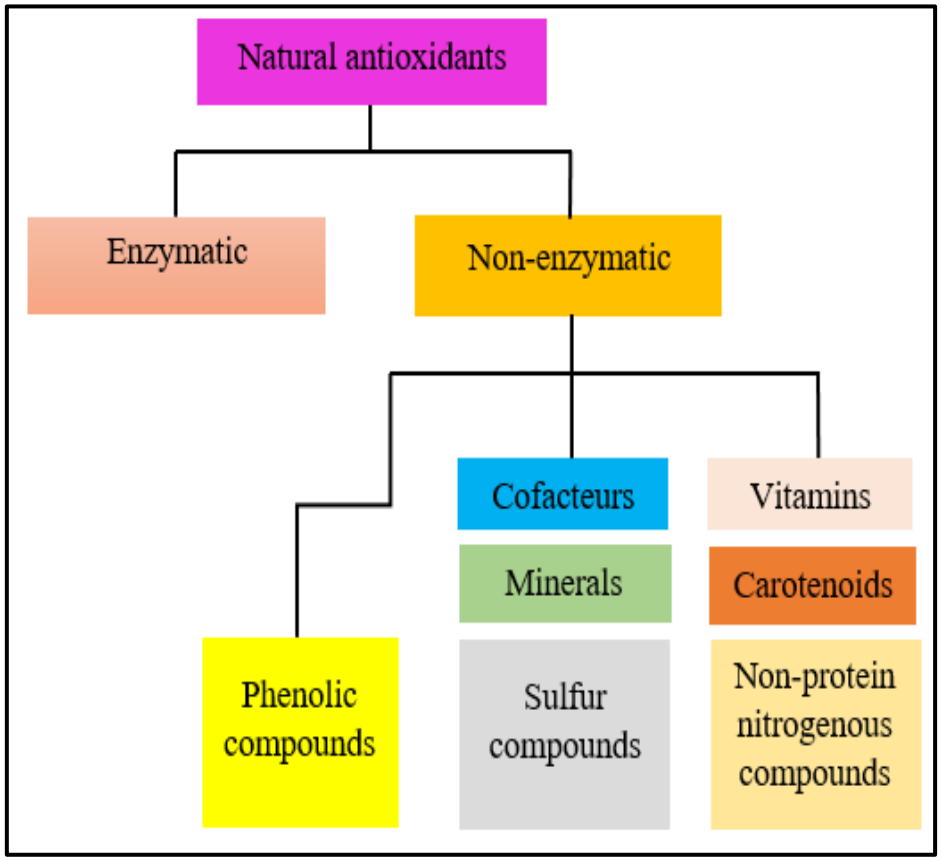

Figure 8: Families of natural antioxidants **(Maurent, 2017)**

3.1.5. Mechanism of action of phenolic antioxidants

Phenols readily lose H^+ protons to form phenoxy ions. Hydrogen: proton + electron loss produces strongly stabilized radical formation by mesomerism. This chemical reactivity gives phenolic compounds an antioxidant nature. By providing hydrogen to LOO lipid peroxides, they stabilize them as LOOH hydroperoxides and inhibit oxidation chain progression reactions. This type of mechanism involves primary antioxidants or free radicals **(San Miguel-Chávez, 2017).**

3.2. Inflammation and anti-inflammatory activity

Inflammation is a complex physiological defense process the body uses following foreign, vascular, and tissue attacks to eliminate or isolate the attacker and maintain the integrity of infected tissue. It is a pathological condition characterized by the following cardinal signs: heat, pain, redness, and swelling of the diseased part **(Stankov, 2012; Diallo, 2019).**

3.2.1. Types of inflammation

Inflammation is classified into two categories acute and chronic, depending on the duration and kinetics of the inflammatory process **(Stankov, 2012; Chen et al., 2018).**

Many tissue injuries trigger an acute inflammatory reaction, but some lesions can immediately cause a chronic inflammatory reaction (e.g., viral infections, reaction to foreign bodies, yeast infection). Acute inflammation can disappear or lead to scarring, but it can develop into chronic inflammation **(Stankov, 2012).**

3.2.2. Therapeutic drugs for inflammation

Anti-inflammatory drugs counteract inflammatory processes, i.e., chemicals that fight general inflammatory phenomena of various origins (infections, burns, irritations, metabolic disorders, etc.) by inhibiting cyclooxygenase (COX), in particular COX_2.

There are generally two categories of anti-inflammatory drugs: some are hormonal (steroidal anti-inflammatory drugs AIS), and others are not (non-steroidal anti-inflammatory drugs NSAIDs), and there are natural anti-inflammatory drugs (herbal) **(Bacchi et al., 2012).**

- **Non-steroidal anti-inflammatory drugs (NSAIDs)**

Non-steroidal anti-inflammatory drugs are weak lipophilic acids and group molecules that, despite structural heterogeneity, have the same mode of action. NSAIDs are a group of drugs used to treat inflammatory reactions and rheumatic diseases. All NSAIDs have varying degrees of anti-inflammatory, analgesic, and antipyretic properties regardless of route of administration. They reduce vasodilation capillary permeability, the migration of leukocytes, and disrupt the energy responses necessary for the inflammatory process. In addition, they all work by inhibiting a membrane enzyme, cyclooxygenase (COX), which reduces the production of prostaglandins E2 and I2, essential mediators of inflammatory phenomena **(Bacchi et al., 2012).**

- **Steroidal anti-inflammatory drugs (AIS) or glucocorticoids**

Steroidal anti-inflammatory drugs (AIS) are a large drug family derived from cortisol. Glucocorticoids (GC) are potent anti-inflammatory agents, cross-free cell membranes, bind to specific receptors belonging to the nuclear steroid receptor superfamily, migrate to the nucleus and act directly on DNA by binding to specific sequences; is Glucocorticoid Response Element (GRE). This complex is involved in regulating of the transcription of target genes by reducing the capillary permeability, production of chemotactic factors, phagocytosis, which blocks the release of serotonin, histamine, and bradykinins **(Jedziniak et al., 2016).**

In addition, GC can increase the transcription of anti-inflammatory genes and inhibit the action of certain trans-nuclear activator proteins, including NF-κB and activator protein-1 (AP-1), thereby inhibiting the expression of many cytokines. Pro-inflammatory (IL-1, IL-6, IL-2, TNF-α), receptors and adhesion molecules, and production of phospholipase A2 **(Jedziniak et al., 2016).**

They work by inhibiting the inflammatory response by inactivating membrane phospholipase, preventing the release of arachidonic acid, the precursor to prostaglandins, and inducing the formation of a protein called lipocortin, which binds to phospholipase and renders it inactive. However, their side effects can be significant, linked to long-term use or an increase in the dose that induces various disorders (stomach ulcers, high blood pressure, hyperglycemia, etc.) **(Jedziniak et al., 2016).**

- **Natural anti-inflammatory drugs (herbal)**

Medicinal plants have played a key role in human health and have contributed to modern therapeutic drugs. They are often used to relieve inflammatory diseases such as rheumatoid arthritis, asthma, bronchitis, eczema, osteoarthritis, gout, allergic rhinitis, stomach ulcers, and duodenal ulcers. The anti-inflammatory activity of these plants is due to their content in secondary metabolites equipped with biological activities: polyphenols, sterols, alkaloids, saponins, coumarins, terpenes, polysaccharides, etc. **(Yatoo et al., 2018).**

The most prominent points for cellular regulation affected by flavonoids are the effect on eicosanoid-generating enzymes such as phospholipases A2 (PLA2), cyclooxygenase (COX), lipoxygenase (LOX), and the effects on pro-inflammatory suppressor molecules such as protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) **(Yatoo et al., 2018).**

3.2.3. Factors that trigger inflammation

The factors that trigger inflammatory phenomena can be different:

- Physical elements, such as heat (burns), cold (frostbite), and ionizing radiation, will cause tissue damage and release degradation products such as collagen.
- Exogenous or endogenous solids, such as microbial pathogens, insect bites or microcrystals (urate crystals), chemicals (acidic, basic, toxic), biological products (toxins, tissue degradation products), compounds derived from the immune reaction (immune complexes, cytotoxic antibodies).
- Regardless of the nature of the stimulus, the manifestations of the inflammatory response will be the same. It is the intensity of the manifestation and their duration that changes conditions the positive or harmful effects of the inflammatory reaction **(Chen et al., 2018).**

3.2.4. Inflammation mediators

The local changes that occur at the site of inflammation are the result of the formation and/or sequential release of pro- and anti-inflammatory mediators of various kinds: amine (histamine and serotonin), lipid mediators (prostaglandins and leukotrienes), and cytokines of peptide, protein or glycoprotein nature **(Table 1) (Abdulkhaleq et al., 2018).**

Table 1: Mediators of inflammation **(Abdulkhaleq et al., 2018).**

	Plasma mediators
Fibrinolysis coagulation system	If this activated system is able to synthesize thromboxane A2 that ensures effective vasoconstriction and activation of Hageman factor (factor XII) triggers coagulation and results in the formation of fibrin.
Complement system	Since the complement system is involved in the nonspecific defense against infectious agents, it comprises three activation pathways, the classical pathway, the alternate pathway and the lectin pathway, resulting in a common end effector unit, the CAM.
Kinins system	The inactive kinogen precursors of kinins are activated by kallikrein, or by Hageman's factor, and increase heat and pain. Bradykinin acts on nerve endings by promoting pain.
	Cellular mediators
Lipid mediators	When a cell (phagocyte or mast cell) detects a danger signal, phospholipase A2 is activated by TNFα. This enzyme converts fatty acids in the plasma membrane of cells into leukotrienes, prostaglandins and PAF.
Vasoactive amines	Histamine and serotonin are stored in mast cells, polymorphonuclear basophils and platelets, produce vasodilation and vascular permeability.
Free radicals (FR)	Activated phagocytic cells (polymorphonuclear neutrophils and macrophages) produce toxic derivatives of oxygen (H_2O_2, $O2^1$), generate hypochlorous acid (HOCL) and secrete toxic (NO).
Cytokines and Chemokines	They are proteins secreted by (lymphocytes and monocyte-macrophages); they allow the orientation of the response according to the nature of the signal detected. Some cytokines are pro-inflammatory (IL1, IL6, TNFα) and others are anti-inflammatory (IL4, IL10, IL13). Chemokines are peptides related to cytokines that allow the recruitment of monocytes and polymorphonuclear cells during inflammation.

3.2.5. Pathological implication of inflammation

Many inflammatory diseases are related to mechanisms considered dysimmune, namely systemic and localized autoimmune diseases, auto-inflammatory diseases, and inflammatory conditions of unknown mechanism, especially iatrogenic or paraneoplastic diseases whose mechanism is not autoimmune **(Zerbato, 2010; Chen et al., 2018).** Inflammatory reaction is illustrated in **Figure 9**.

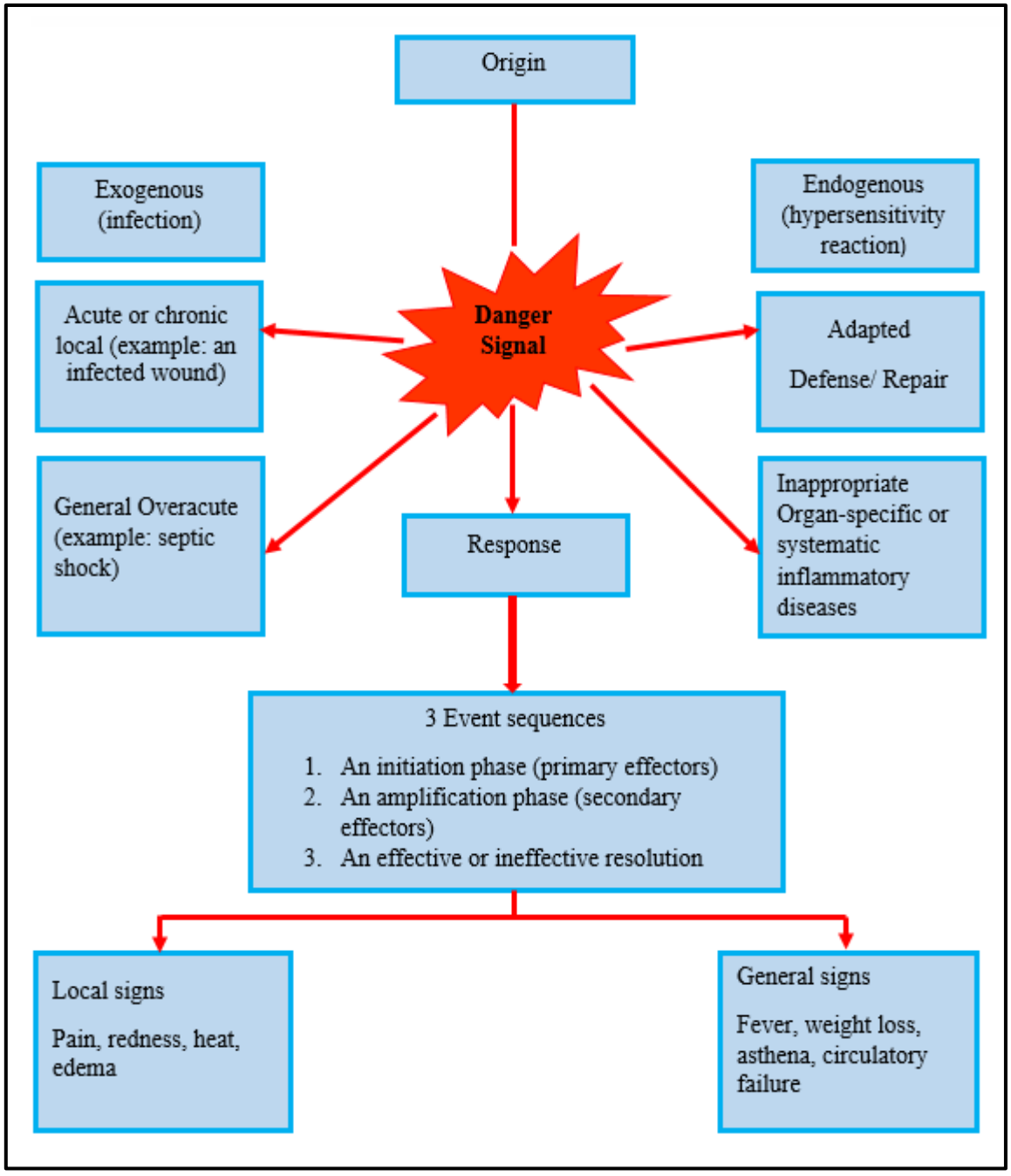

Figure 9: Schematized inflammatory reaction **(Zerbato, 2010)**

3.2.6. Inflammation and protein denaturation

A non-immune inflammatory response may lead to immune inflammation after the denaturation of endogenous proteins that have become autoantigenic **(Du Clos, 2012).**

Exposure of a cell to heat shock causes alteration of proteins, response to heat shock, and eventual recovery or elimination of altered proteins **(Du Clos, 2012; Agarwal et al., 2019).**

- **The first step (protein change)**

Albumin undergoes structural changes with loss of its three-dimensional shape and exposure to specific hydrophobic sites (such as cysteine residue 34), which in the normal physiological case are inaccessible (functional protein native). These hydrophobic regions can interact and form aggregates that are harmful to the cell **(Du Clos, 2012; Agarwal et al., 2019).**

- **Second phase (reaction to heat shock)**

This step involves the intervention of heat shock proteins (HSP or heat shock proteins) that recognize the regions normally buried in the molecule (albumin) and which will be available later denaturation or degradation **(Du Clos, 2012; Agarwal et al., 2019).**

The HSP-denatured protein interaction promotes their degradation or transport in the various organelles and their collection and activation of T lymphocytes after the presentation of autoantigens in the presence of MHC molecules (larger histocompatibility complex) and differentiation of monocytes into macrophages **(Du Clos, 2012; Agarwal et al., 2019).**

- **Elimination phase**

It eliminates abnormal proteins and prevents the formation of aggregates or allows renaturation of proteins if the possibilities for cell repair allow it **(Serhan et al., 2010; Du Clos, 2012).**

Albumin is a small 66.5 kDa globular protein and the most abundant in plasma. It represents approximately 60% of plasma proteins and has 585 amino acids with a thiol group at the level of its cysteine 34 in reduced form, which plays a role in the aggregation of albumin under increased temperature. This protein synthesized by the liver is regulated by nutritional status, insulin, cortisol, glucagon, or thyroid hormones. It is distributed in the blood and organs such as skin, muscles, intestines, and subcutaneous tissue. Its medium life is 15 to 20 days. It is catabolized by the reticuloendothelial system **(Du Clos, 2012).**

3.2.7. Inflammation Cells

The cells involved in inflammation **(Table 2)** are called immunocompetent; they are derived from a common precursor, the pluripotent hematopoietic stem cells in the bone marrow **(Serhan et al., 2010).**

Table 2 : Inflammation Cells **(Serhan et al., 2010).**

Type	Function
Phagocytic cells	Composed of polynuclear neutrophils and monocytes/macrophages, they are the first cells to migrate to lysate focus and are able to eliminate phagocytosis attacks.
Lymphocytes	They are cells with specific, humoral and cellular immunity, type B, T or NK. Among the T lymphocytes, some are called helper (CD4), others cytotoxic (CD8) secrete cytokines, plasma cells (maturation of the B line) secrete antibodies, and NK lymphocytes may have a cytotoxic effect.
Polynuclear basophils	They are the major players in the allergic reaction and various immune and inflammatory events; they act by release of vasoactive compounds (histamine).
Polynuclear eosinophils	They are key cells in allergic inflammation. Indeed, they have in their granules histaminase, an enzyme that acts on histamine and neutralizes it.
Mast cells	Mast cells contain granules that contain the chemical mediators of inflammation, histamine, heparin and serotonin. They are able to send signals to the system immune in the presence of a bacteria or parasite and trigger an immediate response
Platelets	They also called thrombocytes, are small cells devoid of a nucleus. Platelets play an essential role in the coagulation process. They suppress bleeding when a breach appears in the lesion tissue.
Fibroblasts	These cells produce collagen; plays an important role in healing.

3.3. Coagulation and anticoagulation activity

3.3.1. Blood clotting

Blood clotting is a complex process in which liquid blood forms a gel. It results from hydrolysis of protein metabolism leading to the chain activation of plasma coagulation factors, which are distributed in the form of inactive precursors (zymogens) **(Palta et al., 2014).**

This phenomenon is found at the site of the vascular difference because these responses, despite self-reinforcement, are limited and controlled by different systems of physiological inhibitors. The balance between coagulation and restrictive methods is fundamental, rupture causing a risk of bleeding (factor deficiency) or thrombosis (in excess of activated factors or lack of inhibitors) **(Palta et al., 2014).**

3.3.2. Route of coagulation

3.3.2.1. Internal route

In this coagulation process, all the components necessary for coagulation are present in the plasma without external intake. This pathway is initiated by activating factor XII (Hageman) by this contact with electron activating basic epithelium (collagen, sulfate, glycosaminoglycans), an activation, which then leads to the activation of kallikrein, which can reactivate F XII. Activated F XII promotes the transformation of the zymogenic form of factor XI into an activated protein acid form, which in turn activates factor IX. The latter binds to the surface of platelet anion phospholipids (F3P) with calcium ions and forms, in the presence of its coefficient, coagulation factor VIII, which is responsible for the activation of factor X (the Stuart coefficient). The latter, together with its co-effects, coagulation factor V (proaccelerin), platelet phospholipids, and the calcium ion prothrombinase complex that stimulates the conversion of prothrombin (coagulation II) to thrombin **(Figure 10) (Grover and Mackman, 2019).**

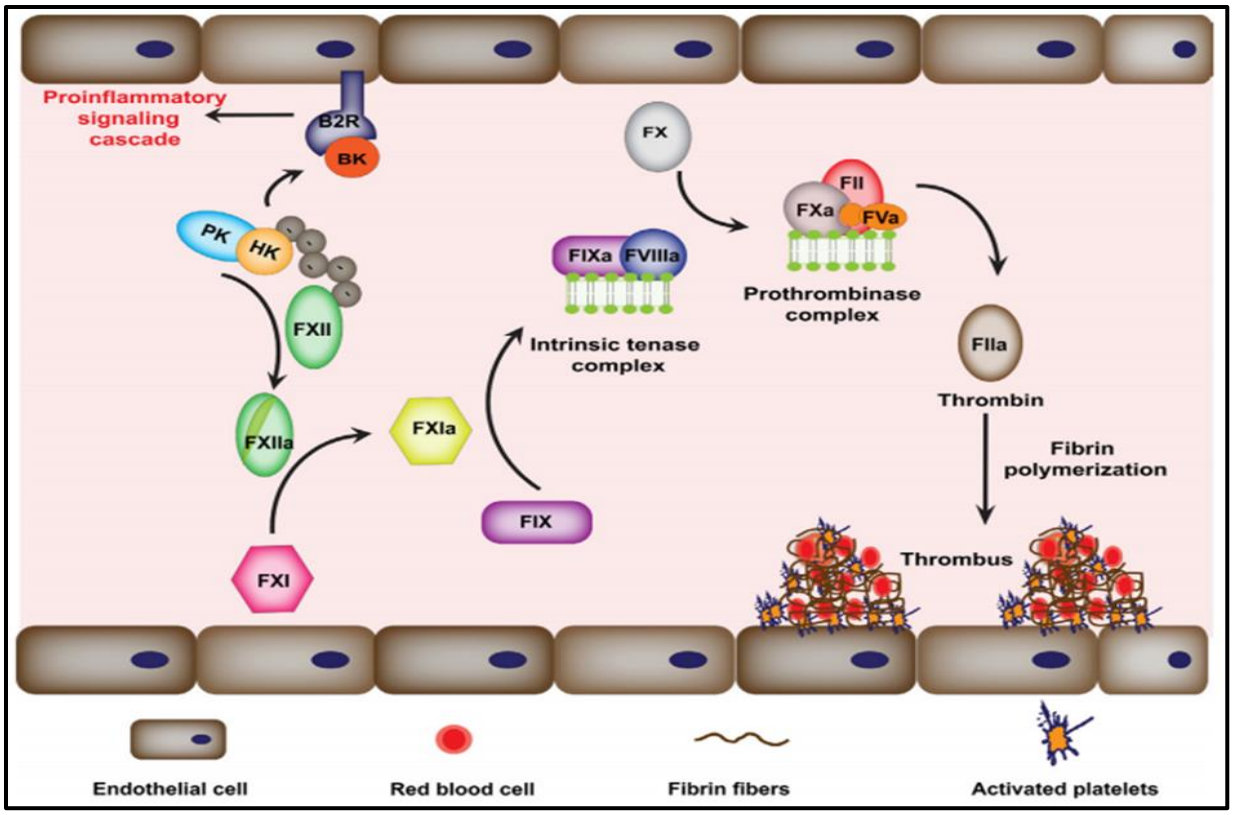

Figure 10: The intrinsic clotting pathway. Intrinsic pathway (contact-clotting pathway) is initiated when artificial surfaces are exposed to blood. Trimolecular assembly of FXII, high molecular weight kininogen (HK) and PK bound to the surface of the foreign material generates FXIIa, and leaves PK to generate active kallikrein. Active kallikrein produces BK from HK and activates FXII. Binding of BK to kinin B2 receptor (B2R) activates proinflammatory-signaling cascade. FXIIa activates FXI to FXIa, which in turn activates FIX. FIXa: FVIIa complex (intrinsic tenase complex) will generate prothrombinase complex leading to thrombin generation and fibrin clot **(Kalathottukaren and Kizhakkedathu, 2018).**

3.3.2.2. External route

The external route is the simplest and fastest route than the internal route; it involves a limited number of factors. This pathway is activated by non-plasma components, which is a tissue component, membrane glycoprotein expressed on the surface of the epithelial cells and submucosal cells. During vascular rupture, the tissue element is exposed to plasma, allowing the interaction with coagulation factor VII (pro converting) to form a substrate of enzyme complex (Tissue Factor-FVII). This complex is responsible for activating factor X and factor IX and consequently from prothrombin to thrombin. The blood-clotting cascade and its regulation are illustrated in **(Figure 11**).

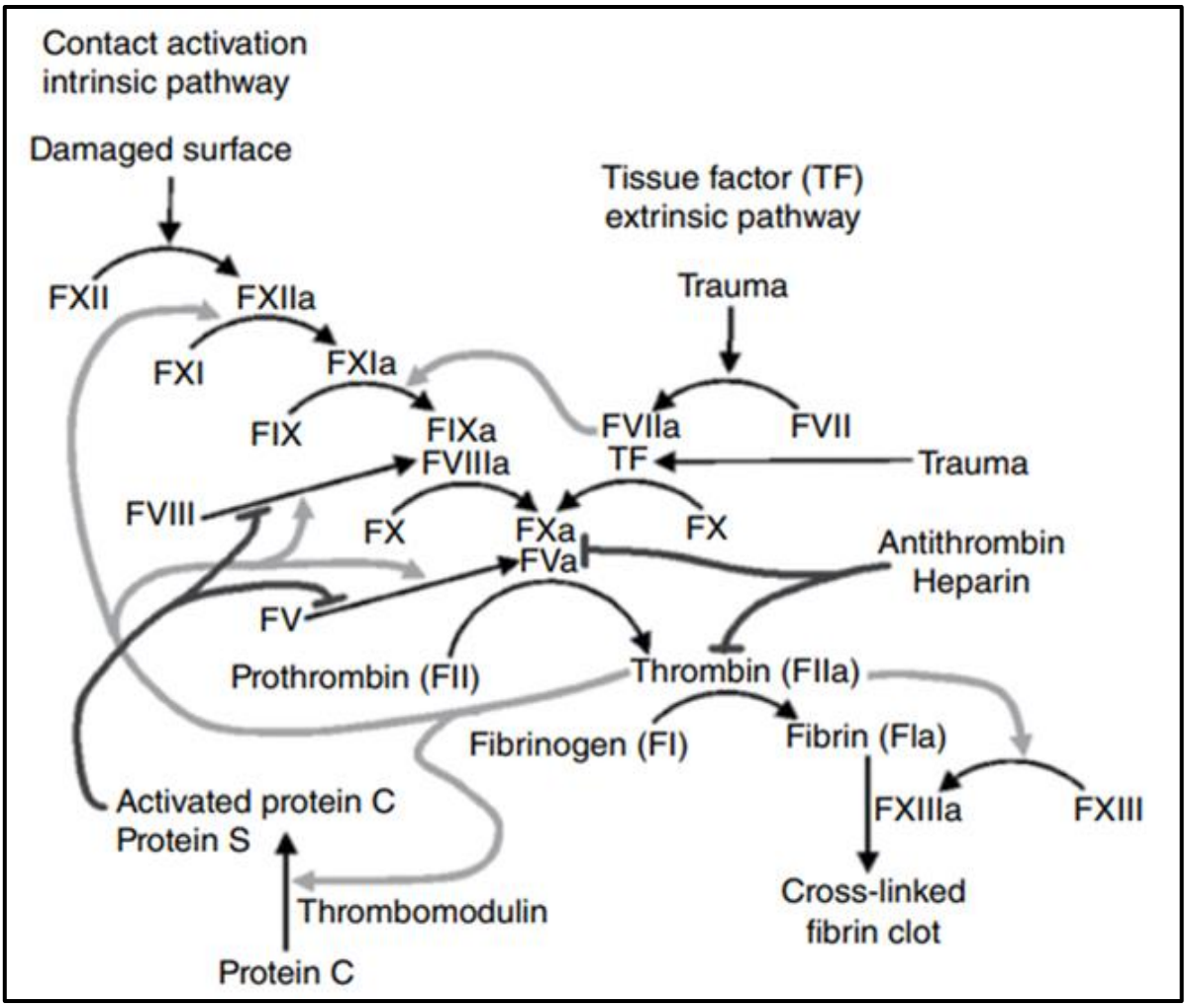

Figure 11: The blood-clotting cascade and its regulation. TF, tissue factor; FV(a), factor V(a); FVII(a), factor VII(a); FVIII(a), factor VIII(a); FIX(a), factor IX(a); FX(a), factor X(a); FXI(a), factor XI(a); FXII(a), factor XII(a); FXIII(a), factor XIII(a). Lines with arrows indicate stimulation; lines ending in a T inhibition **(Schreuder and Matter, 2020).**

Both pathway-induced thrombin induces the conversion of fibrinogen to fibrin monomers, which combine through hydrogen bonds to form an unstable fibrin network, in which factor XIIIa (a fibrin-blocking factor) previously activated by thrombin is involved in the coagulation of fiber in the relationship between different fibrin molecules **(Chaudhry et al., 2018).** The extrinsic clotting pathway is shown in **Figure 12**.

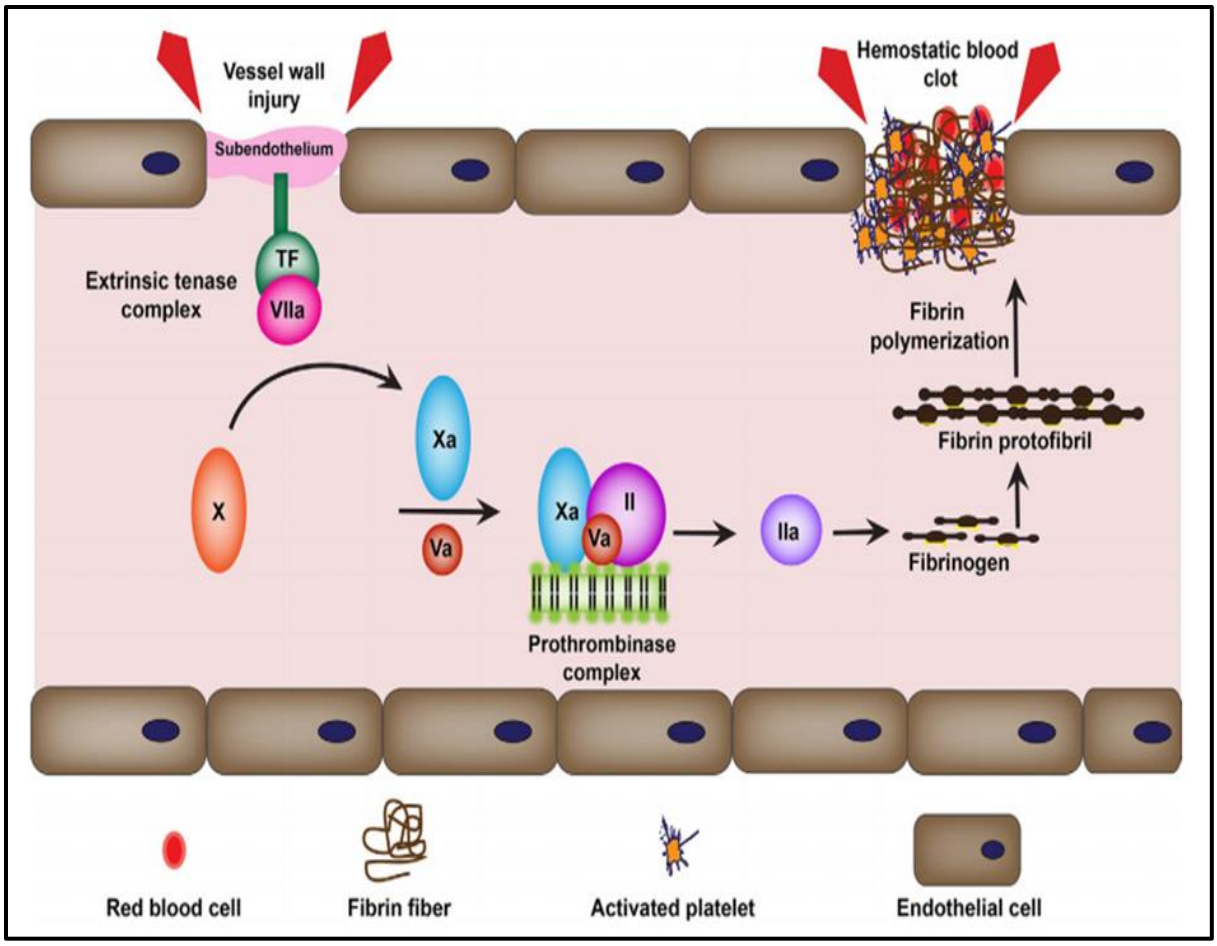

Figure 12: The extrinsic clotting pathway. Upon injury, TF present on the subendothelium is exposed to blood. TF binds to FVIIa and forms the extrinsic tenase complex (TF: FVIIa). The extrinsic tenase complex activates FX into FXa. FXa will then bind to cofactor FVa to form the prothrombinase complex. The prothrombinase complex will convert prothrombin into thrombin. Thrombin polymerizes fibrin to form the stable hemostatic blood clot **(Kalathottukaren and Kizhakkedathu, 2018).**

3.3.3. Coagulation tests

A few simple coagulation tests were used to identify hemorrhagic diathesis in plasma. In the case of a reduced Rapid test, F VII (exogenous system) or the cascade downstream of FX is altered or even disrupted by vitamin K antagonists, and the active partial thromboplastin time (APTT) is added to the plasma citrate (excluding calcium) cephalins and kaolin (a substitute for contact activation), and measure the corresponding coagulation time. If it is long-lasting, the deficiency is related to endogenous activation or return to the common terminal part of FX **(Wang et al., 2019).**

3.3.4. Thrombosis

Thrombosis is the formation of a blood clot (or thrombus) that forms in an artery or vein, so two types of thrombosis differ in risk factors, pathophysiological mechanisms, and clinical manifestations: arterial thrombosis and venous thrombosis.

The consequences of thrombosis are many such as pulmonary embolism, stroke (CVA) in the brain, and it can also lead to a heart attack **(Grover and Mackman, 2019).**

3.3.4.1. Arterial thrombosis

Arterial thrombosis is an embolus or white thrombosis consisting of platelets connected to a fibrous network, and it usually forms after erosion or rupture of atherosclerotic lesions associated with the development of atherosclerosis; this thrombosis is known as atherothrombosis **(Schreuder and Matter, 2020).** Atherothrombosis is currently considered the leading cause of death worldwide; it causes dangerous clinical complications grouped into three categories: acute coronary syndrome, stroke, and acute ischemia of the lower extremities **(Grover and Mackman, 2019).**

Before describing the physiological mechanism that contributes to the appearance of arterial thrombosis, the anatomy of the artery wall is illustrated in **(Figure 12).** The arterial wall consists of three tunics, from the inside to the outside:

- The intima consists of a continuous single cell layer of endothelial cells separated from the sub-endothelium by the basement membrane of the collagen and acts as a molecular "sieve", endothelial cells are antithrombotic. The intimate is separated from the means by means of the inner elastic limiter **(Schreuder and Matter, 2020).**

- Media is more or less developed according to blood vessel type. It is rich in muscle fibers that allow vasoconstriction and in fibroblasts. It is separated from the adventitia by the outer elastic limiter.

- Adventitia will make the connection with other vascular tissue structures and contain neural endings **(Schreuder and Matter, 2020).**

- **Physiological Mechanism**

Atherosclerosis is defined by the World Health Organization (WHO) as "a variable association of changes in the intima of large and medium-sized arteries, consisting of local accumulation of lipids, complex carbohydrates, blood and blood products, fiber tissue and

calcium deposits; a whole is accompanied by modifications of media" **(Grover and Mackman, 2019).**

It is a multifaceted pathology due to the combination of unchanging risk factors such as age, male gender and genealogy, and modifiable environmental factors such as a diet rich in fat and sugar, inactivity, and smoking. Such living conditions favor the development of arterial hypertension, type II diabetes, dyslipidemias and obesity, pathologies that accelerate plaque formation and its complications **(Palta et al., 2014).**

It is considered the main generator of arterial thrombosis; it is a chronic inflammatory disease that affects the large and medium-sized arteries, which are primarily the coronary, peripheral, cerebral arteries, carotid arteries, and aorta. Arteriosclerosis generally has the same meaning but affects only small arteries and does not contain fat **(Belkheiri, 2010; Palta et al., 2014).**

It is mainly due to aging. Therefore, atherosclerosis causes plaque formation that develops inside the artery; these plaques can block the artery **(Figure 12).** Inflammatory and bloody phenomena escape the events, as atherosclerosis can quickly generate the formation of blood clots that will block the artery **(Schreuder and Matter, 2020).**

3.3.4.2.Venous thrombosis

Phlebitis or deep vein thrombosis refers to the formation of a blood clot (or thrombus) that blocks a blood vessel. It most often occurs in a vein in the legs, but it can occur in almost any vein in the body (arms, brain, digestive tract, kidneys, etc.). In addition, the superficial veins under the skin can also be affected by phlebitis, called superficial phlebitis **(Wolberg et al., 2015).**

- **Physiological mechanism**

Mechanism Phlebitis often begins in the veins of the legs at the level of small valves or valves that prevent the blood from flowing back **(Wolberg et al., 2015).** Once formed, the embolus can become large and block the entire length of the veins in a bone **(Figure 12).** Phlebitis is also known as venous thrombosis. Blood clots, or thrombi, will block the vein, completely or partially, and prevent blood from circulating normally in the vein. It should know that there are two types of phlebitis: superficial phlebitis, the blood clot that forms in a superficial vein; this often occurs in people who already have varicose veins and deep phlebitis. Embolus in the vein in the bone can separate, travel upward through the heart's veins, cross it,

and reach the arteries in the lungs; this is a pulmonary embolism or venous thromboembolism (VTE). Pulmonary embolism is the severity of phlebitis because if the emboli migrated into the pulmonary arteries are numerous and/or large and can cause suffocation in the patient **(Belkheiri, 2010; Wolberg et al., 2015).**

3.3.5. Treatment of thrombosis

There are two classes of antithrombotic pharmacological agents useful, anticoagulants and antiplatelet drugs **(Aubry et al., 2010).**

3.3.5.1. Antiplatelet drugs

Antiplatelet (aspirin, ticagrelor, clopidogrel, etc.) currently represents the standard treatment for arterial thrombosis, but anticoagulants are also given in combination with antidepressants and fibrinolytic drugs for acute coronary heart disease infarction **(Aubry et al., 2010).**

3.3.5.2. Anticoagulant drugs

Anticoagulants are the main treatment for venous thromboembolic disease. Many anticoagulants that act at different levels of coagulation cascade are used and are grouped into three classes, two classes of classical anticoagulants (heparin and antivitamin K) and the class of newer anticoagulants **(Gómez-Outes et al., 2012).**

- **Classical Anticoagulants**
  - **Heparins**

There are two types of heparins that can be used either with intravenous or subcutaneous, unfractionated heparin (UFH) and low molecular weight heparins (LMWH). Fractionated heparin consists of a heterogeneous mixture of sulfate-polysaccharide chains of various sizes and structures. The molecular weights range from 5 000 to 35 000 Daltons, while low molecular weight heparins are derived from the depolymerization of UFH polysaccharide chains by chemical or enzymatic processes; their molecular weights vary from 3 000 to 5 000 Daltons. UFH and LMWHs form a complex with the physiological anticoagulant antithrombin III, potentiating its effect on the inactivation of various coagulation factors. In addition, the UFH antithrombin III complex inactivates most factors Xa and thrombin (factor IIa), but to a lesser extent factors IXa, XIa, and XIIa, while the LMWH antithrombin III complex mainly inhibits factor Xa and, to a lesser extent degree thrombin **(Gómez-Outes et al., 2012).**

- **Anti-Vitamin K**

Vitamin K is an essential element in the synthesis in the liver of four coagulation factors, prothrombin II, proconvertin VII, Stuart factor X, and antihemophilic factor B (factor IX). In addition, it is involved in the carboxylation of glutamic acid molecules at the -N terminus of the glycoprotein chain by all these factors. This carboxylation is necessary for the biological activity and binding of these factors to the platelet phospholipid surfaces **(Gómez-Outes et al., 2012).**

- **New anticoagulants**

New anticoagulants are currently being used in conjunction with conventional anticoagulants. These anticoagulants are divided into two categories according to their mode of action:

- **Indirect inhibitors:** acting by potentiating the activity of antithrombin III, and among them, fondaparinux and hydraporin, which are indirect and selective synthetic inhibitors of factor Xa and consist of 5 saccharide units capable of altering conformation by increasing the natural inhibitory activity of antithrombin I III on factor Xa **(Gómez-Outes et al., 2012).**
- **Direct inhibitors**: acting directly on factor Xa or thrombin and among those, DX-9065a, Hirudin, Argatroban, etc. DX-9065a is a synthetic derivative of propanoic acid that directly inhibits the free factor Xa and factor Xa in the prothrombinase complex, while Hirudin is a 65 amino acid peptide and it directly and irreversibly prevents thrombin. Argatroban is a synthetic derivative of carboxylic acid that directly inhibits thrombin **(Gómez-Outes et al., 2012).**

Part 2:
Experimental Part

Chapter 1: Material and Methods

1. Material and methods

The experimental part of this study was carried out at the campus level of the educational laboratories of the University Abbes Laghrour, Khenchela, and the Laboratory of Arid and Oasis Cultures, Institute of Arid Regions (IRA) Medenine, Tunisia. The anticoagulant activity was performed in the central laboratory at Ahmed Ben Bella Hospital, Khenchela. Chromatography analysis (LC-MS) of the phenolic extracts of OMW was carried out in the central laboratory of the Institute of Arid Regions Medenine, Tunisia.

1.1. Material

1.1.1. Biological material

- **Origin of the olive mill wastewaters**

During the olive harvest season, fresh samples of OMW were taken in a modern cold extraction oil mill which the temperature does not exceed 15 ° C **(Annex 2)**, located in Baghai Wilaya of Khenchela in the northeast of Algeria (35°31'19" N, 7°6'52"E, 886m a.s.l), in January 2019. The climate of Khenchela is semi-arid. More precisely, according to the Köppen-Geiger classification, it is a dry and cold steppe climate. It is very harsh in winter with freezing temperatures, and in summer it is more moderate and dry with warm but tolerable temperatures. The studied OMW was from Abani and Zlitni olives **(Annex 1)** and their mixture. The samples were collected from the liquid effluent collecting basin immediately after the olive press, stored in clean closed glass bottles washed with the OMW to be analyzed, without any alteration or treatment. They are stored at room temperature for 12 months in the dark to conduct physicochemical and biological analyses. Each test was done three times. Before determining various physicochemical characteristics, the sample is filtered using a colander and centrifuged (3000 g/min at 4 ° C). Then, the sample is utilized without filtration or centrifugation to determine pH, DM, MM, VM, and TSS percent.

This study was conducted throughout five storage periods: (t0) immediately following sample collection, (tM1) one month later, (tM2) two months later, (tM6) six months later, and (tM12) twelve months later.

- **Blood samples**

Blood samples were taken at the central laboratory of Ahmed Ben Bella Hospital in Khenchela, Algeria. The blood from healthy donors is obtained on either acid citrate dextrose (ACD) or heparin.

1.1.1. Equipment and apparatus

The devices used are as follows:

-High Performance Liquid Chromatography coupled with Mass Spectrometry ((Shimadzu UFLC XR System (Kyoto, Japan);

-Rotary evaporator (HAHNSHIN SCIENTIFICCO);

-Spectrophotometer UV Visible (SPECORD210plus);

-Spectrophotometer (mindray BA-88A);

-pH meter (AdwaAD1000);

-Kjeldahl apparatus;

-Conductivity meter (inoLab wtw);

-Oven (memmert);

-Refrigerated centrifuge (SIGMA 2-16K);

-Bain-Marie (MEMMERT);

-Precision scale (Kalstein, E6028);

-Freeze dryer (CHRIST GAMMA 1-20);

-Muffle furnace (Nabertherm);

-BOD meter (VELP Scientifica);

-Thermostatically controlled enclosure (thermostat schrank, thermostat cabinet) (AQUALYTIC);

-COD meter (VELP Scientifica);

-Spectrophotometer DCO (HACH DR/4000U);

-Automatic coagulation analysis system (CoaDATA 4004);

-Ultrasonic bath model LM Cleaner 25E-MT (Branson Ultrasonics Corporation, Dambury, CT, EUA);

-Centrifuge (ROTOFIX 32A, Hettich ZENTRIFUGEN);

-Cuvettes Micro with Mixer 1.0×4.0 mm (REF, Germany);

-Mixer Micro 1.0×4.0 mm (E6028);

-Desiccator (DURAN);

-Hotplate Stirrer (Lab Tech);

-Vortex (VELP SCIENTIFICA Zx^3);

- Refrigerator -18 (LIEBHERR).

1.1.2. Products and reagents

DPPH, ABTS, aluminum chloride $AlCl_3$, mercuric sulfate, Mohr salt, Ferron, Boric acid, quercetin, gallic acid, vanillin, tannic acid, catechin, Trolox, BHT, Ascorbic acid,

potassium dichromate, bovine serum albumin BSA, potassium sulfate, ammonium persulfate, Folin-Ciocalteu reagent, sulfuric acid (H_2SO_4), CO_3NO_2, $NaNO_2$, $CuSO_4$, K_2SO_4, H_2O_2, Soda NaOH, selenium, hydrochloric acid, iron sulfate $FeSO_4$, salicylic acid, potassium persulfate, Tris-HCl buffer, Alsever solution, Iso-saline solution, phosphate-buffered saline solution, sodium oxalate (NaOX), calcium chloride (NaCl), sodium citrate, Heparin, $CaCl_2$, NaCl, phenolic compounds standards: (Quinic acid, Gallic acid, Protocatechuic acid, Catechin (+),Caffeic acid, Syringic acid, 1,3-di-o-caffeoyquinic acid, Epicatechin, P-coumaric acid, Rutin, Trans frolic acid, Hyperoside (quercetin-3-o-galactoside), Luteolin-7-o-glucoside, 3,4-di-o-caffeoyquinic acid, Naringin, Rosmarinic acid, 4,5-di-o-caffeoyquinic acid, Quercetrin (quercetin-3-o-rhamnoside), Apegenin-7-o-glucoside, O-coumaric acid, Salviolinic acid, Kampherol, Quercetin, Trans cinnamic, Naringenin, Apegenin, Luteolin, Cirsiliol, Cirsilineol, Acacetin, Chlorogenic acid).

1.1.3. Solvents

Sulfuric acid, methanol, ethanol, ethyle acetate, hexane, DMSO, chloridric acid.

1.1.4. Consumable

Laboratory glassware, filter paper, micropipettes, microfilter at 0.45 µm, matrass, porcelain capsules, NaOH pastilles, cellulose paper containing sodium sulfate, the kaolin partial thromboplastin time and quick time, citrate tube, heparin tube.

1.2. Methods

The different stages carried out in this study are the physicochemical characterization, the extraction of polyphenols and their quantitative and qualitative characterization, then the *in vitro* evaluation of the antioxidant, anti-inflammatory, and anticoagulant activities of OMW.

Physicochemical Characteristics

1.2.1. Physicochemical Characteristics

To evaluate the physicochemical properties of olive oil mill wastewaters (OMW), seventeen (17) criteria were studied.

1.2.1.1. Potential of Hydrogen (pH)

It assesses the degree of acidity or basicity of a solution by measuring the concentration of an aqueous proton solution, which is dictated by the amount of free hydrogen ions in the liquid. After calibrating the pH meter (AdwaAD1000) with pH buffer solutions 4, 7, and 10, the measuring electrode is extended in a beaker containing 30 mL of OMW, and the indicated pH is recorded **(Rodier, 1996).**

1.2.1.2. Electrical Conductivity (EC)

It is measured the samples filtered and centrifuged without being diluted. After rinsing the conductivity meter probe (inoLab wtw), it is immersed in a beaker containing 30 mL of OMW to be studied, taking care to submerge the electrodes thoroughly. The OMW is agitated to ensure that the ion concentration between the electrodes matches the ambient liquid and that any air bubbles are removed. Finally, the value is read while the measurement temperature is considered. The results are expressed in mS/cm **(Rodier, 1996).**

1.2.1.3. Lipid content

20 mL OMW is mixed with 30 mL n-hexane (3.30 mL), the mixture is vigorously mixed in a 100 mL separating funnel with a vortex type (VELP Scientifica ZX3), then centrifuged for 5 min at 3000 t/min in a centrifuge type (SIGMA 2-16K) **(De Marco et al., 2007).** The three-hexane phases are mixed, and the solvent is evaporated at 79 °C in a rotary evaporator (HAHNSHIN SCIENTIFICCO), dried in an oven at 100 ° C, and chilled in a desiccator for 30 minutes. The drying process is repeated until the desired content weight is achieved.

1.2.1.4. Water content (H_2O%)

The humidity of the samples is measured by drying in an oven type (Memmert), according to **(Gortzi et al., 2008).** First, 10 g of effluents are weighed on a precision scale (Kalstein) and dried at 103 °C for 24 hours. Allow cooling for 30 minutes in a desiccator after drying, then reweigh. The weight difference between the wet and dry samples determines water loss.

1.2.1.5. Dry Matter (DM)

The weight difference between the OMW (100%) and the content determines the dry matter rate (%H_2O) **(Gortzi et al., 2008).**

$$DM\% = 100\% - \% H_2O$$

1.2.1.6. Total Suspended Solids (TSS%)

The total suspended solids (TSS%) are determined by centrifuging fresh OMW at 4000 g for 15 minutes and drying the solid residue overnight at 105 °C **(Assas et al., 2000).** Then, filtration via glass filters, drying for 4 hours at 105 °C, and weighing is used to determine the suspended particles **(Assas et al., 2000).**

1.2.1.7. Organic Matter (OM)

The organic matter content was calculated by subtracting the dry weight of the OMW from its weight after calcination (mineral matter) **(Pauwels et al., 1992).**

1.2.1.8. Mineral Matter (MM)

It was carried out following the method of **(Pauwels et al., 1992),** with minor changes. First, the dried OMW sample is calcined for 24 hours in a muffle furnace (Nabertherm) at 550 °C. Then, after cooling in a desiccator, the ashes are weighed and constitute the mineral matter.

It was determined using the following formula:

$$C = (M_2 - M_0) / V \times 1000$$

With:

C: ash content (g/L),

M_0: empty capsule mass (g),

M_2: capsule mass with calcined dry matter (g),

V: volume of the test sample (mL).

1.2.1.9. Volatile Matter (VM)

It was calculated as the difference between the dry matter obtained and the ash residue after 24 hours of calcination at 550 ° C in a muffle furnace. It is measured in grams per liter (g/L) in relation to the dry weight **(Pauwels et al., 1992).**

1.2.1.10. Total Organic Carbon (TOC)

According to **AFNOR (1975),** it was calculated from the organic matter rate using the formula:

$$TOC\% = \% OM / 1.725$$

1.2.1.11. Total Kjeldahl Nitrogen (TKN)

It is calculated using the AFNOR standard **AFNOR (1975).** The nitrogen was determined using the Kjeldahl technique, with slight modifications.

In a matrass, combine 5 mL of raw OMW with a 7.5 g catalyst ($CuSO_4 + K_2SO_4$) and a trace of selenium. As an anti-foaming agent, it is combined 10 mL of H_2SO_4 with 10 mL of 30% oxygenated water (H_2O_2). As a shock absorber, it is placed some glass balls. After that, it

is heated to 100 ° C for a few minutes to prevent the foam from spilling, and then continue heating until the foam evaporates and the contents are burned. Then, it is raised the mineralization temperature to 400 ° C until a clear and limpid green coloring appears, and it is continued heating for 30 minutes before allowing it cool. In an automated distillation device, soda (32%) and distilled water are distilled. The distilled ammonia was trapped in an Erlenmeyer flask containing 20 mL of boric acid 4 percent, and the pH of the boric acid was immediately titrated with sulfuric acid (H_2SO_4) (N/50) up to the original pH of the boric acid. The nitrogen level was determined using the following formula:

$$\%N_2\ (g) = (V_1 \times 0.014 \times 100 \times N) / V_0$$

With:

N: normality of the sulfuric acid N/50.

V_0: sample volume in mL (5 mL).

V_1: volume in mL of the sulfuric acid solution used for the titration.

1.2.1.12. Carbon to Nitrogen mass ratio (C/N)

It is determined according to the method of **(AFNOR, 1975).** The carbon to nitrogen ratio indicates an organic product's potential to decompose more or less fast in soil.

C/N > 25: Microorganisms will draw on soil reserves instead of releasing them.

C/N < 25: Microorganisms will release excess nitrogen available to plants.

1.2.1.13. Biological Oxygen Demand (BOD_5)

The biological oxygen demand BOD_5 is measured using a BOD meter type (VELP Scientifica) in a thermostatically controlled enclosure type (AQUA LYTIC) (AFNOR T90-103). To decrease the harmful impact of the polyphenols on the microorganisms; the samples are diluted with 1/100e with distilled water, then two NaOH pastilles are added to the lid, which yields the amount of oxygen utilized by the bacteria for five days at 20 ° C and in the dark **(Rodier, 1996).**

1.2.1.14. Chemical Oxygen Demand (COD)

The chemical oxygen demand is the amount of oxygen used by compounds in water that are oxidizable under certain working circumstances. The chemical oxygen demand COD is measured on raw OMW samples using a COD meter type (VELP Scientifica) diluted to 1/1000th due to the high organic matter load, in accordance with the AFNOR NF T90-101 standard. The principle entails oxidizing the organic content in the OMW for two hours in an acid medium with an excess of potassium dichromate and the presence of potassium sulfate as a catalyst. This excess is dosed with Mohr salt in the presence of Ferron. To avoid chloride interaction with organic matter, they are complexed with mercuric sulfate **(Rodier, 1996).**

1.2.1.15. Total Oxidizable Matter (TOM)

It accounts for the majority of the biodegradable portion of the organic pollutants produced **(Rodier, 1996)**; it is calculated using the formula:

$$\mathbf{TOM\ (g/L) = (2\ BOD_5 + COD)\ /3}$$

1.2.1.16. Biodegradability Index (BI)

It is found by calculating the COD/BOD_5 ratio. It indicates the significance of pollutants with little or no biological degradation **(Rodier, 1996),** if:

BI > 6 hardly biodegradable substrates

3 < BI < 6 partially (or less easily) biodegradable substrates

BI < 3 Very easily biodegradable substrates.

1.2.1.17. BOD_5/COD

BOD_5/COD is an indicator that determines the degree of pollutants produced by liquid effluents. If:

BOD/COD > 0.5 effluent treatable by biological processes.

0.2 < BOD/COD < 0.6 feasibility of treatment using selected microbial strains.

BOD/COD < 0.2 biological treatment impossible **(Radoux, 1995).**

Quantification and Qualification of Polyphenols

1.2.2. Extraction and quantification of phenolic compounds

1.2.2.1. Polyphenol extraction method

Sample preparation is of utmost importance for any reliable analysis. The procedures for preparing samples for the analysis of phenolic compounds vary greatly depending on the nature of the compounds to be analyzed.

The protocol for extracting polyphenols from OMW is based on the method described by **Uysal et al. (2019)** with some adjustments, the main steps of which are summarized in the diagram shown in **Figure 13**. It is an extraction using maceration in a polar solvent (pure methanol) after a step of drying the OMW.

One (01) g of dried olive oil mill wastewater (OMW) was mixed with 10 mL of pure methanol, the mixture was vortexed for 15 min, and then let macerated in the dark overnight at 4 °C. After maceration, filtration using filter paper is carried out. The macerate was collected, and then OMW was added to 10 mL of pure methanol for a second time. The mixture is vortexed for 15 min and left to macerate for 1 hour. The two filtrates are combined and filtered through cellulose paper containing sodium sulfate. Then this solution was concentrated at 40 ° C in a rotary evaporator type (HAHNVAPOR), and the dry residue was stored in 3 mL of methanol in dark bottles at -18 ° C **(Gueboudji et al., 2022b) (Figure 13).**

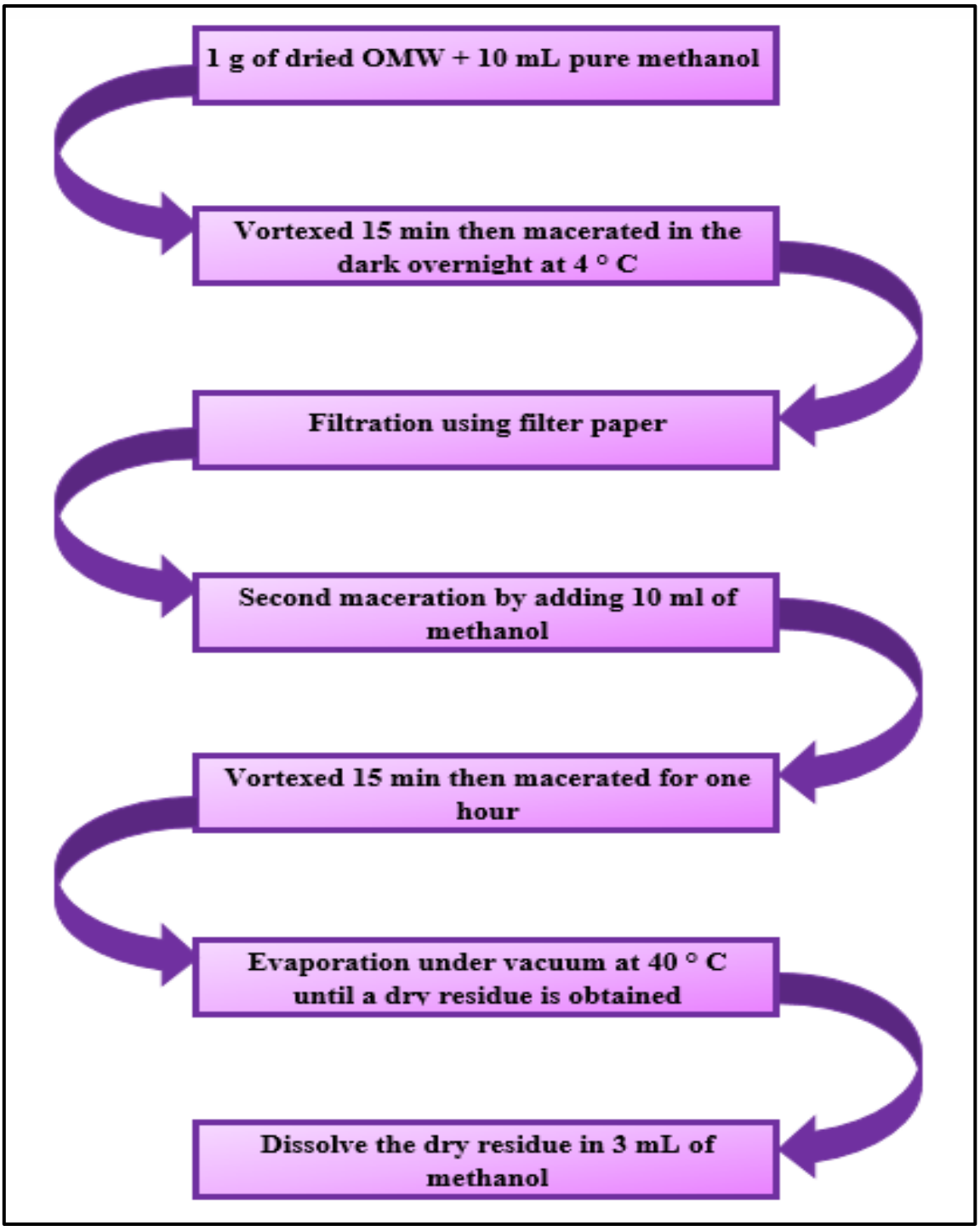

Figure 13: Diagram of the extraction of polyphenols from OMW

1.2.2.2. Quantitative and qualitative characterizations of polyphenols

1.2.2.2.1. Quantitative characterization of extracts

1.2.2.2.1.1. Total phenolic content (TPC)

The determination of total polyphenols by the method using the Folin-Ciocalteu reagent as described in 1965 by Singleton and Rossi. Since then, its use has grown widely to characterize plant extracts of more diverse origins.

Folin Ciocalteu's reagent is a yellow acid consisting of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). It is reduced during the oxidation of phenols to a mixture of blue oxides of tungsten and molybdenum. The coloration produced, whose maximum absorption at 765 nm is proportional to the quantity of polyphenols present in the plant extracts **(Muller et al., 2010).**

Polyphenols were determined by the reagent method of Folin Ciocalteau **(Müller et al., 2010).** In an alkaline medium, polyphenols reduce Folin Ciocalteau's reagent (FCR) to tungsten and molybdenum oxide of blue color. The intensity of this blue color reveals the level of total polyphenols in the mixture. Taking 125 µL of the extract diluted 100 times is put in the presence of 500 µL of distilled water, and 125 µL of the FCR. After stirring and standing for 3 min, 1250 µL of a 7% CO_3Na_2 solution was added to the mixture. The volume of the mixture was adjusted to 3 mL with ultrapure water and then left in the dark at room temperature for 90 min. The absorbance reading was taken at 760 nm. The standard range was prepared with gallic acid at concentrations of 50, 100, 200, 300, 400, and 500 mg / mL **(Figure 14).** The polyphenol content is expressed in micrograms of gallic acid equivalent per milliliter (µg GAE/mL). All samples were analyzed in triplicate.

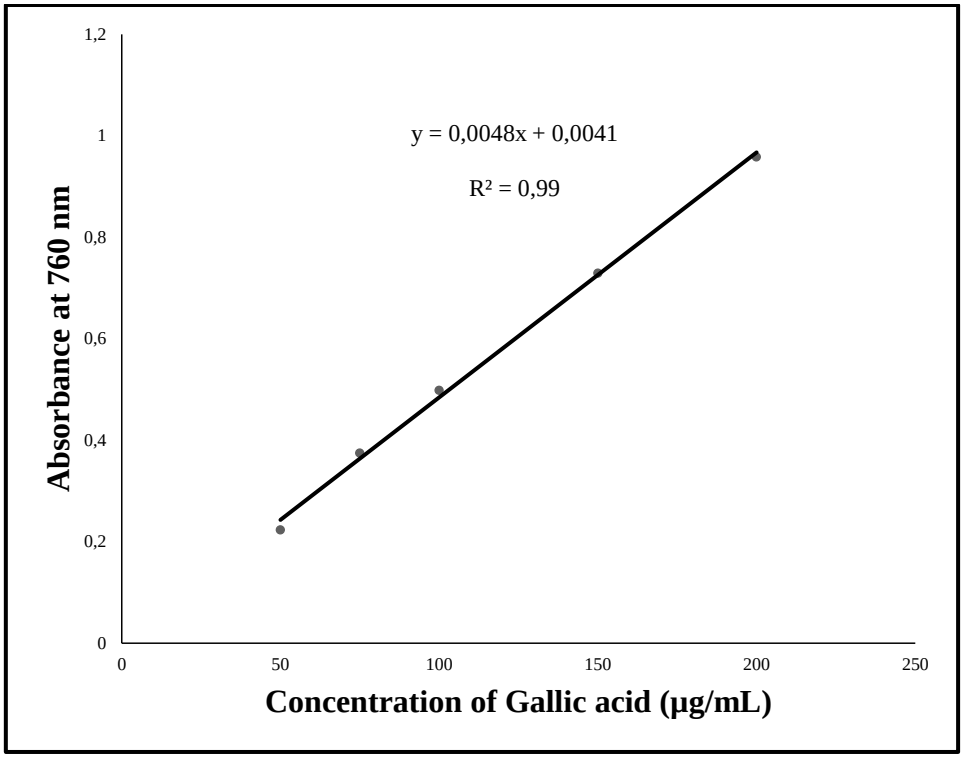

Figure 14: Gallic acid-based calibration curve for the determination of total polyphenols

1.2.2.2.1.2. Total flavonoid content (TFC)

The total flavonoid content of the extract studied is evaluated according to the method of **Topçu et al. (2007).** By means of a colorimetric test using aluminum chloride as a specific reagent. The principle of this method is based on the formation of a complex between flavonoids and aluminum chlorides. The presence of the free hydroxyl (OH) group in the C3 or C5 position in the flavonoids causes the formation of flavonoids-aluminum complexes, which by chelation of the Al^{+3} ion gives a yellowish coloration measurable at 448 nm. The yellow coloration produced is proportional to the amount of flavonoids present in the extract. To a dose of 250 µL of the extract diluted 100 times with methanol, 75 µL of a 5% $NaNO_2$ solution is added. After 6 min of incubation at room temperature, 150 µL of an aluminum chloride solution $AlCl_3$ was added to the mixture. After 5 min of incubation at room temperature, 500 µL of sodium hydroxide was added to the mixture, and then the volume was adjusted to 2500 µL with distilled water. The absorbance reading was taken at 510 nm. A standard range based on quercetin was prepared at concentrations of 50, 100, 200, 300, 400, and 500 µg / mL **(Figure 15).** The flavonoid content is expressed in micrograms of quercetin equivalent per milliliter (µg EQ/mL). All samples were analyzed in triplicate.

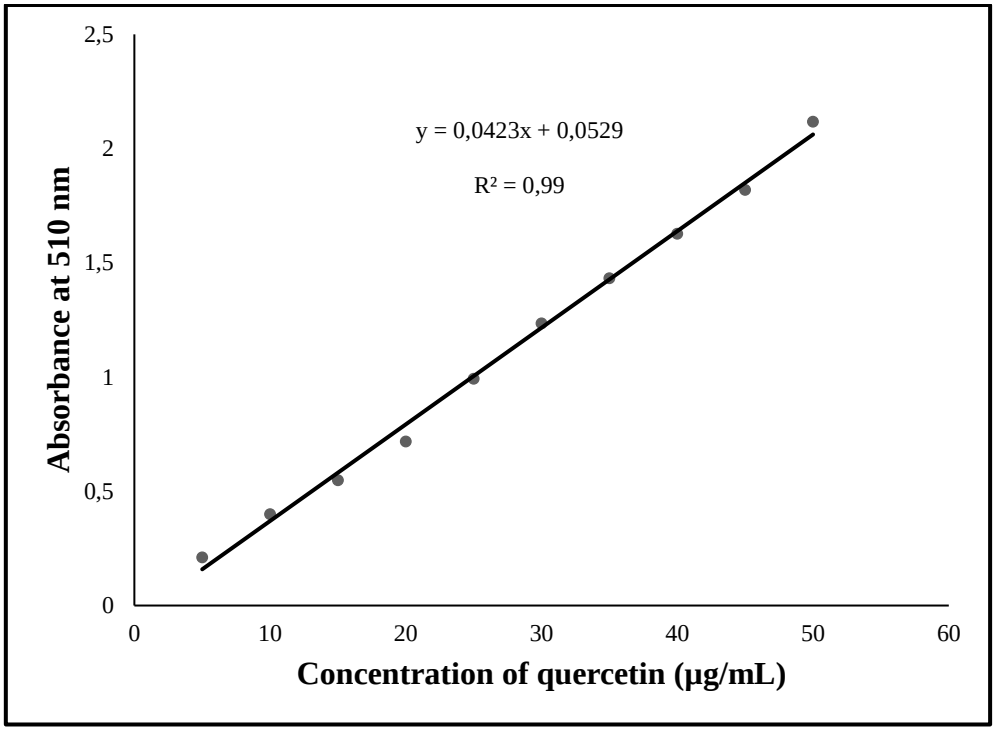

Figure 15: Quercetin-based calibration curve for the determination of flavonoids

1.2.2.2.1.3. Condensed tannin content (CTC)

Condensed tannin content is measured in the presence of concentrated sulfuric acid. CTC depolymerize and, by reaction with vanillin, are transformed into anthocyanidins of specific red color, measurable by spectrophotometry at 500 nm **(Hagerman, 2002).**

A volume of 0.5 mL intake of the suitably diluted extract is mixed with 2 mL of 1% vanillin and then added with 2 mL of concentrated sulfuric acid. After homogenization, the mixture is incubated at room temperature. Absorbance is measured at 500 nm against a blank containing pure methanol.

The content of condensed tannins is determined by referring to a standard range of tannic acid 3.12, 6.25, 12.5, 25, 50, 100, 200 µg / mL **(Figure 16).** The condensed tannin content is expressed in micrograms of tannic acid equivalent per milliliter (µg TAE / mL).

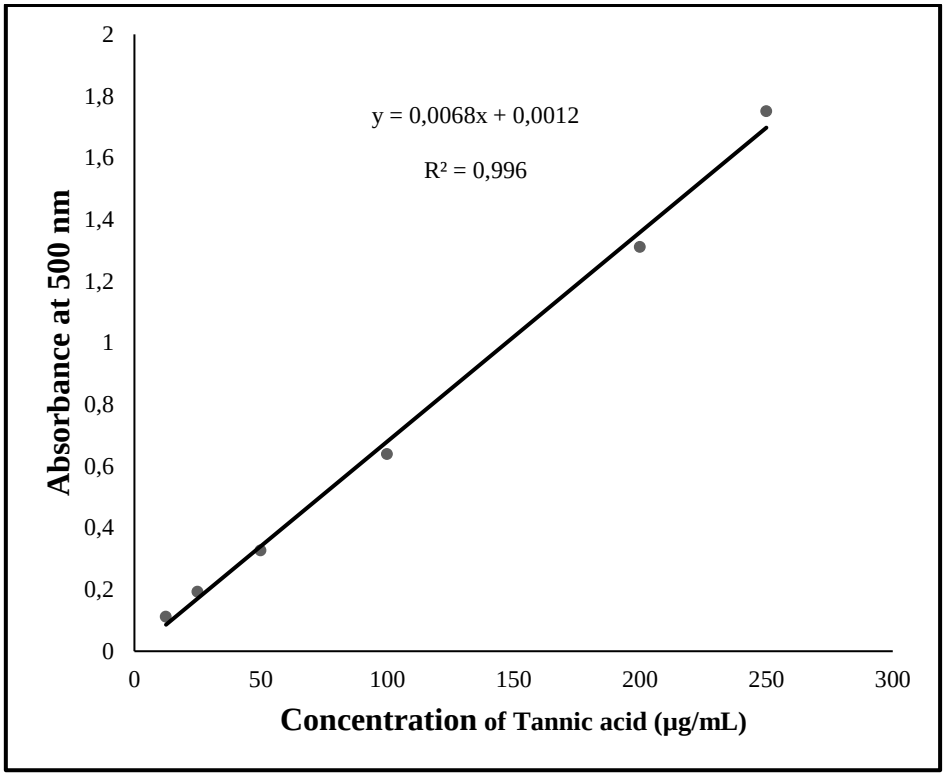

Figure 16: Tannic acid-based calibration curve for the determination of Condensed tannins

1.2.2.2.1.4. Total tannin content (TTC)

Vanillin reacts with free flavan3-ols and the terminal units of proanthocyanidins, giving a red color whose intensity is proportional to the levels of flavonols present in the medium and which exhibits maximum absorption at 500 nm wavelength.

In an Erlenmeyer flask, 1 mL of the extract is added to 5 mL of the analysis reagent (2.5 mL of the 1% vanillin solution mixed with 2.5 mL of the 8% HCl solution (8 mL HCl made up to 100 mL with methanol), the mixture is stirred vigorously. After 1 min, 5 mL of 4% HCl solution are added. The Erlenmeyer flask is then introduced into a water bath at 30 ° C and left for 20 min. Absorbance is read at 500 nm, respecting the time interval of one minute. Pure catechuic acid (1 mg/mL) was used as standard with different concentrations **(Figure 17)**. Blank consists of all reagents except the extract. The content is expressed in micrograms of catechuic acid equivalent per milliliter (µg CAE / mL) **(Hagerman, 2002).**

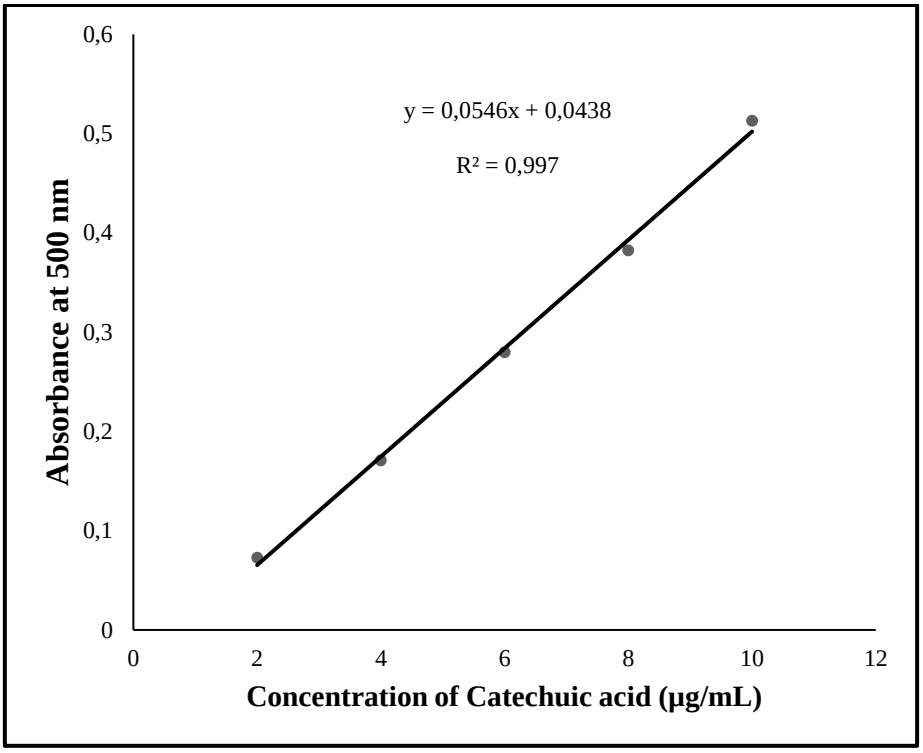

Figure 17: Catechuic acid-based calibration curve for the determination of total tannins

1.2.2.2.2. Qualitative characterization by High-performance liquid chromatography-mass spectrometry (LC-MS)

It was determined according to the method described by **Emwas et al. (2019).** Briefly, each crude phenolic extract was dissolved in 0.5 mL of the methanol. Before use, the solvents are filtered through a 0.45 µm membrane filter and degassed for 15 min in an ultrasonic bath model LM Cleaner 25E-MT (Branson Ultrasonics Corporation, Danbury, CT, EUA).

Figure 18 represents the chromatographic system used. The analysis of phenolic compounds of OMW was carried out on a Shimadzu UFLC XR system (Kyoto, Japan), equipped with a SIL-20AXR autosampler, a CTO-20 AC column oven, an LC- binary pump. 20ADXR and a 2020 quadrupole detection system. This instrument was equipped with a Discovery BIO Wide Pore C18 5 µm column (S250 × 4.0 mm id). The column temperature was set at 40 ° C, and the injection volume was 5 µL with a flow rate of 0.5 mL/min. Water + 0.1% FA and MeOH + 0.1% FA were used as mobile phases A and B, respectively. Analysis was performed using a linear gradient programmed as follows: 0.01-14 min, from 10% to 20% B; 14-27 min, 0 from 20% to 55% B; 27 to 37 min, 55% to 100% B; 37-45 min, 100% B; 45-50 min 10% B. The temperature of the dissolving line was 280 ° C, the nebulizing gas flow rate was 1.50 L/min, and the drying gas was set at 15.00 L/min. LC-ESI (-) MS [M-H] mass spectra

were acquired using Lab Solutions software. Phenolic compounds were identified by comparison with the retention time of standards for phenolic compounds.

Figure 18: Original photo of the chromatographic system used

- **Preparation of standards**

The standards are dissolved in methanol except for ellagic acid, which was dissolved in dimethyl sulfoxide then in methanol (final ratio 1/4). Thirty one (31) standards have been prepared, which are quinic acid, gallic acid, protocatechuic acid, catechin (+), caffeic acid, syringic acid, 1,3-di-o-caffeoyquinic acid, epicatechin, p-coumaric acid, rutin, trans frolic acid, hyperoside (quercetin-3-o-galactoside), luteolin-7-o-glucoside, 3,4-di-o-caffeoyquinic acid, naringin, rosmarinic acid, 4,5-di-o-caffeoyquinic acid, quercetrin (quercetin-3-o-rhamnoside), apegenin-7-o-glucoside, o-coumaric acid, salviolinic acid, kampherol, quercetin, trans cinnamic, naringenin, apegenin, luteolin, cirsiliol, cirsilineol, acacetin, and chlorogenic acid. The concentration of each standard is 330 µg/mL. A volume of 20 µL of the standard solution and the extract are injected onto the column of the HPLC.

Biological Activities

1.2.3. Biological activities of phenolic extracts of OMW

1.2.3.1. Antioxidant activity *in vitro*

Antioxidant power can only be measured indirectly from its effects. Most methods of measuring antioxidant activity are based on the use of systems generating a wide variety of radicals. These are mainly so-called "inhibition" methods in which a chemical species capable of generating free radicals is used with a substance capable of detecting these species. The sample whose antioxidant power is to be measured can inhibit the generation of radicals. In order to evaluate the antioxidant activity of the extracts of the polyphenols from OMW studied, four *in vitro* tests were used: DPPH, ABTS, FRAP, and H_2O_2. All used methods of the four-antioxidant tests of the extract were compared to synthetic antioxidants (ascorbic acid, Trolox, and BHT).

1.2.3.1.1. DPPH free radical-scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH •) **(Figure 19)** molecule is a stable free radical; the solution has a purple coloration and a characteristic absorption at 517 nm. Furthermore, when a solution of DPPH • is mixed with a substance that donates hydrogen atoms and antioxidant, the reduced form will be formed; this causes the change from purple coloration to yellow coloration characterized by a visible absorption band at 517 nm **(Ozgen et al., 2006).**

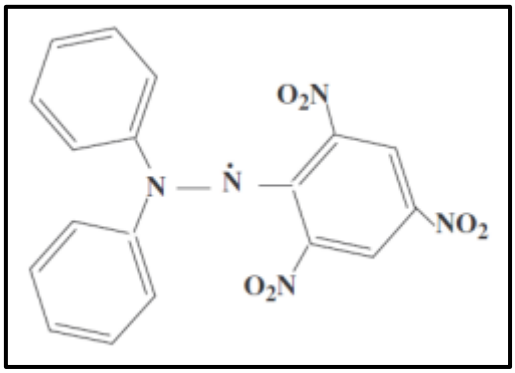

Figure 19: Chemical structure of the DPPH• radical

To measure this activity, a test sample of 0.5 mL of the extract at different concentrations is mixed with 0.5 mL of a solution of DPPH (0.2 mM in methanol). After vigorous shaking of the mixture, it is left to stand for 30 minutes in the dark. The same protocol was followed for the three standards (ascorbic acid, Trolox, and BHT).

The study of the variation of the anti-radical activity as a function of the concentration of the extracts makes it possible to determine the concentration that corresponds to 50%

inhibition (IC_{50}), the lower value of IC_{50}, which also corresponds to a low absorbance and the extract is powerful towards free radicals.

The absorbance is measured at 517 nm using a visible UV spectrophotometer, with reference to control without extract. For each concentration, the test is repeated three times. The anti-radical activity is estimated as a percentage of inhibition using the following formula:

$$\mathbf{IC50\%\ (DPPH\ \bullet) = [(A_0 - A_1) / A_0] \times 100}$$

With:

IC50% (DPPH •): percent inhibition;

A_0: absorbance of control;

A_1: absorbance of the test.

The effective concentration (EC_{50}), which is the concentration of extract, of the crude product or the standard antioxidant responsible for 50% inhibition of the DPPH $^{\bullet}$ radicals present in the reaction medium, is determined by a graph representing the percentage of inhibition of $DPPH^{\cdot}$ depending on the concentrations of the samples tested or the standard antioxidants used.

1.2.3.1.2. $ABTS^{+}$ free radical scavenging activity

This method is based on the ability of compounds to trap the radical-cationic $ABTS^{+}$, (ammonium salt of 2,2 '-azinobis (3-ethylbenzothiazothiazoline) -6-sulfonic acid) **(Figure 20),** by reacting with persulfate potassium ($K_2S_2O_8$), ABTS forms the radical $ABTS^{\cdot+}$, blue to green in color. The addition of an antioxidant causes the reduction of this radical and thus the discoloration of the reaction mixture. The discoloration of the radical, measured by spectrophotometry at 734 nm, is proportional to the concentration of antioxidants. This test is used in many laboratories to study antioxidant activity because the $ABTS^{\cdot+}$ radical reacts quickly with antioxidants and can be used over a wide pH range. The cationic radical $ABTS^{\cdot+}$ is formed by tearing an electron (e-) from a nitrogen atom of the ABTS. In the presence of an H^{+} donor antioxidant, the nitrogen atom concerned traps an H^{+}, leading to $ABTS^{+}$, leading to the solution's discoloration **(Ozgen et al., 2006)**.

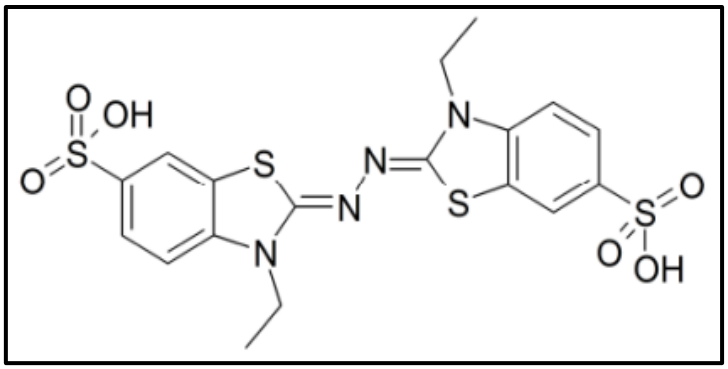

Figure 20: Chemical structure of the $ABTS^{\bullet+}$ radical

The anti-radical power against the cationic radical $ABTS^{\cdot+}$ was determined according to the method of **(Ozgen et al., 2006).** A solution of ABTS at 7 mM and 2.45 mM of potassium persulfate is prepared in 25 mL of distilled water; this solution is incubated in the dark for 12 h at room temperature. This period allows the formation of the radical $ABTS^{\cdot+}$. The solution thus obtained is blue-green and stable; it can be stored at room temperature **(Ozgen et al., 2006).**

A volume of 10 µL of the extract is added to 990 µL of ABTS solution. The discoloration relative to the control, containing $ABTS^{+}$ and the solvent (ethanol), is measured with a spectrophotometer at 734 nm after 30 min of incubation in the dark.The same protocol was followed for the three standards (ascorbic acid, Trolox, and BHT).

The antioxidant activity is expressed as percentage inhibition of the ABTS radical according to the following formula:

% Inhibition of ABTS = (Ac - As / Ac) × 100

Ac: Absorbance of the control;

As: Absorbance of the sample.

The effective concentration (EC_{50}), which is the concentration of extract, the crude product, or of the standard antioxidant responsible for 50% inhibition of the ABTS radicals present in the reaction medium, is determined by a graph representing the percentage of inhibition of ABTS depending on the concentrations of the samples tested or the standard antioxidants used.

1.2.3.1.3. Ferric reducing antioxidant power FRAP

This method is based on the ability of extracts to reduce ferric iron (Fe) to ferrous iron (Fe^{2+}), established by **Ozgen et al. (2006).** The mechanism is known to be an indicator of electron donor activity, characteristic of the antioxidant action of polyphenols. It consists of mixing 1 mL of each solution of extracts or the standard antioxidant (ascorbic acid, Trolox, and BHT) at different concentrations (0 - 50 µg / mL) with 1 mL of phosphate buffer (0.2 M, pH 6, 6) and 1 mL of a 1% solution of potassium ferricyanide [$K_3Fe(CN)_6$]). The mixture obtained is incubated at 50 ° C for 20 min, and then 1 mL of 10% trichloroacetic acid (CCl_3COOH) is added to stop the reaction. The mixture is centrifuged at 2000 g for 10 min. To 1 mL of the supernatant are added 1 mL of distilled water and 0.5 mL of 0.1% iron chloride ($FeCl_3$). The reaction medium is incubated at room temperature for 10 min. The absorbance of the reaction mixture is read at 700 nm against a blank, which contains all reagents except $FeCl_3$. The same protocol was followed for the three standards (ascorbic acid, Trolox, and BHT).

The effective concentration (EC_{50}), which is the concentration of extract, the crude product, or of the standard antioxidant responsible for 50% inhibition of FRAP radicals present in the reaction medium, is determined by a graph representing the percentage of inhibition of FRAP depending on the concentrations of the samples tested or the standard antioxidants used.

The increase in absorbance in the reaction medium indicates an increase in the reducing power of the extracts tested. The study of the variation of the reducing activity of iron as a function of the concentration of the extracts makes it possible to determine the concentration which corresponds to 50% inhibition (IC_{50}), the lower the value of IC_{50}, the more the extract has significant reducing power; the IC_{50} is an index used to compare and express the power of reducing capacities of bioactive substances.

The reducing power of iron in the samples tested compared to the standards used is calculated according to the following formula:

$$\textbf{Reducing power (\%)} = [(A_0 - A_1 / A_0)] \times 100$$

A_0: Absorbance of $FeCl_3$;

A_1: Absorbance of $FeCl_3$ in the presence of the extract or standard.

1.2.3.1.4. Scavenger activity on hydrogen peroxide H_2O_2

The ability of the phenolic extracts of OMW to scavenge hydrogen peroxide is measured according to the protocol reported by **Benkeblia (2005).** The principle consists of monitoring the decrease in the concentration of H_2O_2 by spectrophotometry.

The samples are prepared at different concentrations (from 0 to 0.4 g/L) in distilled water. Two hundred (200) µL of each solution is mixed with 200 µL of a $FeSO_4$ solution (5 mM). After homogenization by vortex, 200 µL of H_2O_2 (1%) are added, then the mixture is vortexed before being incubated at room temperature for 60 min. 1 mL of distilled water is then added. After stirring (vortex), the absorbance is read at 510 nm. The same protocol was followed for the three standards (ascorbic acid, Trolox, and BHT).

The percentage of anti-hydroxyl radical activity is calculated according to the equation:

$$\% H_2O_2 \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

A_0: Absorbance of control

A_1: Absorbance of the sample.

The effective concentration (EC_{50}), which is the concentration of extract, the crude product, or of the standard antioxidant responsible for 50% inhibition of H_2O_2 radicals present in the reaction medium, is determined by a graph representing the percentage of inhibition of H_2O_2 depending on the concentrations of the samples tested or the standard antioxidants used.

1.2.3.2. Anti-inflammatory activity *in vitro*

The evaluation of the *in vitro* anti-inflammatory activity of phenolic extract of OMW was performed using two tests: Inhibition of protein denaturation (IPD) and Membrane stabilizing potential (MSP) **(Gueboudji et al., 2022a).**

1.2.3.2.1. Inhibition of protein denaturation (IPD)

The *in vitro* anti-inflammatory activity of phenolic extract of OMW was performed using the protein denaturation inhibition method. It was done according to the method described by **Kandikattu et al. (2013).** The method consists of preparing four solutions.

The test solution (0.5 mL) composed of 0.45 mL of 5% aqueous bovine serum albumin (BSA) solution and 0.05 mL of phenolic extract with a concentration of 250 µg / mL.

The control test solution (0.5 mL) was composed of 0.45 mL of the aqueous solution of 5% bovine serum albumin (BSA) and 0.05 mL of distilled water. The product control solution (0.5 mL) was composed of 0.45 mL of distilled water and 0.05 mL of the aqueous extract with a concentration of 250 µg / mL.

The test standard solution (0.5 mL) consists of 0.45 mL of the 5% BSA aqueous solution and 0.05 mL of the diclofenac sodium standard solution with a concentration of 250 µg / mL.

All the above solutions were adjusted to pH 6.3 with HCl (1N) solution, and the samples were incubated at 37 ° C for 20 min. Then temperature was increased to keep the samples at 57 ° C for 3 min. After cooling the tubes, 2.5 mL of the phosphate buffered saline solution (PBS) (pH = 6.3) was added to the above solutions, the absorbance was read by the UV visible spectrophotometer at 416 nm, and the percent inhibition of protein denaturation was calculated as follows:

$$\%\text{Inhibition} = 100 - [(OD_{\text{test solution}} - OD_{\text{control}}) / OD_{\text{test control}}] \times 100$$

Where:

OD: optical density.

The control represents 100% of the denatured proteins, and the results are compared with diclofenac sodium (250 µg / mL).

The extract/drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage inhibition with respect to control against treatment concentration.

1.2.3.2.2. Membrane stabilizing potential (MSP)

It was measured according to the method described by **Murugan and Parimelazhagan (2014)** with some adjustment**.** The protocol is illustrated in **Figure 21**. Briefly, an equal volume of blood was drawn from a healthy human volunteer who had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for two weeks before blood collection and was mixed with an equal volume of sterilized Alsever solution. This blood solution was centrifuged at 3000 rpm for 10 min, the packed cells were separated, and then washed with iso-saline solution, and a 10% v/v suspension was prepared with iso-saline. The dosage mixture contains 1 mL of phosphate-buffered saline (PBS), 0.5 mL of 10% blood suspension, 0.5 mL of phenolic extract of OMW with different concentrations, and 2 mL of hypotonic saline. All

tests mixtures were incubated at 37 °C for 30 min and then centrifuged at 3000 rpm for 20 min. The supernatant was separated, and the hemoglobin content was estimated by spectrophotometric reading at 560 nm. Distilled water was used as a negative control. The positive control was diclofenac sodium at the final concentration in between. The percentage of stabilization or protection of the membrane was calculated using the following formula:

$$\% \text{ of protection} = [100 - (OD_{sample} - OD_{control})] \times 100$$

Where;

OD_{sample}: Optical Density of the hypotonic-buffered saline solution alone;

$OD_{control}$: Optical Density of the test sample (phenolic extracts and diclofenac) in the hypotonic medium.

The half-maximal inhibitory concentration (IC_{50}) was calculated from a graph defining inhibition against the different concentrations.

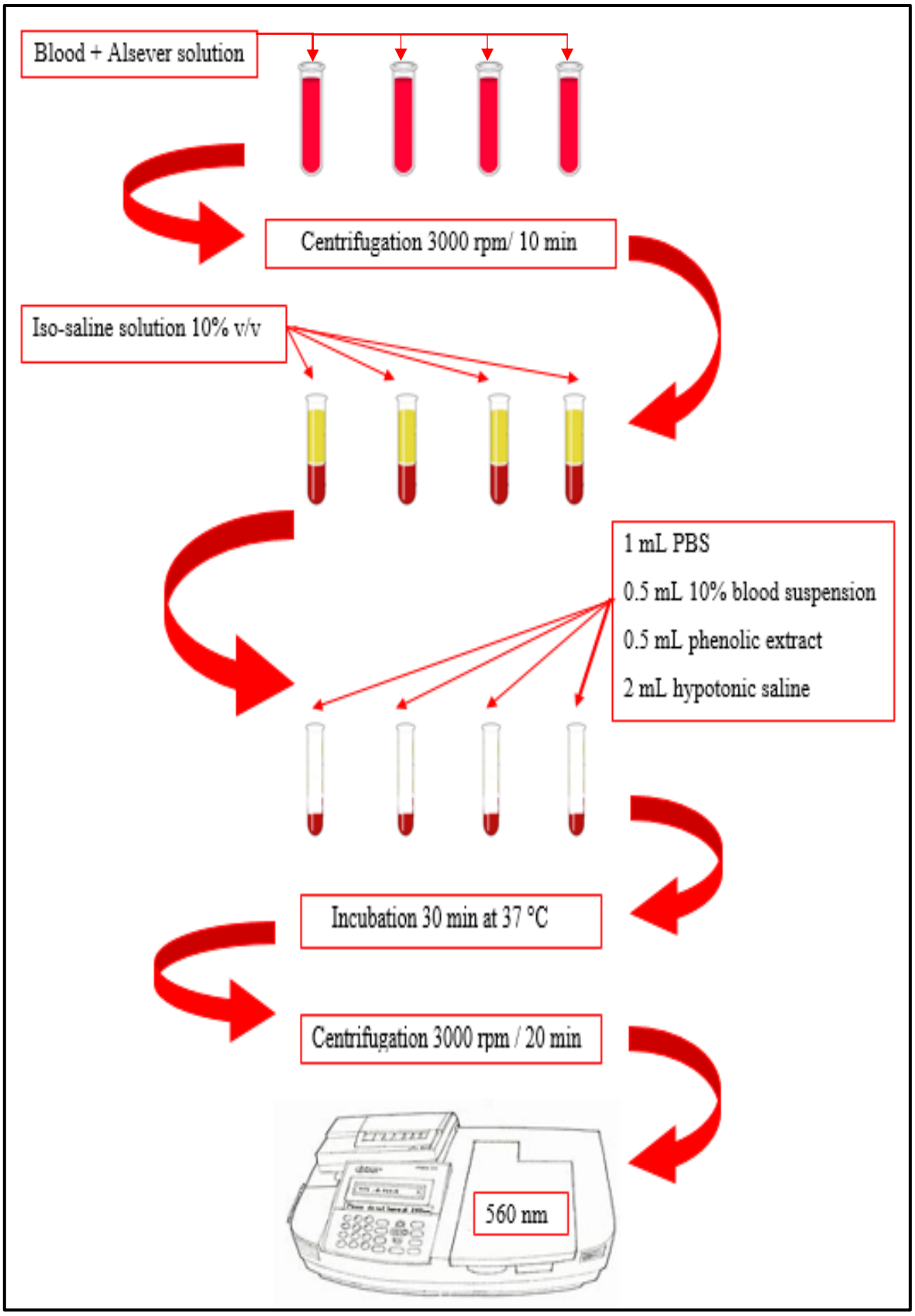

Figure 21: Protocol of membrane stabilizing potential test

1.2.3.3. Anticoagulant activity *in vitro*

The anticoagulant effect of phenolic extract of OMW was measured *in vitro* with respect to the exogenous and endogenous coagulation pathways. This activity was on a pool of normal plasmas depleted and using two (02) global and chronometric tests, the Quick prothrombin time (QT) or also called Prothrombin time (PT), and Kaolin clotting time (KCT) or also called activated partial thromboplastin time (APTT) **(Wang et al., 2015).**

- **Platelet plasma pool preparation (standard)**

The depleted plasma pool is a mixture of plasma depleted from healthy untreated adult volunteers, whose prothrombin time (PT) and Kaolin clotting time (KCT) are normal and comparable.

Each volunteer's blood was taken by venipuncture in a plastic tube on an anticoagulant solution of sodium citrate at 3.2%, at a rate of 1 volume for 3 volumes of blood. The blood is then centrifuged for 10 minutes at 3000 rpm to obtain a plasma poorly in platelets **(Figure 22).**

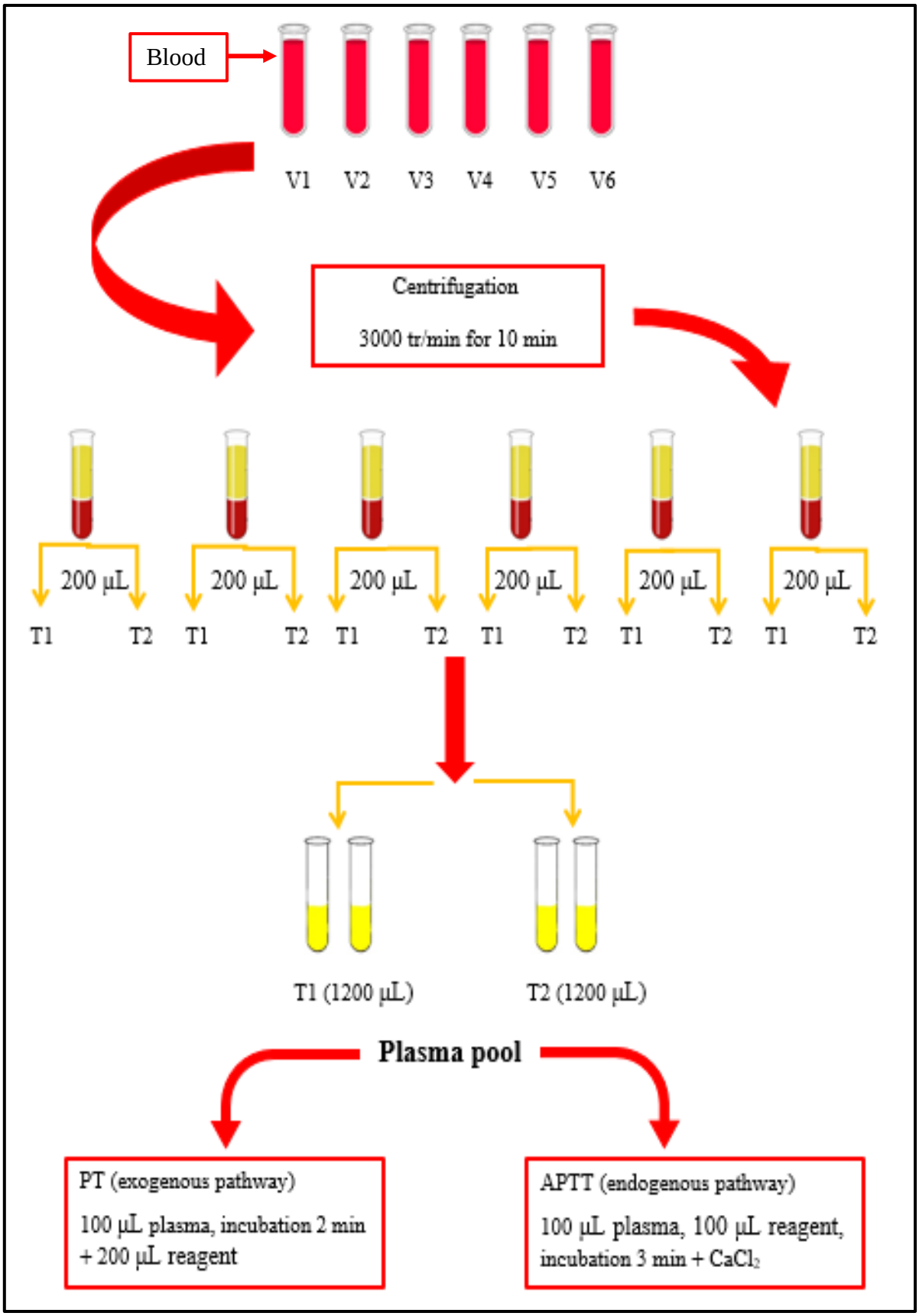

Figure 22: Protocol for Plasma Pool Preparation

1.2.3.3.1. Endogenous coagulation pathway (APTT)

This test consists of measuring the clotting time at 37 °C in platelet-poor plasma in the presence of a mixture of tissue factors, phospholipids (thromboplastin), and calcium. The time is measured until a clot forms. Compared to the negative control, the extended clotting time explains why OMW's polyphenols have an anticoagulant effect versus this coagulation pathway **(Wang et al., 2015).**

The mean normal KCT value in adults is 30 to 34 seconds habitually. Therefore, control time must always be provided to allow interpretation of the test.

Activating partial thromboplastin time (APTT) was determined according to **Wang et al. (2010).** A pool of platelet plasma consisting of a plasma mixture of 10 healthy untreated volunteers whose APTT and PT are normal. The activity of the phenolic extract is established on a volume of 100 µL which 90 µL of plasma is mixed with 10 µL of extract. After 15 min of incubation at 37 °C 100 µL, kaolin cephalin is added to the mixture, which is re-incubated for 3 min with agitation at 37 °C. The coagulation time is determined using a coagulometer by adding 100 µL of preheated calcium chloride (0.025 M). In parallel, positive control of Calciparine (unfractionated heparin) and a negative control test (substitution of the samples with a 0.9% NaCl solution are carried out under the same conditions **(Figure 23).**

An elongation of APTT in the presence of the polyphenols relative to the negative control indicates an anticoagulant effect at the level of this pathway. Clotting time was determined by an automatic coagulation analysis system (Coa DATA 4004). The results are expressed by the coagulation in second (s).

1.2.3.3.2. Exogenous coagulation pathway (PT)

This test consists of measuring the clotting time at 37 ° C of a poor plasma in platelet in the presence of a mixture of tissue factors and phospholipids (thromboplastin). Therefore, the factors of the exogenous pathway are activated, and then the time until the clot formation is measured. An extended coagulation time compared to that of the negative control explains that the polyphenols in vegetable waters exert an anticoagulant effect with respect to this coagulation pathway **(Wang et al., 2015).**

When an excess of thromboplastin or tissue factor is added to the plasma in the presence of calcium, normally the clot forms in 12 to 13 seconds, which represents Quick time,

the QT explores the factors of the exogenous pathway of coagulation: factor VII, factor X, factor V, factor II, fibrinogen **(Caquet, 2011).**

A longer clotting time compared to that of the negative control explains why the sample exerts an anticoagulant effect on this coagulation pathway.

Prothrombin time (PT) is determined according to the protocol described by **Wang et al. (2010).** This activity consists in measuring the coagulation time of citrated plasma in the presence of an excess of calcium thromboplastin, using platelet-poor plasma in the presence of calcium thromboplastin. 100 µL of platelet-poor plasma preheated for 2 min at 37 °C is mixed with the phenol extract prepared in dimethyl sulfoxide **(**DMSO) (90 and 10 µL by order). After 15 min of incubation at 37 °C, 200 µL of calcium thromboplastin preheated at least 15 min at 37 °C is added to the mixture. Coagulation time was determined by an automatic coagulation analysis system (CoaDATA 4004). The results are expressed by the coagulation in second (s) **(Figure 23).**

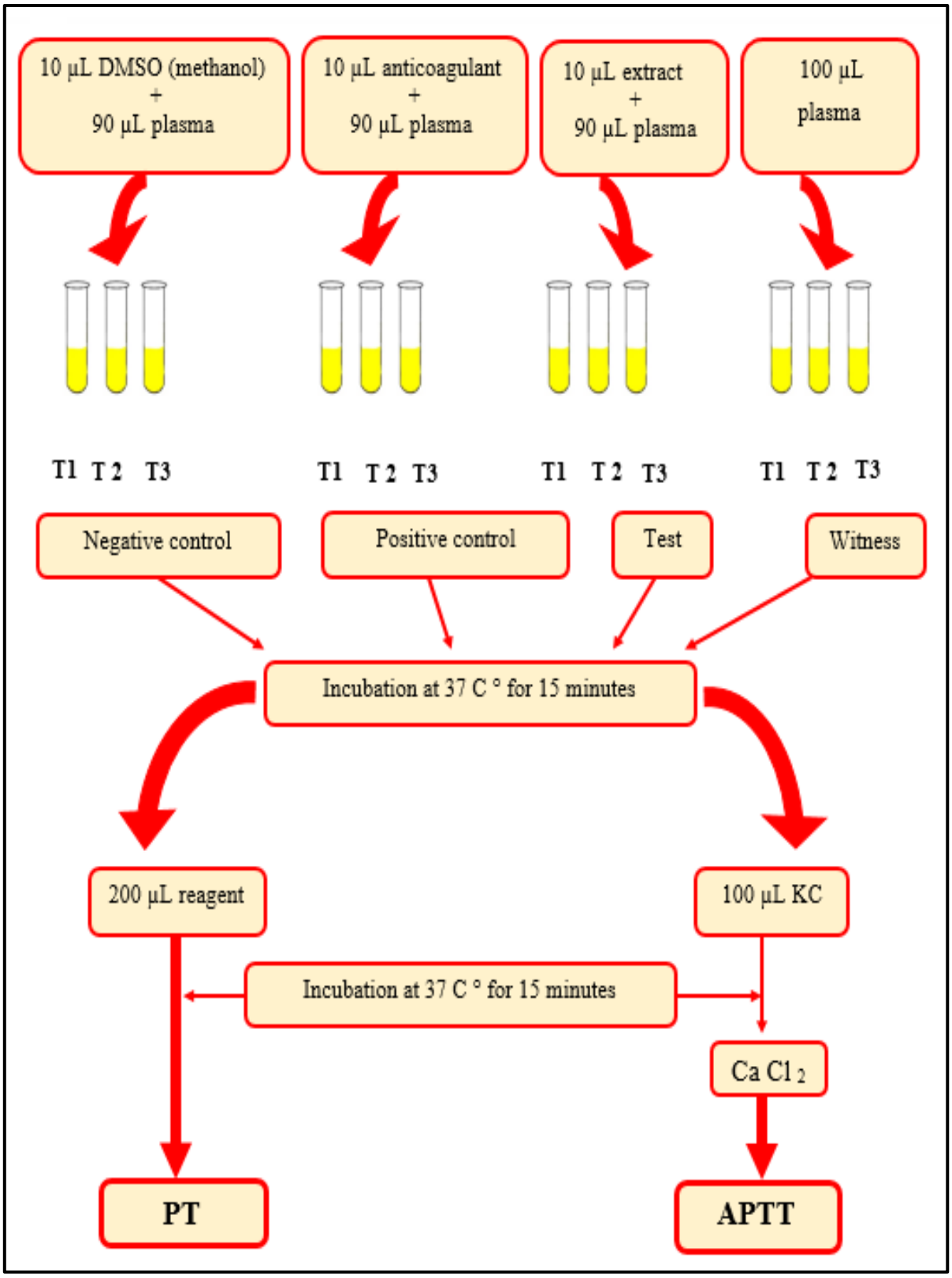

Figure 23: Protocol of the anticoagulant activity of the phenolic extract of OMW

1.2.4. Statistical processing of data

The curves and the histograms are plotted by Microsoft Excel 2013. To promote the work carried out, all results obtained are subjected to an analysis of variance followed by an analysis of the averages based on the least significant difference (LSD) (5%) in order to highlight the homogeneous groups. The statistical analyses of the physicochemical characteristics were carried out by the SPSS 25 software (version 2019), and those of the qualitative and quantitative characterizations as well as the biological activities were carried out by the SAS 9.1.3. The results of the tests carried out are expressed as the mean ± SD standard deviation. The IC_{50} values (50% inhibitory concentration) are calculated by the linear regression method from the curve [% inhibition = f (concentration)]. The difference between the control and the various tests is determined by the Student test for single comparisons or Analysis of variance (ANOVA) followed by the Dunnett / Tukey test for multiple comparisons and the determination of the significance rates. The values of $p \leq 0.05$ are considered statistically significant. The principal component analysis and the correlations of all the measured parameters are carried out by the software EXCEL STAT (version 2014).

Chapter 2: Results and Discussions

Physicochemical Characteristics

2. Results and Discussions

2.1. Physicochemical characterization of OMW

2.1.1. Two ways ANOVA analysis of the variety, time and their interaction on the measured parameters.

Results of analysis of variance (ANOVA) of the variety, time and their interaction effects on the measured parameters were presented in **(Table 3).**

Table 3: Results of variance analysis of the variety, time and their interaction effects on the measured parameters.

ANOVA	Variety (V)	Time (T)	V × T	Error
pH	***	***	***	0.000
EC	***	***	***	0.000
Lipids	***	***	***	0.001
TSS	***	***	**	0.001
Water	***	***	***	0.005
DM	***	***	***	1.508
MM	*	***	*	1.777
VM	***	***	ns	3.225
OM	ns	**	ns	9.535
TOC	ns	**	ns	3.204
TKN	***	***	*	0.017
C/N	***	***	ns	8.918
COD	***	***	*	432.822
BOD_5	***	***	ns	118.803
BI	ns	***	ns	0.092
TOM	***	***	*	142.061
BOD_5/COD	ns	***	ns	0.004

ns: not significant, *: significant, ***: very highly significant at 5%

Analysis of variance (ANOVA) **(Table 3)** revealed the presence of a very highly significant Variety effect on the measured parameters (pH, EC, Lipids, TSS, Water, DM, VM, TKN, C/N, COD, BOD_5, and TOM) with ($p < 0.001$), significant on MM with ($0.01 < p < 0.05$) and not significant on the parameters (OM, TOC, BI, and BOD_5/COD) with ($p > 0.05$). In addition, the time effect is very highly significant for all of the measured parameters (pH, EC,

Lipids, TSS, Water, DM, VM, TKN, C/N, COD, BOD_5, MM, BI, TOM, and BOD_5/COD) with ($p < 0.001$) and except for the two parameters OM, TOC showed a significant effect at ($0.001 < p < 0.01$).

A very high significance interaction effect (V × T) on the parameters measured (pH, EC, Lipids, Water and DM) with ($p < 0.001$). This interaction gave a significant effect to $0.001 < p < 0.01$ on TSS and to $0.01 < p < 0.05$ on the parameters (MM, TKN, COD, and TOM).

On the other hand, an insignificant effect was shown by the (V × T) interaction on the measured parameters (VM, OM, TOC, C/N, BI, and BOD_5/COD). The means of the measured parameter BI showed a very high significance with the duration of storage for the varieties Zlitni, Abani, and their mixture. However, it is not significant for the variety effect and interaction (V × T). The rate of the biodegradability of OMW is not constant during storage time because OMW constitutes a favorable medium for the survival of microorganisms that they present because of their organic and mineral charges. Microorganisms find the organic matter that consumes it with the oxygen dissolved in this liquid effluent. In addition, changes also stimulate and influence microbial habitat while organic degradation with various enzymatic activities is already in progress **(Bustamante et al., 2010).**

The OMW appears as an aqueous and viscous residual liquid, reddish-brown to black, with a strong olive odor. It contains various organic and mineral compounds of very different nature and concentrations. This variation is mainly due to the following factors: Olive-ripening stage, soil and climate conditions, variety of olives, cropping system, duration, and storage conditions of the olives before crushing, olive oil extraction process, which represents the most determining factor **(Battista et al., 2016).**

In the early days of assembling OMW, it was found that they represent a reddish-brown coloration, which becomes increasingly dark during storage, with a cloudy appearance and strong smell reminiscent of olive oil, which increases with storage time.

2.1.2. Mean effect of the variety on the measured physicochemical parameters

Results of the mean effect of the variety on the measured physicochemical parameters were presented in **Table 4**.

Table 4: Mean effect of the variety on the measured physicochemical parameters

Variety	Abani	Zlitni	Mixture
pH	4.82 ± 0.78 [a]	4.40 ± 0.59 [c]	4.42 ± 0.71[b]
EC (mS/cm)	14.72 ± 0.59 [a]	12.83 ± 0.68 [c]	14.33 ± 0.89 [b]
Lipids (%)	1.58 ± 0.76 [a]	1.33 ± 0.70 [c]	1.45 ± 0.76 [b]
TSS%	1.02 ± 0.14 [a]	0.87 ± 0.12 [c]	0.92 ± 0.12 [b]
H_2O%	88.48 ± 6.87 [c]	89.38 ± 6.49 [a]	88.86 ± 6.61 [b]
DM (g/L)	117.17 ± 65.74 [a]	107.70 ± 62.72 [c]	111.43 ± 66.14 [b]
MM (g/L)	23.07 ± 3.74 [a]	21.85 ± 2.38 [b]	22.86 ± 3.77 [a]
VM (g/L)	94.10 ± 62.23 [a]	85.85 ± 60.67 [c]	88.57 ± 62.71 [b]
OM (g/L)	81.61 ± 2.20 [a]	81.98 ± 3.02 [a]	82.44 ± 4.71 [a]
TOC%	47.31 ± 1.28 [a]	47.52 ± 1.75 [a]	47.79 ± 2.73 [a]
TKN %	1.90 ± 0.40 [b]	1.71 ± 0.31 [c]	2.20 ± 0.41 [a]
C/N	26.19 ± 6.44 [b]	28.78 ± 6.21 [a]	22.56 ± 5.00 [c]
COD (g O_2/L)	209.8 ± 88.64 [b]	157.8 ± 72.78 [c]	237.87 ± 92.54 [a]
BOD_5 (g O_2/L)	81.92 ± 21.45 [b]	62.27 ± 12.95 [c]	93.6 ± 21.80 [a]
BI	2.48 ± 0.61 [a]	2.43 ± 0.71 [a]	2.47 ± 0.59 [a]
TOM (g/L)	124.55 ± 42.36 [b]	94.12 ± 32.16 [c]	141.69 ± 44.09 [a]
BOD_5/COD	0.43 ± 0.11 [a]	0.45 ± 0.14 [a]	0.43 ± 0.11 [a]

[a, b, c]: homogeneous groups

The comparison of the averages of the physicochemical parameters for the three samples of OMW, Abani, Zlitni, and the mixture, was distributed in the homogeneous groups. It revealed 3 groups on pH, EC, lipids, TSS%, DM, and VM. The first group is characterized by the highest values recorded in the Abani variety. In addition, it revealed 3 homogeneous groups on H_2O, C/N, TKN, BOD_5, COD, and TOM. The highest values of H_2O and C/N were recorded in the Zlitni variety and in the mixture for TKN, BOD_5, COD, and TOM. MM recorded 2 homogeneous groups; the first was recorded in the Abani variety. OM, TOC, BI, and BOD_5/COD revealed one group.

2.1.3. Mean effect of the time on the measured physicochemical parameters

The comparison of the average values of the physicochemical parameters for the five storage times was distributed in the homogeneous groups, as shown in **Table 5.** It revealed 5 homogeneous groups on pH, EC, lipids, H_2O, DM, MM, VM, COD, TOM, and BOD_5. The first group is recorded in t0 for pH, EC, lipids, DM, MM, VM, COD, BOD_5, and TOM, while for H_2O, the first group is recorded in tM12. In addition, it revealed 4 groups on TSS, TOC, and OM, which the first group recorded in t0, and it revealed 3 groups on BI, TKN, C/N, and BOD_5/COD. For BI and TKN, the first group recorded in t0 and in tM12 for C/N and BOD_5/COD.

Table 5: Mean effect of the Time on the measured physicochemical parameters

Time	t0	t (1M)	t (2M)	t (6M)	t (12M)
pH	5.4 ± 0.26 [a]	5.02 ± 0.25 [b]	4.64 ± 0.45 [c]	4.14 ± 0.08 [d]	3.54 ± 0.11 [e]
EC (mS/cm)	14.69 ± 0.86 [a]	14.49 ± 0.87 [b]	14.29 ± 0.87 [c]	13.45 ± 0.86 [d]	12.86 ± 0.93 [e]
Lipids (%)	2.16 ± 0.17 [a]	1.92 ± 0.15 [b]	1.74 ± 0.12 [c]	1.29 ± 0.06 [d]	0.15 ± 0.09 [e]
TSS%	1.05 ± 0.08 [a]	1.03 ± 0.08 [a]	0.97 ± 0.07 [b]	0.91 ± 0.06 [c]	0.72 ± 0.06 [d]
H_2O%	82.67 ± 0.49 [e]	83.66 ± 0.51 [d]	84.89 ± 0.57 [c]	95.19 ± 0.28 [b]	98.12 ± 0.20 [a]
DM (g/L)	173.28 ± 4.94 [a]	163.37 ± 5.06 [b]	151.07 ± 5.69 [c]	48.14 ± 2.84 [d]	24.64 ± 4.11 [e]
MM (g/L)	25.50 ± 1.36 [a]	25.08 ± 1.47 [ba]	24.07 ± 1.80 [b]	20.3 ± 1.82 [c]	18.01 ± 1.34 [d]
VM (g/L)	147.78 ± 4.14 [a]	138.28 ± 4.30 [b]	127.00 ± 4.65 [c]	27.84 ± 3.86 [d]	6.63 ± 3.64 [e]
OM (g/L)	85.16 ± 1.21 [a]	81.29 ± 2.80 [bc]	82.94 ± 1.95 [b]	79.19 ± 5.16 [c]	81.46 ± 1.48 [bc]
TOC%	49.37 ± 0.70 [a]	47.13 ± 1.63 [bc]	48.08 ± 1.13 [b]	45.91 ± 2.99 [c]	47.22 ± 0.86 [bc]
TKN %	2.25 ± 0.26 [a]	2.19 ± 0.26 [a]	2.15 ± 0.27 [a]	1.70 ± 0.23 [b]	1.39 ± 0.27 [c]
C/N	22.16 ± 2.50 [c]	21.73 ± 2.55 [c]	22.69 ± 3.19 [c]	27.51 ± 3.97 [b]	35.11 ± 6.06 [a]
COD (g O_2/L)	309.33 ± 34.25 [a]	270.78 ± 50.84 [b]	184.22 ± 58.29 [c]	153.67 ± 40.17 [d]	91.11 ± 18.09 [e]
BOD_5 (g O_2/L)	97.44 ± 15.23 [a]	90.46 ± 18.58 [b]	82.47 ± 25.09 [bc]	72.89 ± 14.33 [c]	53.06 ± 10.72 [d]
BI	3.20 ± 0.22 [a]	3.01 ± 0.31 [a]	2.24 ± 0.31 [b]	2.11 ± 0.34 [b]	1.73 ± 0.22 [c]
TOM (g/L)	168.07 ± 21.33 [a]	150.57 ± 28.19 [b]	116.39 ± 34.98 [c]	99.82 ± 21.48 [d]	65.74 ± 12.58 [e]
BOD_5/COD	0.31 ± 0.02 [c]	0.33 ± 0.03 [c]	0.45 ± 0.06 [b]	0.49 ± 0.07 [b]	0.59 ± 0.08 [a]

[a, b, c, d, e]: homogeneous groups

2.1.4. Mean effect of the variety time interaction on the measured parameters

The physicochemical characterizations of OMW generally depend on the varieties and the extraction system. The wastewater compositions of the Zlitni, Abani varieties and their mixture used in this work are presented as average values. The mean effect of the variety, time and their interaction on the lipids contents, TSS% and MM revealed 11 groups, water content 14 groups, DM 13 groups, VM and C/N 10 groups, on OM 4 groups, on TOC 5 groups, on TKN 12 groups.

2.1.4.1. Potential of Hydrogen (pH)

The obtained results have shown that the pH values of the three samples were increasing progressively during a storage time of 12 months **(Figure 24).**

The rates of decrease of Zlitni, mixture, and Abani varieties are 1.68 units, 1.88 units, and 2.03 units, in order from the less to the more. OMW studied are acidic effluents. According to a comparison of average values, the Zlitni variety is more acidic than the mixture and the Abani variety, with values (4.40, 4.42, and 4.82), respectively. The results obtained are included in the literature and are similar to those of several researchers. There are in the range found by **Zaier et al. (2017)** for OMW from three different regions (North, Sahel, and South) of Tunisia and which are of the order of (4.7 - 5.15). **El-Hajjoujj (2007)** found a value of (5.39) for OMW from a modern three-phase centrifuge olive oil mill located in Marrakech, Morocco. **Ouabou et al. (2014a)** found a value of (5.32) for OMW obtained from an artisanal crushing unit located in Attaouia, central Morocco. The results obtained by **Esmail et al. (2014)** are in the range (4.65 - 5.16) for OMW from two regions (Ouazzane and Fez Boulman) located in Morocco. **Rajhi et al. (2018)** found (5.045) for OMW from Tunisia.

According to literature, the presence of organic acids and fatty acids like palmitic acid, oleic acid, linoleic acid, and arachidonic acid **(Amaral et al., 2008),** as well as the fermentation of sugars and the auto-oxidation and polymerization reactions that transform phenolic alcohols into phenolic acids **(Esmail et al., 2014).** These reactions are manifested by a change in initial coloration on the OMW to a very dark black color **(Assas et al., 2002).** Indeed, the studied effluent are characterized by a reddish-brown coloration, which becomes very dark during storage. Therefore, the gradual decrease in pH during storage is caused by microbial reactions, oxidation, and fermentation.

The role of pH is main for the growth of microorganisms, which generally have an optimum pH varying from 6.5 to 7.5. When the pH is lower than 5 or higher than 8.5, the growth of microorganisms is directly affected and makes the biological treatment of raw OMW very difficult given the conditions for the development of microorganisms **(Esmail et al., 2014).**

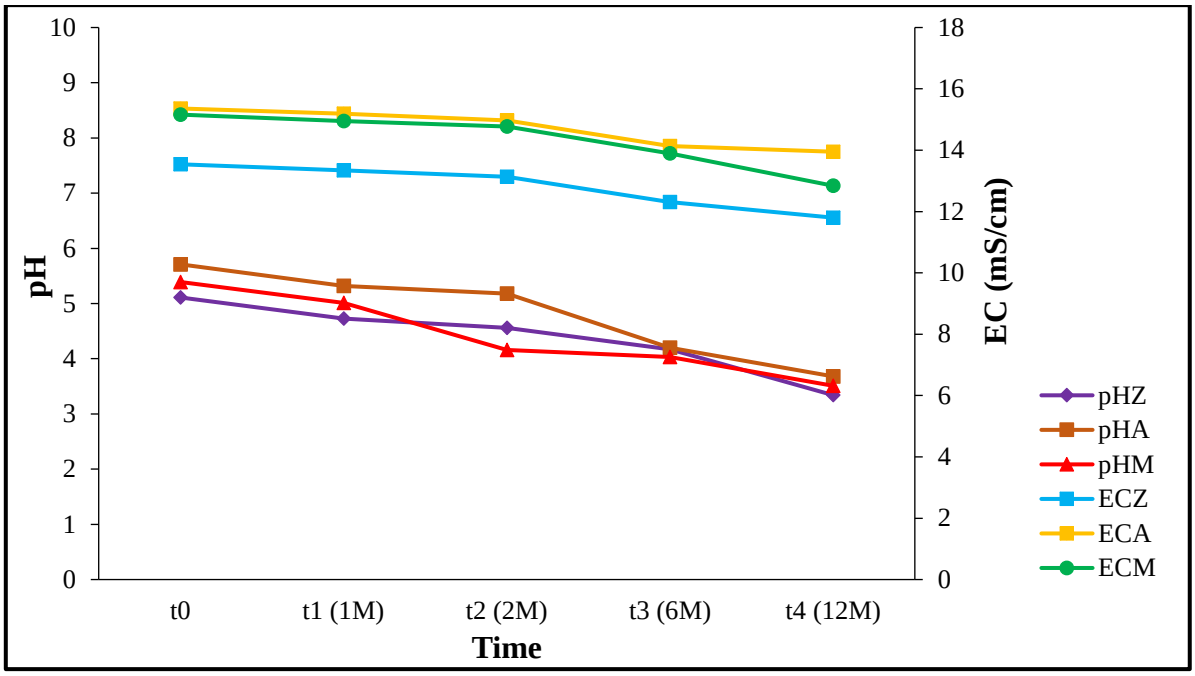

Figure 24: Linear regression representing the pH and EC of the tested OMW within time of the storage

2.1.4.2.Electrical conductivity (EC)

The electrical conductivity of the samples decreased with a storage time of 12 months **(Figure 23).** The rate of decreasing for Abani, Zlitni varieties, and the mixture is successively (90.82%, 87.15%, and 84.7%). The results obtained show that the OMW has high electrical conductivity because of the rich load of minerals **(El-Hajjouji, 2007).** The average values recorded are successive from the more to the less as follows: Abani (14.72 mS/cm), mixture (14.33 mS/cm), and Zlitni (12.83 mS/cm). The results are situated in the range of **Belaid et al. (2002)**, which is (15 - 15.1 mS/cm) for OMW from Tunisia. **Zaier et al. (2017)** for OMW from three different regions (North, Sahel, and South) of Tunisia and which are of the order of (11 - 13 mS/cm). **Ouabou et al. (2014a)** found a value of (16.14 mS/cm) for OMW obtained from an artisanal crushing unit located in the Attaouia region, central Morocco. This difference in electrical conductivity is due to the natural richness of olives in mineral salts **(Aissam, 2003; Achak, 2011).**

According to **Achak (2011),** conductivity is closely related to the concentration of dissolved substances and their nature. In the case of OMW, the values of this conductivity vary between 18 and 50 mS/cm. Therefore, the decrease in the electrical conductivity rate with the storage duration may be due to the bacteria activity qualified as prototrophs degrading the mineral elements contained in OMW.

2.1.4.3.Lipid contents

Figure 25 represents the mean effect of interaction (V × T) on lipids of the tested OMW and their homogenous groups. The lipid content was reduced during 12 months of storage by (96.95%, 88.93%, and 95.34%) for Zlitni, Abani, and the mixture, respectively. The OMW studied are low in lipids. According to the comparison of average values, the highest value of lipids is revealed in OMW of the Abani variety than the mixture and the lowest is in Zlitni with respectively the average values recorded are (1.58%, 1.44%, and 1.33%). Generally, the lipid level in OMW ranges literature from 0.03% to 4.25%. The results obtained are situated in the ranges of several authors. **Esmail et al. (2014)** found a range of (1 - 2.5%) for OMW from two regions (Ouazzane and Fez Boulman) located in Morocco. **Mohawesh et al. (2019)** found a value of (0.97%) for OMW from Jordan. **Khdair et al. (2019)** found values for lipids of (0.015 – 13.13 g/L) for OMW obtained from olive oil mills with traditional, two-phase, and three-phase extraction processes located in Jordan.

A recent study with a range of (0.03 - 1.1%) is found by **Benamar et al. (2020)** for OMW from an oil mill with a traditional extraction process located in Beni Mellal (Morocco). The amount of lipids present in the OMW depends on the olive oil extraction system. The centrifugation process achieves low rates compared to the traditional process. The difference in lipid content is due to the quality of the olives, their degree of ripening, and the method of crushing **(Amaral et al., 2008).**

The OMWs studied have a viscous appearance linked to the presence of the oily fraction. This viscosity is also reduced with storage time. It forms a lipid layer on the surface of OMW, which could limit natural evaporation.

The fatty matter content causes the formation of a layer on the water's surface, preventing its correct oxygenation and the passage of light and barrier to the normal development of flora and fauna within rivers. In addition, fatty acids and their derivatives inhibit spore-forming soil bacteria.

The decrease in the lipid level is maybe due to lipolysis, which degrades the fat gradually throughout the storage.

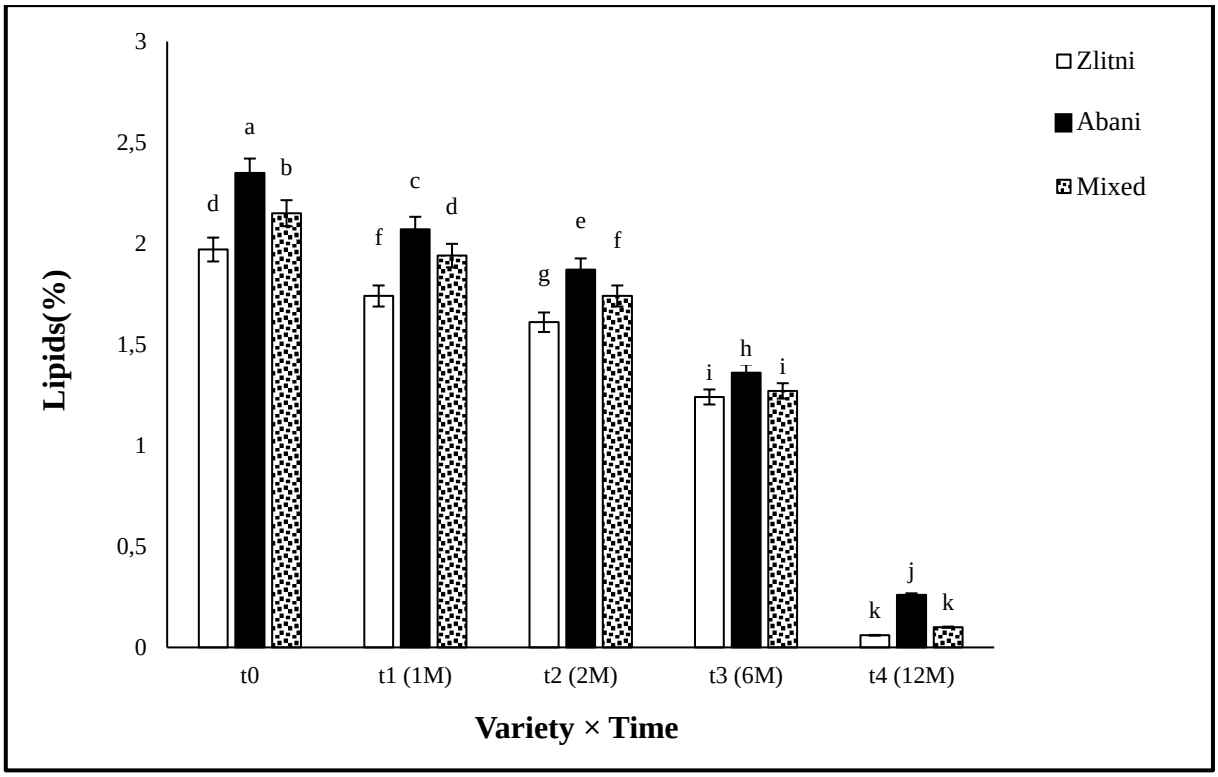

Figure 25: Mean effect of interaction (V × T) on lipids of the tested OMW and their homogenous groups

2.1.4.4.Total Suspended Solids (TSS%)

The mean effect of interaction (V × T) on TSS of the tested OMW and their homogenous groups is illustrated in **(Figure 26).** During 12 months of storage time, values were reduced by 33.67%, 32.45%, and 28.71% for Zlitni, Abani, and the mixture, respectively. According to the results obtained, the effluents studied are not very loaded with TSS. The average values obtained were ordered from more to less charge as follows, Abani (1.02%), mixture (0.91%) and Zlitni (0.87%). They are close to the results of **D'Annibale et al. (2003)** (0.04 - 1.04%) obtained for OMW from Spain. **Khdair et al. (2019)** found results of TSS of (0.18 – 51.59 g/L) for OMW obtained from olive oil mills with traditional, two-phase, and three-phase extraction processes; located in Jordan. **Hamimed et al. (2020)** found a value of (21.2 ± 2.60 g/L) for OMW from Tunisia.

The variations in suspended matter content may be due to climatic and geological parameters, botanical variations, the stage of olive ripening, and the oil extraction process. The TSS of OMW decreases under the effect of settling, and this is probably due to the effect of variety, degree of maturity, and agitation caused at the time of the unloading of the OMW. The decrease in TSS% is probably due to the fermentation reaction and auto-oxidation **(El-Abbassi et al., 2011).**

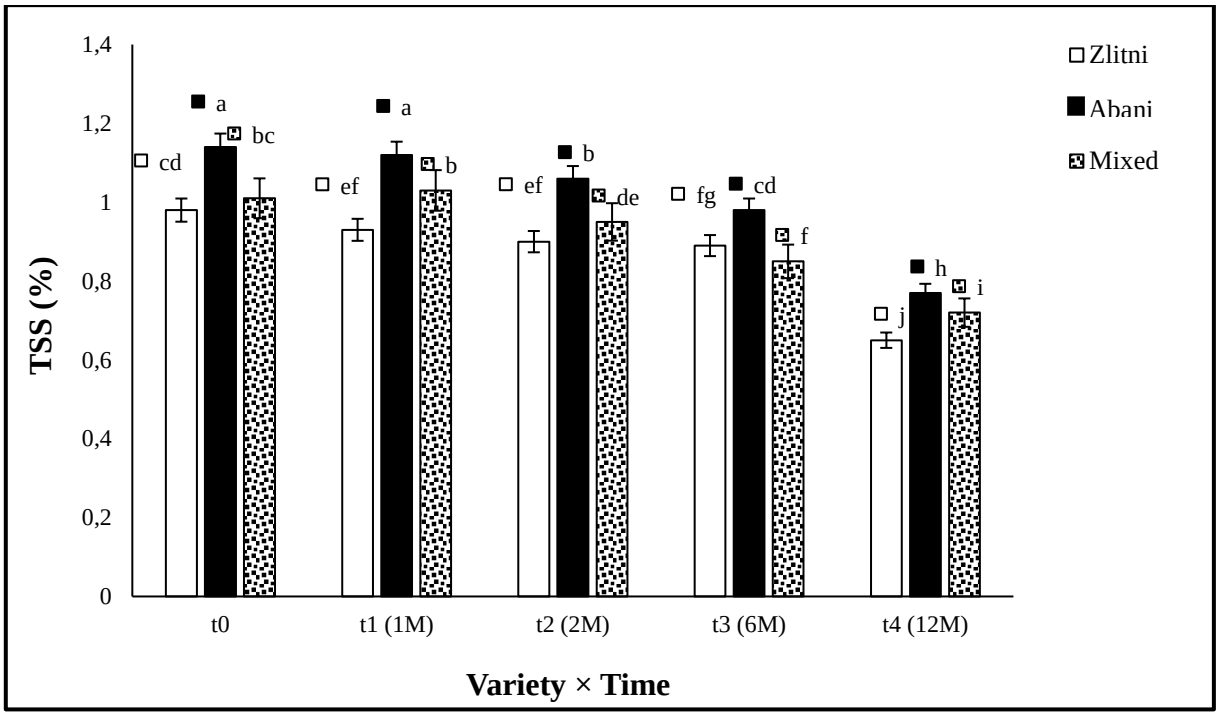

Figure 26: Mean effect of interaction (V × T) on TSS of the tested OMW and their homogenous groups

2.1.4.5.Water content (H_2O)

Figure 27 represents the mean effect of interaction (V × T) on the water content of the tested OMW and their homogenous groups. During a storage time of 12 months, water contents are progressively increased; the rate is increases by 18.13%, 19.38%, and 18.56% for Zlitni, Abani, and the mixture, respectively.

OMW samples studied are very rich in water. The average values obtained ordered from more to less charge in water Zlitni, mixture, and Abani variety with 89.38%, 88.86%, and 88.48%, respectively. Several authors have similar results as **Ouabou et al. (2014a)** found a value of (88%) for OMW obtained from an artisanal crushing unit located in the Attaouia region, central Morocco. In another study, a range of (86 - 87.6%) is found by **Ouabou et al. (2014b)** for OMW three traditional units (Settat, El Kelaa Sraghna, Marrakech), Morocco. A recent study by **Sáez et al. (2021)** found a value of (58%) OMW from an olive oil mill located in Mora, Toledo, Spain.

OMW is composed of 40 to 50% of the vegetable water that comes from the fruit and the rest of the manufacturing water added during the crushing process. The high humidity level of OMW is explained by the water existing in the olives on the one hand and the other hand by water added during the olive oil extraction process **(D'Annibale et al., 2003; El-Hajjouji, 2007; Zaier et al., 2017).**

The increase in humidity is may be due to microbial activities, which release water molecules and H_2 and CO_2 gases during these reactions.

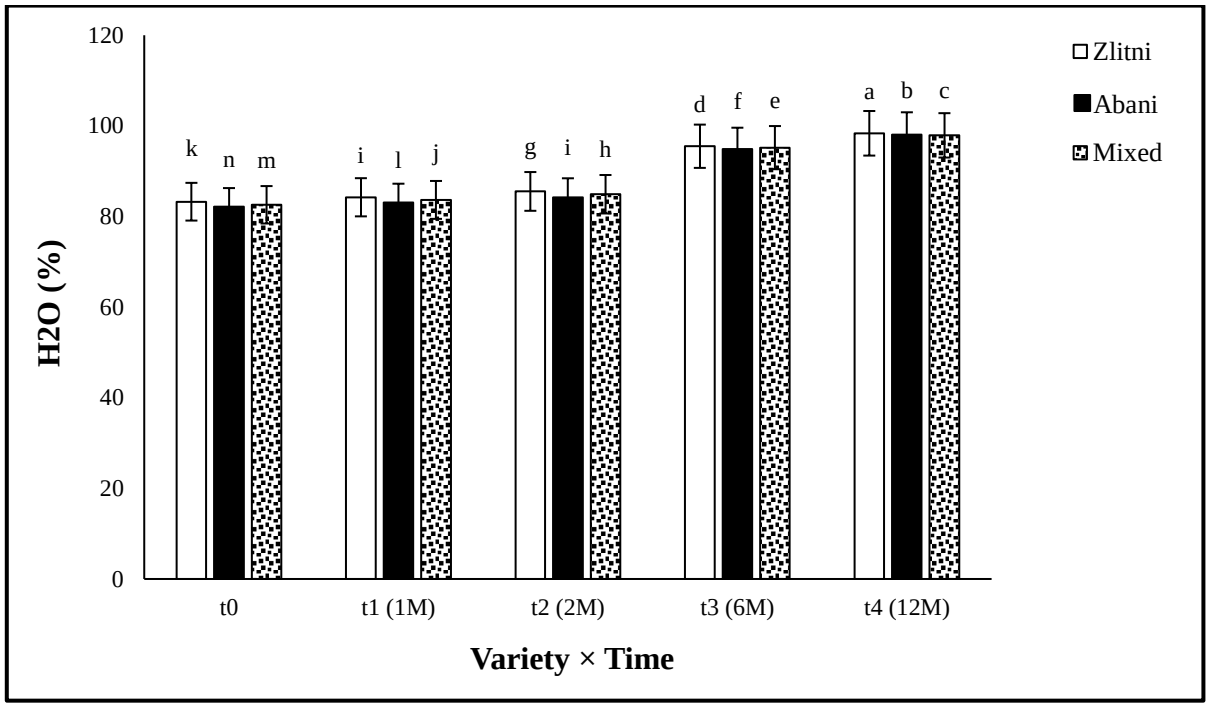

Figure 27: Mean effect of interaction (V × T) on water content of the tested OMW and their homogenous groups

2.1.4.6.Dry Matter (DM)

Figure 28 illustrates the mean effect of interaction (V × T) on DM of the tested OMW and their homogenous groups. During 12 months of storage, dry matter content decreases gradually. It is reduced by 85.65%, 83.68%, and 88.03% for Zlitni, Abani, and the mixture, respectively. The values obtained show that the effluents are rich in dry matter. The Abani variety has the highest average value, followed by the mixture and then Zlitni, with the following values (117.17, 111.43, and 107.7 g/L). The results obtained are close to the results found by **Ouabou et al. (2014a)**, which found a value of (138 g/L) for OMW obtained from an artisanal crushing unit located in the region of Attaouia, central Morocco, and **Ouabou et al. (2014b)** found a range of (138.44 - 240 g/L) for OMW three traditional units (Settat, El Kelaa Sraghna, Marrakech), Morocco.

According to **El-Abbassi et al. (2011),** the variations in dry matter content may be due to climatic and geological parameters, botanical variations, the stage of olive ripening, and the oil extraction process.

This reduction during the storage period is probably due to the degradation of the matter because of the microbial load contained in the olive oil mill wastewater.

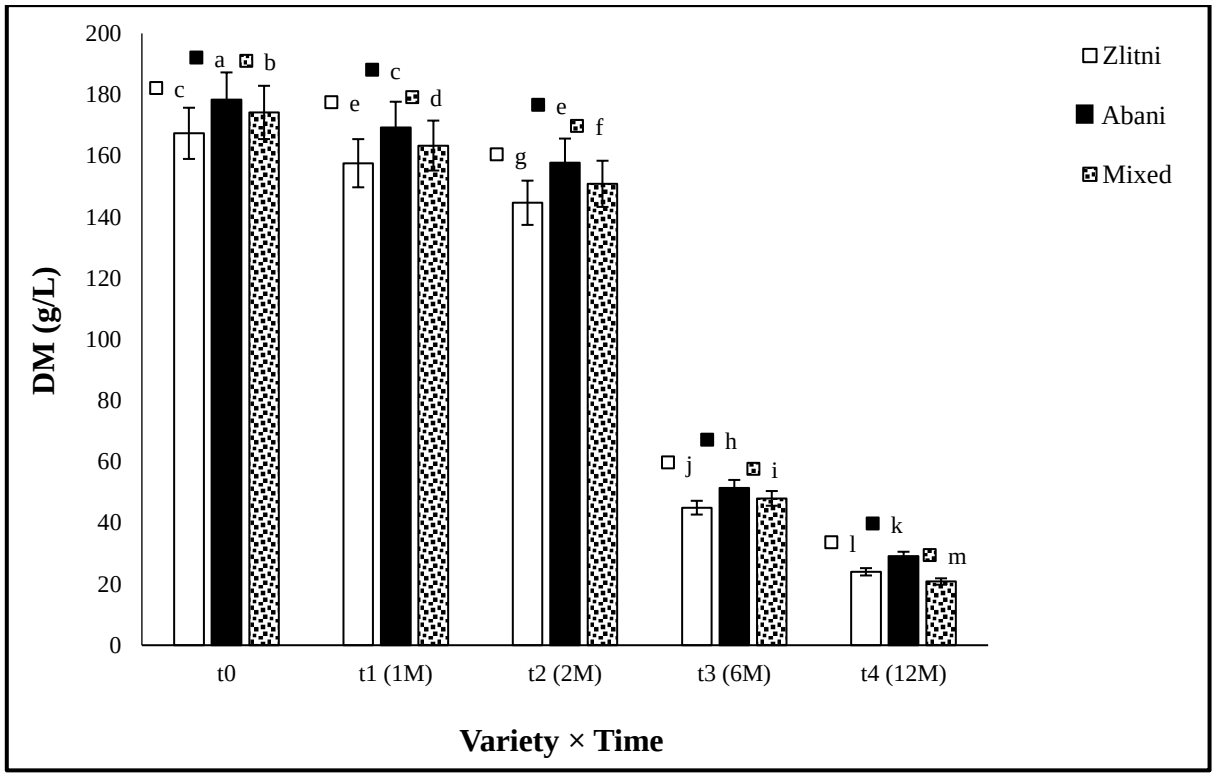

Figure 28: Mean effect of interaction (V × T) on DM of the tested OMW and their homogenous groups

2.1.4.7.Organic Matter (OM)

The results obtained **(Figure 29)** showed that organic matter is decreased with a storage time of 12 months by 3.86%, 5.03%, and 4.14% for Zlitni, Abani, and the mixture, respectively. According to these results, OMW is loaded with organic matter. It is almost the same content for the three samples mixture, Abani, and Zlitni, with the following average values (82.44, 81.61, and 81.98 g/L). The findings are superior to those obtained by **Zaier et al. (2017)**, which were of the order of (41.2 - 65.9 g/L) for OMW from three different regions (North, Sahel, and South) of Tunisia, and closer to those obtained by **Rajhi et al. (2018)** which are found (89.59 g/L) for OMW from Tunisia. A recent study by **Sáez et al. (2021)** found a value of (45.8%) OMW from an olive oil mill located in Mora, Toledo, Spain.

The organic charge is mainly due to the presence of macromolecules such as polysaccharoses, lipids, proteins, and a number of monocyclic and polymeric aromatic molecules **(Aissam, 2003).**

The decrease in the rate of organic matter is due to microbial development, which needs energy provided by the degradation of organic molecules, mainly the slow degradation of macromolecules.

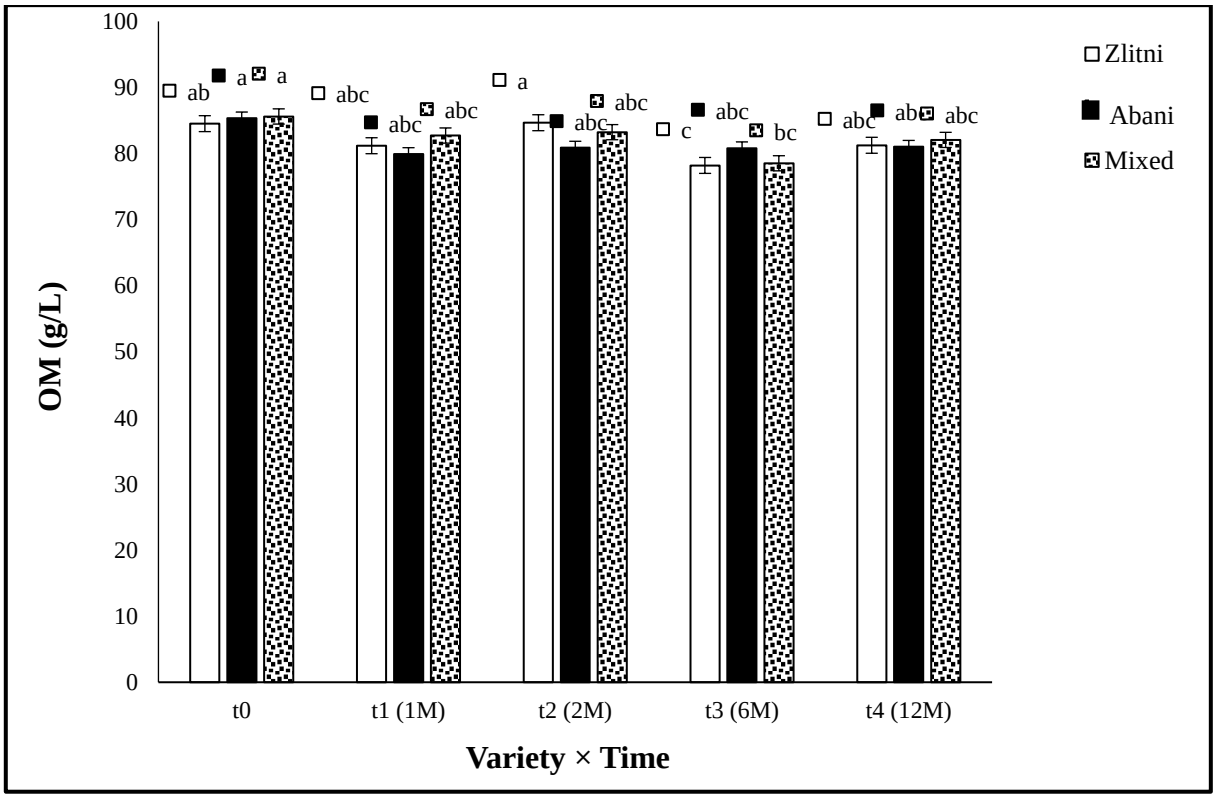

Figure 29: Mean effect of interaction (V × T) on OM of the tested OMW and their homogenous groups

2.1.4.8.Mineral Matter (MM)

With storage for 12 months, the mineral matter is slowly decreasing. The decreasing rates are about 24.57%, 29.90%, and 33.45% for Zlitni, Abani, and the mixture, respectively. The values recorded during the study showed almost similarities for the three samples **(Figure 30).** The average values obtained are in order as follows: Abani (23.07 g/L), mixture (22.86 g/L), and Zlitni (21.85 g/L). These results are in the range (10 - 25 g/L) found by **Belaid et al. (2002)** for OMW from Tunisia. **Ouabou et al. (2014a)** found (24 g/L) for OMW obtained from an artisanal crushing unit located in Attaouia, central Morocco. In addition, a range of (22.69 - 24.35 g/L) is found by **Ouabou et al. (2014b)** for OMW three traditional units (Settat, El Kelaa Sraghna, Marrakech), Morocco.

The difference in the mineral matter is explained by the degree of ripening and the variety of olives **(El-Abbassi et al., 2011).**

The slight decrease may be due to bacteria's weak degradation, which requires mineral elements for growth.

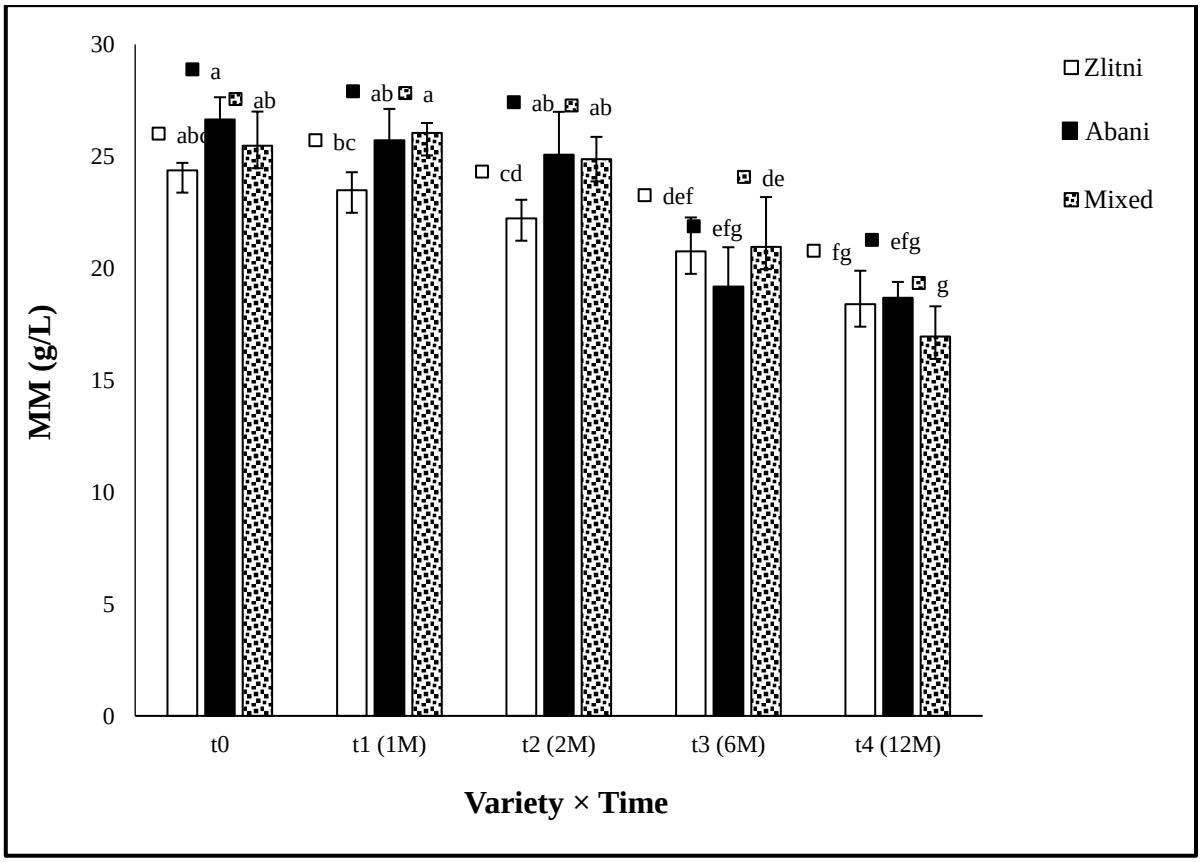

Figure 30: Mean effect of interaction (V × T) on MM of the tested OMW and their homogenous groups

2.1.4.9.Volatile Matter (VM)

The results obtained showed that volatile matter reduced with storage time of 12 months by 96.08%, 93.13%, and 97.40% for Zlitni, Abani, and the mixture, respectively. In addition, the OMWs studied are very loaded with the volatile matter, which shows the organic nature of OMW. The average results are (70.49, 66.20, and 65.56 g/L for Abani, Zlitni, and the mixture, respectively) **(Figure 31).** These results are situated in the range (49.7 - 81.4 g/L) found by **Zaier et al. (2017)** for OMW from three different regions (North, Sahel, and South) of Tunisia. On the other hand, **Belaid et al. (2002)** found a range of (20 - 21.5 g/L) for OMW from Tunisia. This difference is due to climatic conditions, olive oil extraction methods as well as the storage time of the olives before crushing **(El-Abbassi et al., 2011).**

The gradual decrease in the volatile matter may be caused by bacteria, which can degrade many hydrocarbon substances such as organic acids, alcohols, sugars, and polyholosides.

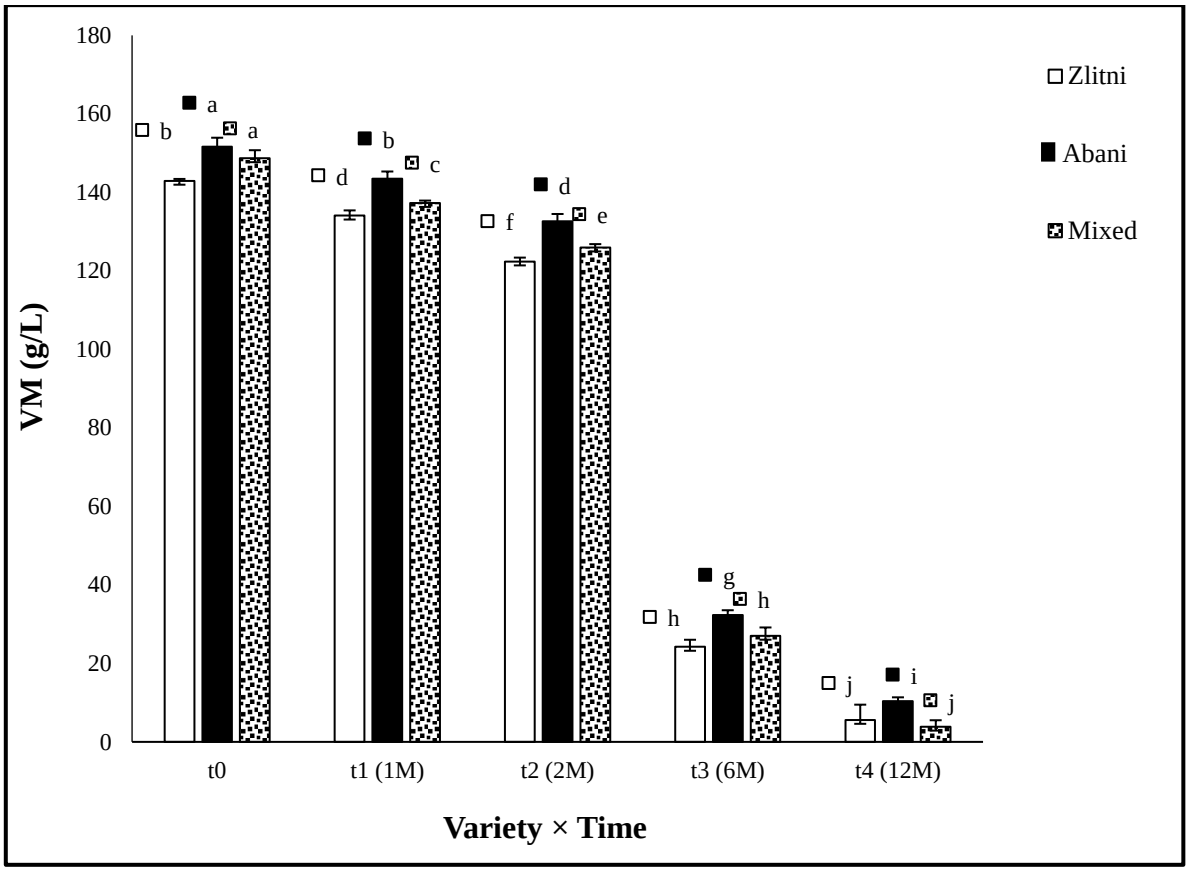

Figure 31: Mean effect of interaction (V × T) on VM of the tested OMW and their homogenous groups

2.1.4.10. Total Organic Carbon (TOC%)

According to the results obtained **(Figure 32),** TOC% decreased during 12 months of storage by 3.85%, 5.03%, and 4.15% for Zlitni, Abani and the mixture, respectively. The values obtained have shown that the TOC is abundant in the liquid effluents studied. Almost similar values were recorded in the three samples. Average values obtained in the order are 47.79, 47.31, and 47.52% for the mixture, Abani, and Zlitni, respectively. These results are similar to those obtained by **El-Hajjouji (2007)**, which recorded a value of (50%) for OMW from a modern three-phase centrifuge olive oil mill located in Marrakech, Morocco, and higher than the results obtained by **Sáez et al. (2021)** which found a value of (30%) for OMW from olive oil mill located in Mora, Toledo, Spain.

These results are may be due to its richness by organic matter and the metabolism that degrades and releases it during CO_2, ethanol, and lactic acid reactions, further due to the polymerization.

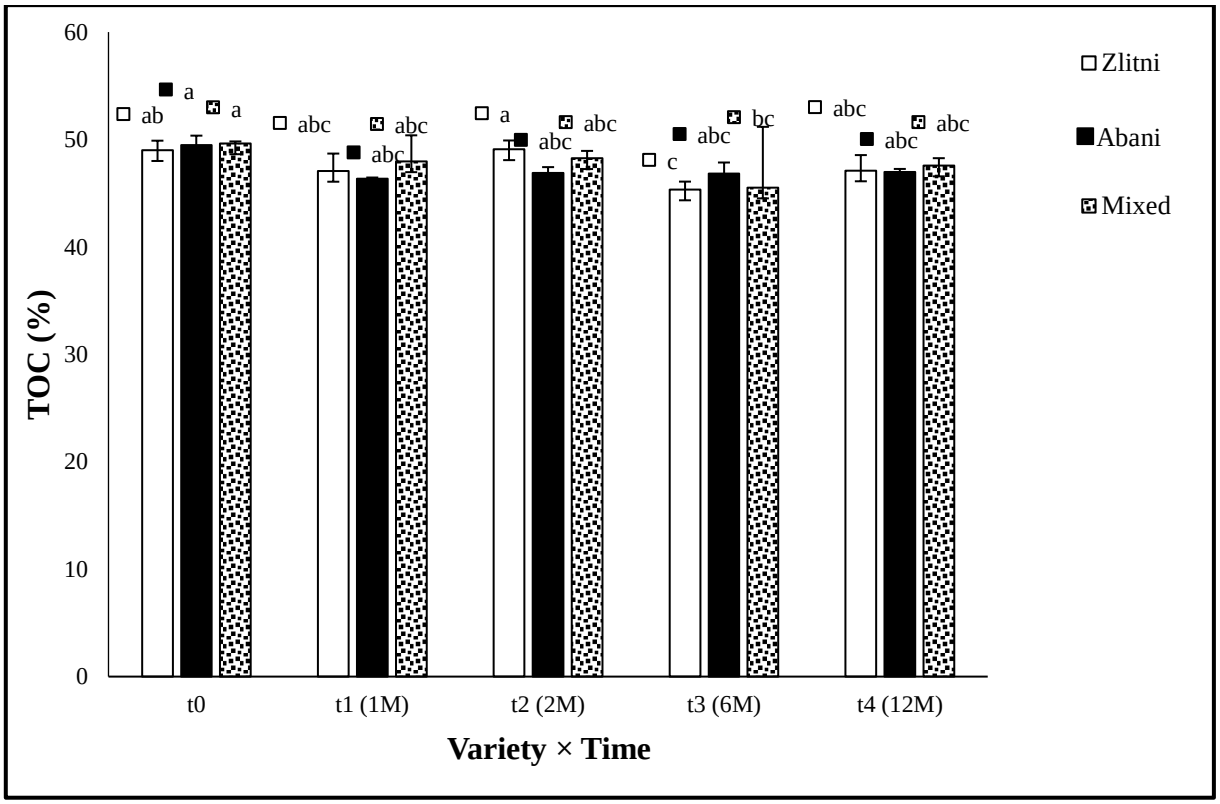

Figure 32: Mean effect of interaction (V × T) on TOC of the tested OMW and their homogenous groups

2.1.4.11. Total Kjeldahl Nitrogen (TKN)

The results obtained are presented in **(Figure 33).** TKN content is decreased during storage of 12 months. It was reduced by 29.60%, 43.50%, and 41.17% for Zlitni, Abani, and the mixture, respectively. According to the results obtained, the studied samples of OMW are very rich in nitrogen. It recorded the high values in the mixture of the two varieties than Abani and Zlitni, respectively, with average values of (2.20, 1.90, and 1.71 g/L). The results are situated in the range cited by **Sáez et al. (2021)**, which found (1.6 g/L) OMW from olive oil mill located in Mora, Toledo, Spain, and lower than those found by **Di Mauro et al. (2017)**, which found a range of (116 - 350 g/L) for OMW from three-phase olive oil mill processing system located in Menfi and Mascalucia, Italy.

This differentiation is perhaps due to the soil's natural richness in nitrogen stored in the fruit or by the use of chemical fertilizers **(Zenjari et al., 2006).**

The continuous decrease is probably due to protein synthesis, which requires nitrogenous substances. For most bacteria, the nitrogen source consists of other inorganic compounds (ammonia, ammonium salts, nitrites, nitrates) or organic sources (amine group of organic compounds).

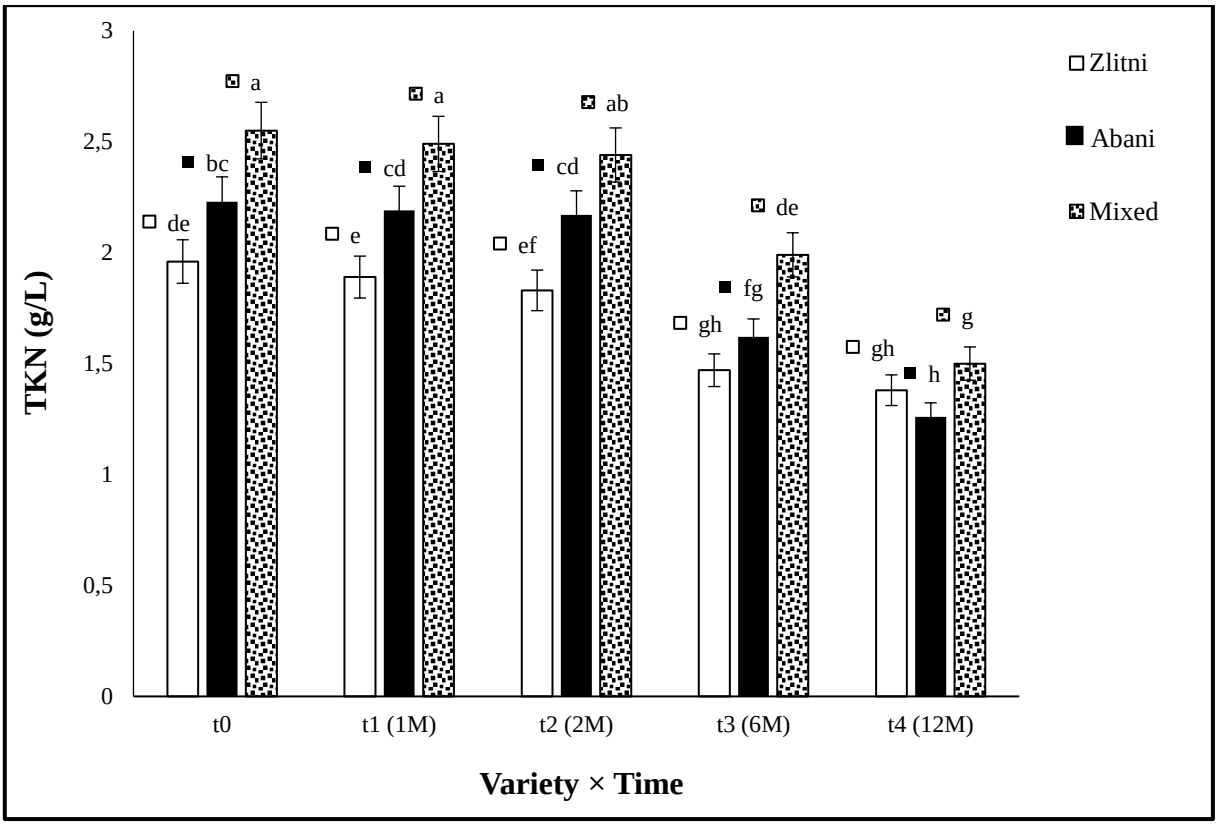

Figure 33: Mean effect of interaction (V × T) on TKN of the tested OMW and their homogenous groups

2.1.4.12. Carbon to Nitrogen mass ratio (C/N)

According to the results obtained **(Figure 34),** this ratio increases gradually with 12 months of storage duration. The increase was with rates of (46.55%, 68.12%, and 62.64%) for Zlitni, Abani, and the mixture, respectively. In addition, the Zlitni variety has the highest average value (28.77) than Abani (26.19) and then the mixture (22.56). These results are close to those found by **Sáez et al. (2021)**, which recorded a value of (C/N = 25) for OMW from an olive oil mill located in Mora, Toledo, Spain, and lower than those of **El-Hajjouji (2007)**, which recorded (C/N = 50.50) for OMW from a modern three-phase centrifuge olive oil mill located in Marrakech, Morocco.

This increase is probably due to the decrease in the biodegradable organic load and the total organic carbon load in the samples.

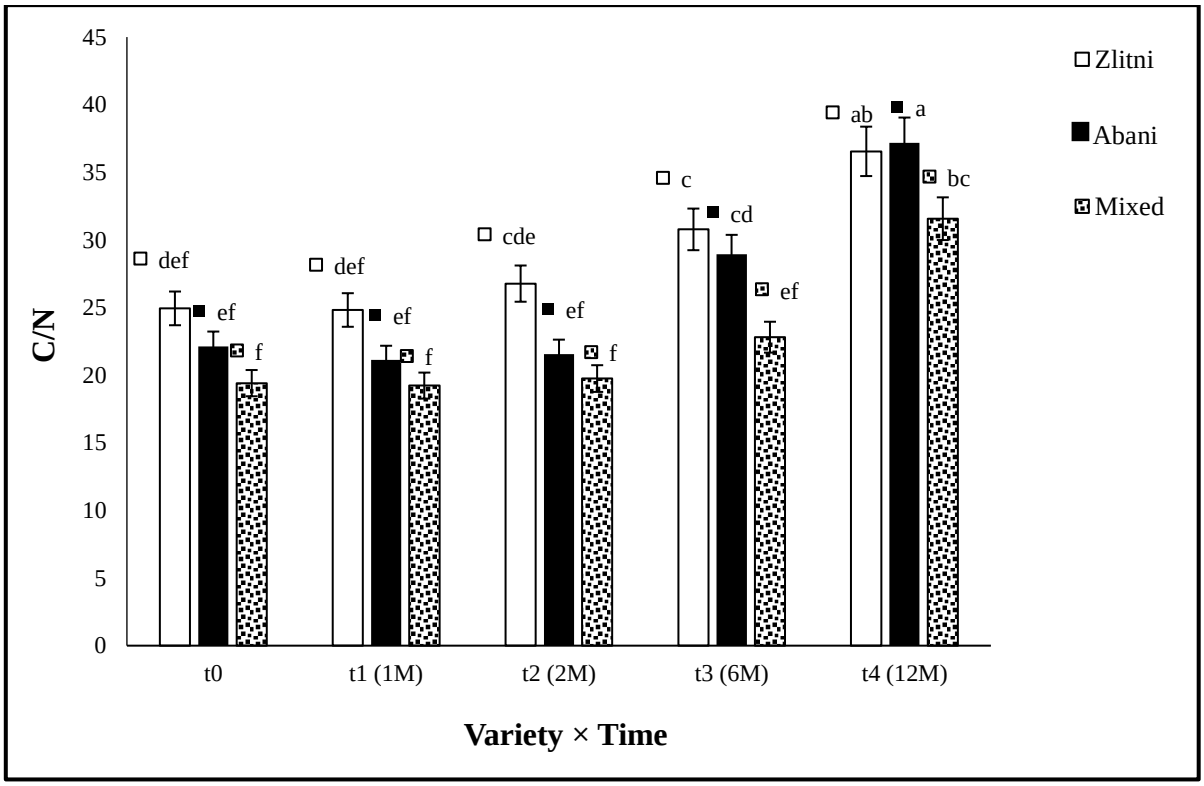

Figure 34: Mean effect of interaction (V × T) on C/N ratio of the tested OMW and their homogenous groups

2.1.4.13. Biological Oxygen Demand (BOD_5)

During 12 months of storage time, the values of BOD_5 gradually decreased by 40.65%, 48.38%, and 46.42% for Zlitni, Abani, and the Mixture, respectively.

The studied OMW were recorded important values of BOD_5, showing that they are very loaded with biologically oxidizable organic matter. Average values showed that the OMW of the mixture has the highest value (93.59 g/L) than Abani and Zlitni (81.92 and 62.27 g/L), respectively **(Figure 35).** The findings are almost situated in the range (65.87 - 86.71 g/L) found by **Zaier et al. (2017)** for OMW from three different regions (North, Sahel, and South) of Tunisia, and in the range (12.50 - 62.50 g/L) recorded by **Ouabou et al. (2014b)** for OMW obtained from three traditional units (Settat, El Kelaa Sraghna, Marrakech), Morocco. **Khdair et al. (2019)** found results of BOD_5 of (0.06 – 41 g/L) for OMW obtained from olive oil mills with traditional, two-phase, and three-phase extraction processes located in Jordan.

It can maybe explain these high values by the abundance of organic matter in the OMW. This progressive decrease during the storage period is may be due to the microbial load and the glucose metabolism that releases gas, CO_2, ATP, H_2, alcohols, and H_2O, and the use of biologically degradable molecules such as H_2O and CO_2.

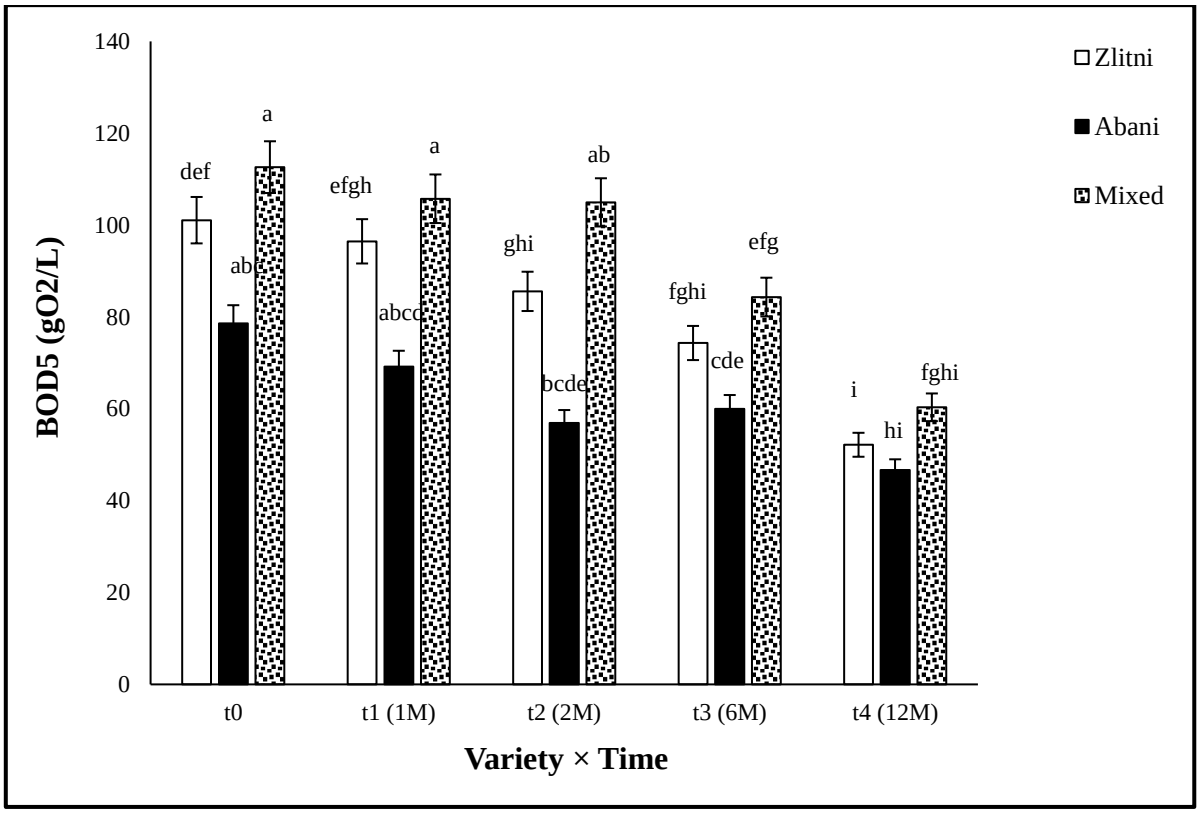

Figure 35: Mean effect of interaction (V × T) on BOD_5 of the tested OMW and their homogenous groups

2.1.4.14. Chemical Oxygen Demand (COD)

The values of COD decreased progressively during the storage period meaning degradation of the organic matter. The rate of decrease is 71.18%, 71.11%, and 69.53% for Zlitni, Abani, and the mixture, respectively.

According to the results found, OMWs have shown a very high oxygen demand for the complete oxidation of organic matter. The highest average values are recorded in the mixture (237.87 g/L), Abani (209.8 g/L), and Zlitni (157.8 g/L), respectively **(Figure 36).** Several researchers found results that are closer to those found in this study. **Khdair et al. (2019)** found results of COD of (0.37 – 105 g/L) for OMW obtained from olive oil mills with traditional, two-phase, and three-phase extraction processes located in Jordan. **Hamimed et al. (2020)** found a value of (189.70 g/L) for OMW from Tunisia, and **Benamar et al. (2020)** found a result of (227.33 g/L) for OMW from an oil mill with a traditional extraction process located in Beni Mellal (Morocco).

The decrease in COD value during storage time is probably due to autoxidation and polymerization reactions, in addition to the decomposition of recalcitrant molecules into biodegradable molecules or mineral compounds such as H_2O and CO_2.

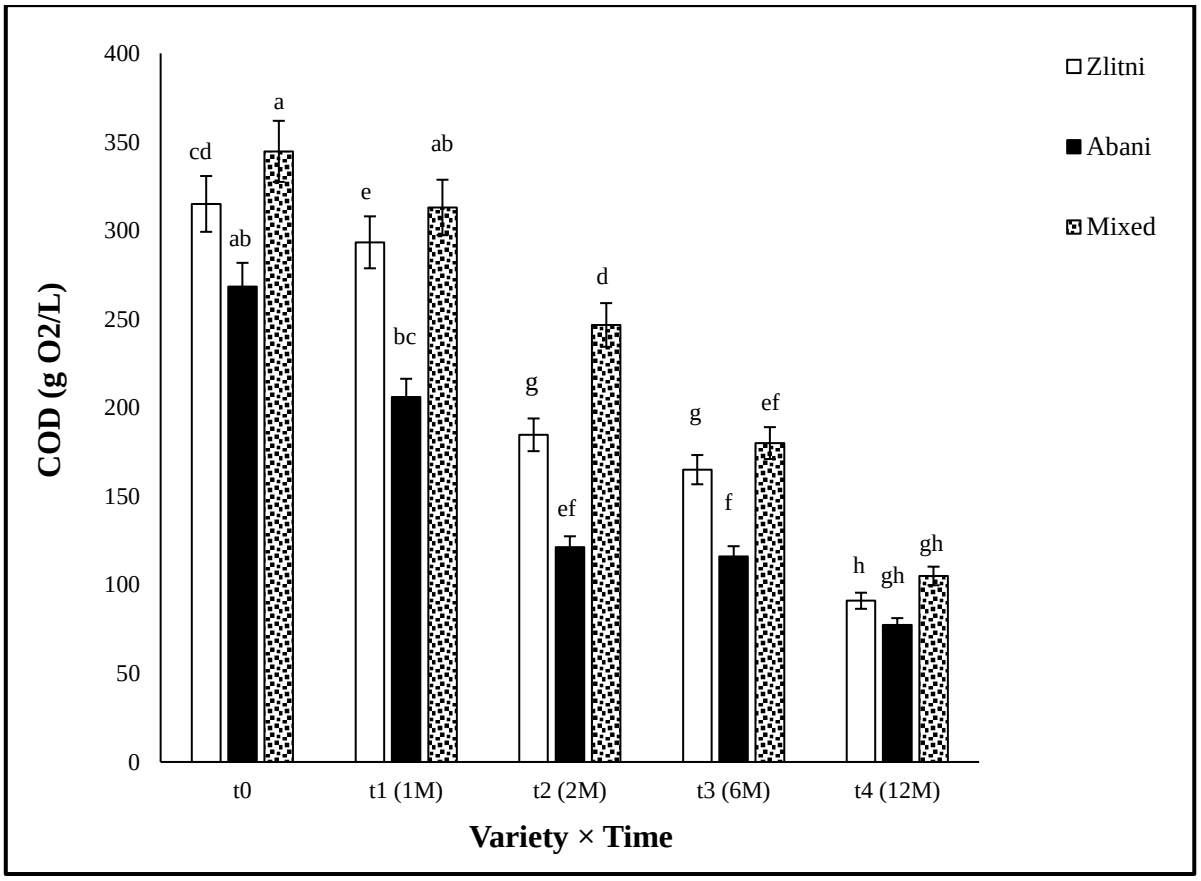

Figure 36: Mean effect of interaction (V × T) on COD of the tested OMW and their homogenous groups

2.1.4.15. Total Oxidizable Matter (TOM)

The results obtained showed that the oxidizable matter degrades during storage time **(Figure 37).** From t0 to t4, total oxidizable matter (TOM) values reduced by 59.90%, 62.22%, and 60.40% for Zlitni, Abani, and the mixture, respectively.

In addition, TOM is highly present in the samples of the olive oil mill wastewater studied. The average values are higher in the mixture (141.68 g/L), Abani (124.55 g/L), and then Zlitni (94.11 g/L) orderly.

The results obtained are higher than the results found by **El-Rhaouat et al. (2014)**, which found a value of (50.91 g/L) for OMW from the city of Sidi Kacem, Morocco.

The high values of TOM obtained in this study are due to the high values of BOD_5 and COD. The decrease in TOM with storage time is caused by the decrease in BOD_5 and COD values during the storage time of olive oil mill wastewaters.

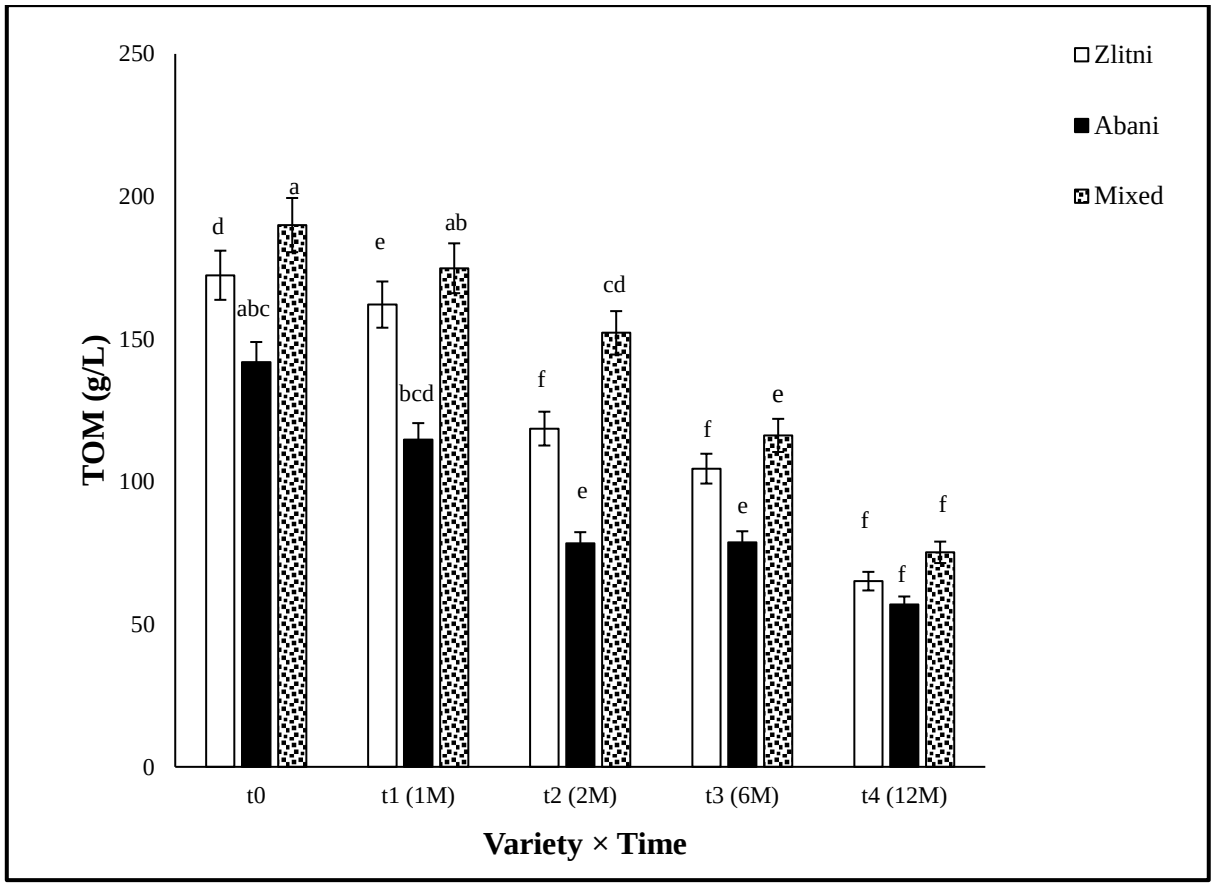

Figure 37: Mean effect of interaction (V × T) on TOM of the tested OMW and their homogenous groups

2.1.4.16. Biodegradability Index (BI)

The results of this ratio constitute an indication of the importance of little or biodegradable pollutants. According to the results obtained **(Figure 38)**, the biodegradability index decreases gradually with the storage duration (12 months) by 50.58%, 43.40%, and 43.13% for Zlitni, Abani, and the mixture, respectively.

In addition, the biodegradability index (BI) is almost the same value in the three samples of OMW obtained from Abani, Zlitni, and the mixture. The average values found are respectively (2.43), (2.46), (2.48) from Zlitni, Mixture, and Abani. They are situated in the range of the results found by **Zaier et al. (2017)**, which are (3.301 - 3.342) for OMW from three different regions (North, Sahel, and South) of Tunisia. Moreover, it is lower than the results found by **El-Rhaouat et al. (2014)**, which is (BI = 4.48), for OMW from the city of Sidi Kacem, Morocco. Therefore, it can be concluded that even the OMWs have a high organic load and is easily biodegradable after one month of storage.

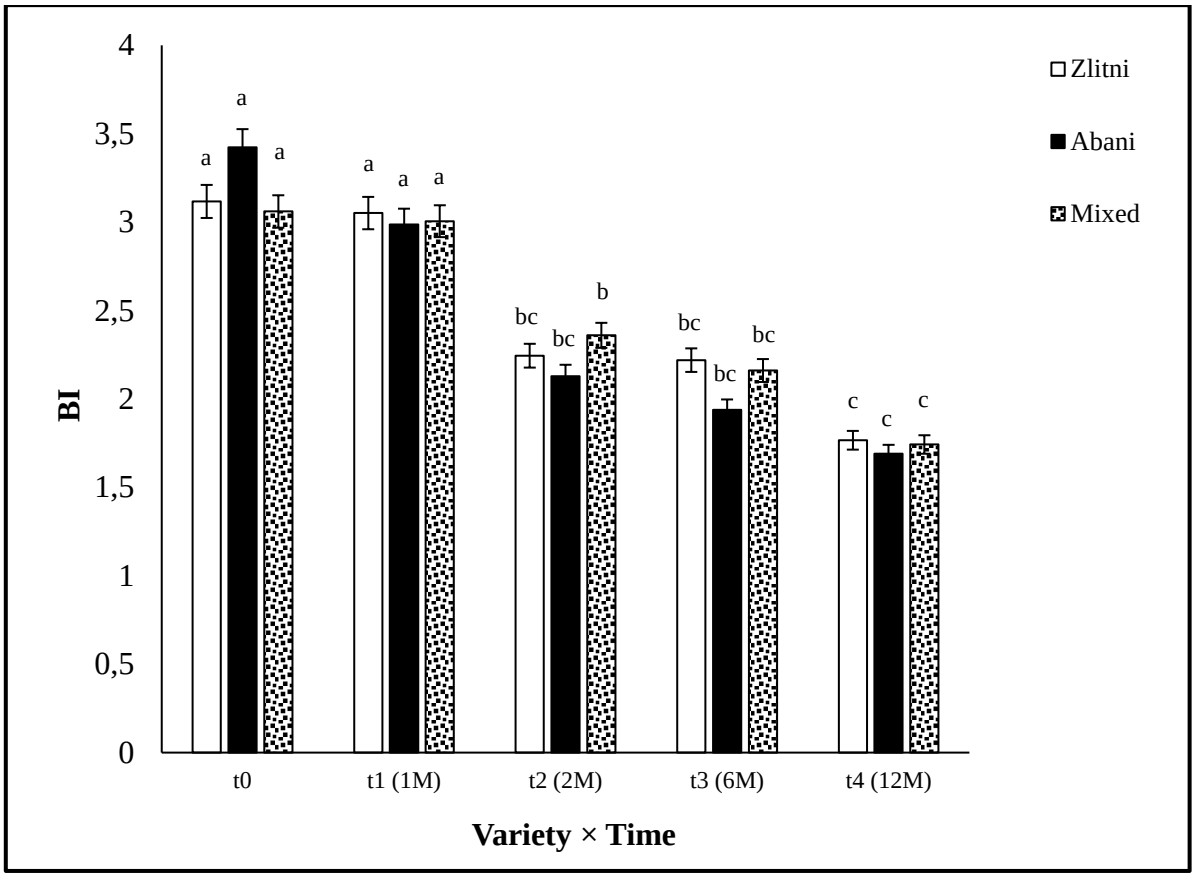

Figure 38: Mean effect of interaction (V × T) on BI of the tested OMW and their homogenous groups

2.1.4.17.BOD_5/COD

The results obtained showed that this ratio (BOD_5/COD) increased during 12 months of storage **(Figure 39).** This rate increased by 110.34%, 110.34%, and 78.12% for Zlitni, Abani, and the mixture, respectively.

The values found showed that this ratio is almost close in all samples. Average values obtained in the order are Zlitni (0.44), Abani (0.43), and the mixture (0.43). The results obtained are higher than those found by **El-Rhaouat et al. (2014)**, which are found a result of (BOD_5/COD = 0.24) for OMW from an olive oil mill located in Sidi Kacem, Morocco.

Based on findings, values of BOD/COD are situated between 0.2 and 0.6. Therefore, the feasibility of treatment using selected microbial strains.

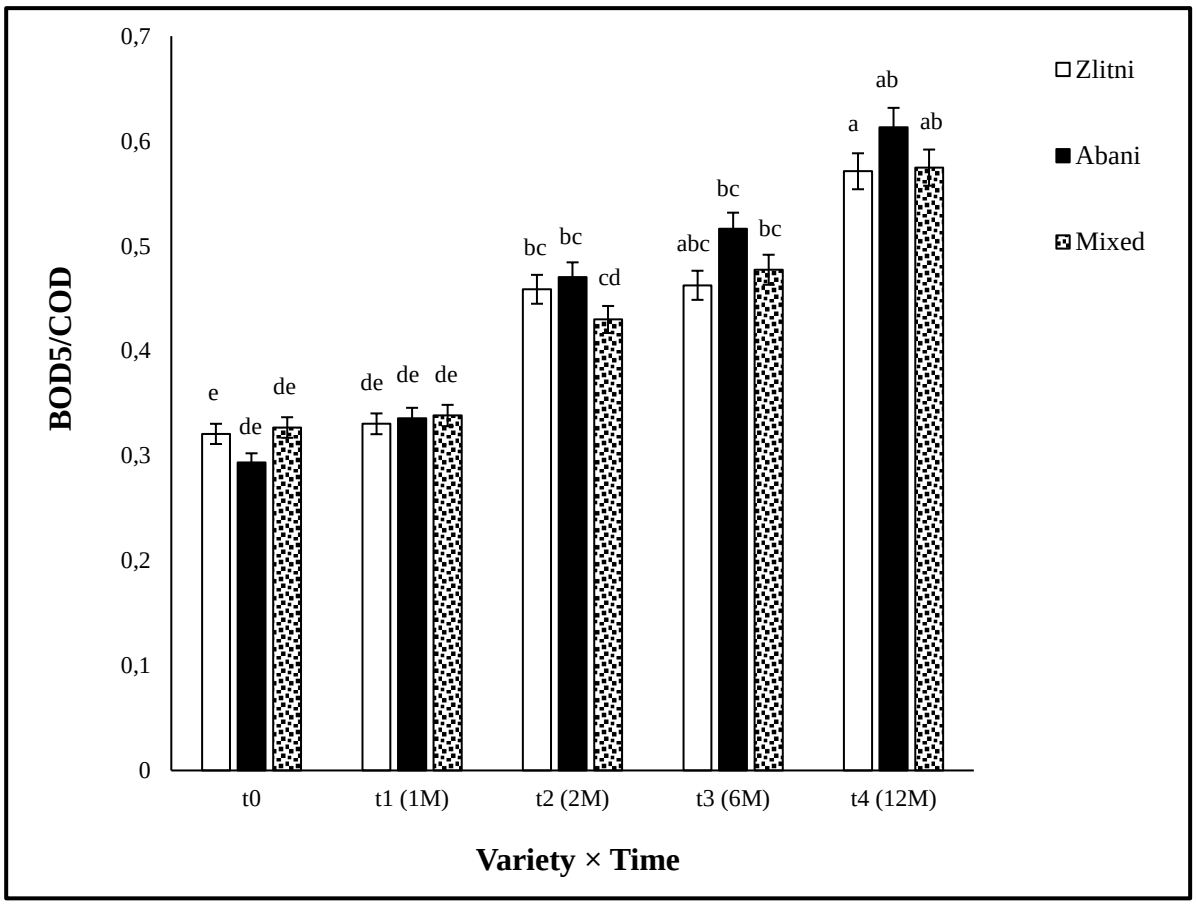

Figure 39: Mean effect of interaction (V × T) on BOD_5/COD of the tested OMW and their homogenous groups

2.1.5. Multivariate analysis (PCA)

The research for links between the variety of OMW and their parameters measured during their storage (after times of 0, 1 month, 2 months, 6 months, and 12 months) is studied using principal component analysis (PCA) **(Figure 40).**

The projection of the variables (the parameters) and the individuals (the variety) was carried out on the two axes, which represent 88.17% of the total variance; the first two axes hold respectively 78.51% and 9.66% of the information.

Axis 1 integrates the information provided by most of the measured parameters (pH, EC, Lipids, TSS, DM, VM, TKN, COD, BOD_5, MM, BI, TOM, OM, and TOC) of the Zlitni variety, Abani variety, and their mixture after 0 months, 1 month, and 2 months of their storage (t0, t1, t2); these parameters are positively correlated with this axis. The group composed exclusively of VM, DM, and BI parameters of the varieties tested in addition to their mixture and which were measured just after their collection is positively correlated to axis 1 and 2 of

the principal component analysis (PCA) and is opposed along these two axes of the PCA and to the group consisting of the water parameter measured after 6 months of storage varieties tested with their mixture.

Axis 2 of the PCA integrates the information provided by two groups. The third group positively correlates with this axis (2) and negatively with axis 1. This group consists of the two parameters C/N and BOD_5/COD of the varieties Zlitni, Abani, and their mixture; these two parameters were measured after one year (12 months) of their storage. This group is opposed along this axis to the group made up of the parameters (pH, EC, Lipids, TSS, DM, VM, TKN, COD, BOD_5, MM, BI, TOM, OM, and TOC) of the Zlitni variety, Abani variety, and their mixture. These parameters were measured after one month of their storage (t1) and which is positively correlated with axis 1 and negatively with axis 2.

The decrease in parameters (pH, EC, Lipids, TSS, DM, VM, TKN, COD, BOD_5, MM, BI, OM, TOM, and TOC) of Zlitni, Abani, and their mixture are positively correlated with storage time, and which was severe after 6 and 12 months, on the other hand, the parameter BOD_5/COD showed an increase during storage. Therefore, this increase is positively correlated with time, and it gave higher values after 6 and 12 months, which explains it.

The biodegradability of OMW is maybe due to the degradation of the organic matter, which constitutes a food contribution for microorganisms that consume oxygen from the medium, which becomes more and more in deficit **(El-Hajjouji, 2007).**

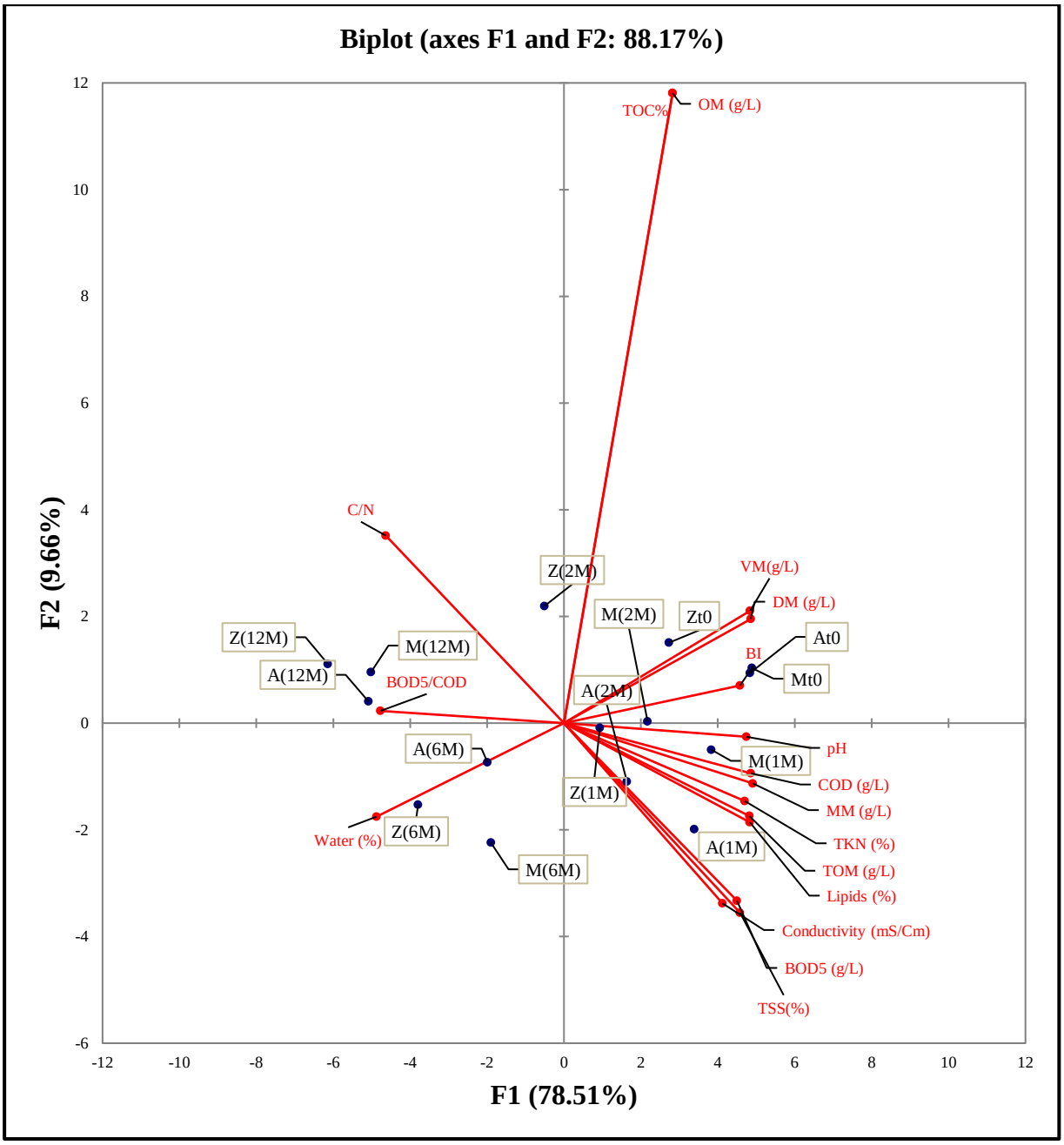

Figure 40: Projection of the interaction (Time × Variety) and parameters measured on the plane (1 × 2) of the principal component analysis (PCA).

Quantification and Qualification of Polyphenols

2.2. Polyphenols of OMW

This work presents the first qualitative and quantitative study of polyphenols from monovarietal OMW of the two varieties of olives (Zlitni and Abani) cultivated in Khenchela, eastern Algeria.

2.2.1. Quantitative study

Total polyphenols, flavonoids, total tannins, and condensed tannins contents in olive oil mill wastewater extracts were estimated using the calibration lines for gallic acid, quercetin, catechuic acid, and tannic acid, respectively **(Figures 15, 16, 17, and 18).** The measurement was being done in triplicate.

2.2.1.1. Effect of storage time on polyphenols

Mean squares of the analysis of variance of the storage effect of olive oil mill wastewater (OMW) on polyphenols were presented in **Table 6**.

Table 6: Mean squares of the analysis of variance of the storage effect on polyphenols from olive oil mill wastewater (OMW)

Source	DF	TPC (µg GAE/mL)	TFC (µg QE/mL)	CTC (µg TAE/mL)	TTC (µg CAE/mL)
Time effect	4	124457.47***	131.26***	1.644 ns	4289.41***
Variety effect	1	7873.20 ns	37.35 *	21.23***	368.39ns
Varieties × Time	4	5550.35 ns	9.22 ns	4.19 ns	286.24 ns
Error	20	7245.10	5.11	2.03	100.89

***: very highly significant at 5%, *: significant, ns: not significant

The two-factor analysis of variance **(Table 6)** revealed a very highly significant difference between time and the amount of polyphenols, flavonoids, and total tannins, but no significant difference between time and the content of condensed tannins. However, there is a very highly significant difference between the varieties studied in terms of condensed tannins, a significant difference between the varieties studied in terms of flavonoids, and a non-significant difference between the same varieties in terms of total polyphenols and total tannins. The ANOVA test of the (time × variety) interaction revealed that there are no significant difference in the content of polyphenols, flavonoids, total tannins, and condensed tannins between the varieties tested and the storage time.

2.2.1.2.Study of the time effect on polyphenols

Table 7 showed that TPC decreases from t0 to t1, and then marginally increases at t2. After that, it drops to t6 and eventually rises to the highest value at t12. TFC's content steadily diminishes over time up to t2 and then marginally increases. TTC rises little until t1, decreases until t6, and then rises slightly. For CTC, the content is nearly constant during the storage duration.

The comparison of the averages revealed that the polyphenol values are divided into 4 homogeneous groups (LSD = 102.51), the first of which is characterized by the highest level of polyphenols and which was recorded after 0 and 12 months of storage of the vegetable waters (923.51 ± 39.00 and 938.26 ± 49.36 µg GAE / mL respectively). Followed by the group which includes the polyphenols after 2 months of storage with (717.76 ± 88.15 µg GAE / mL), the third group comprises the content of total polyphenols 705.11 ± 80.09 µg GAE / mL obtained after 1 month of storage and finally the fourth group with the lowest content obtained after 6 months of storage of vegetable waters (613.12 ± 99.20 µg GAE / mL).

Regarding flavonoids (LSD = 2.72), the best content was recorded before storage (0 months) with (27.06 ± 4.35 µg QE / mL) followed by the content obtained after 1 month and 12 months with (20.33 ± 1.48 and 18.65 ± 1.13 µg QE / mL) respectively and finally the flavonoid contents obtained after 6 months and 2 months with (15.81 ± 0.56 and 15.58 ± 1.47 µg QE / mL) respectively.

The averages of the contents of condensed tannins are in the same group and do not show any significant difference.

The comparison between the averages of the total tannin contents revealed 3 homogeneous groups (LSD = 12.097). The first includes the total tannin contents of non-stored vegetable waters and those obtained after 1 month with (78.12 ± 10.35 and 78.48 ± 15.72 µg CAE / mL) respectively, followed by the group which includes total tannin contents obtained after 2 months of storage with (59.30 ± 2.72 µg CAE / mL) and the third group contains the total tannin contents obtained after 6 months and 12 months with (24.21 ± 3.03 and 26.33 ± 2.89 µg CAE / mL) respectively.

Table 7: Study of the time effect on polyphenols

Time	TPC (µg GAE/mL)	TFC (µg QE/mL)	TTC (µg CAE/mL)	CTC (µg TAE/mL)
t0	923.51 ± 39.00 [a]	27.06 ± 4.35 [a]	78.12 ± 10.35 [a]	6.10 ± 0.74
t1 (1M)	705.11 ± 80.09 [bc]	20.33 ± 1.48 [b]	78.48 ± 15.72 [a]	5.75 ± 1.05
t2 (2M)	717.76 ± 88.15 [b]	15.58 ± 1.47 [c]	59.30 ± 2.72 [b]	6.60 ± 1.45
t3 (6M)	613.12 ± 99.20 [c]	15.81 ± 0.56 [c]	24.21 ± 3.03 [c]	6.69 ± 1.92
t4 (12M)	938.26 ± 49.36 [a]	18.65 ± 1.13 [b]	26.33 ± 2.89 [c]	5.49 ± 0.40

[a, b, c]: homogeneous groups

2.2.1.3.Study of the effect of variety on polyphenols

The comparison of the means of the varieties studied revealed 2 homogeneous groups in the condensed tannins (LSD = 1.08) and two groups for the flavonoids (LSD = 1.72) on the other hand, the total polyphenols (LSD = 64.83) and the total tannins (LSD = 7.65) of the two varieties are in the same groups, which shows that there are no significant differences between the varieties studied with regard to the contents of total polyphenols and total tannins. The results obtained are presented in **(Table 8).**

Table 8: Study of the variety effect on polyphenols

Variety	TPC (µg GAE/mL)	TFC (µg QE/mL)	TTC (µg CAE/mL)	CTC (µg TAE/mL)
Abani	795.75 ± 53.73	18.37 ± 1.75 [b]	49.78 ± 4.78	6.97 ± 1.39 [a]
Zlitni	763.35 ± 88.59	20.60 ± 1.85 [a]	56.79 ± 9.11	5.29 ± 0.83 [b]

[a, b]: homogeneous groups

2.2.1.4.Study of the effect of the time variety interaction on polyphenols

The results obtained showed that the content of total polyphenol (TPC) and total flavonoid (TFC) does not remain constant throughout storage. Total tannin (TTC) and condensed tannin (CTC), on the other hand, change slightly. The comparison of the means of the interaction (time × varieties) showed that the means of each parameter measured with regard to the contents of total polyphenols, flavonoids, total tannins and condensed tannins is in the same group and that it there are no significant differences between the months of storage and the varieties studied.

2.2.1.4.1. Total phenolic content (TPC)

The results **(Figure 41)** showed that the content of total polyphenols in the vegetable waters of the Abani variety varies from (595.31 ± 42.69 µg GAE / mL) to (961.11 ± 65.96 µg GAE / mL) and for the variety Zlitni varies from (630.94 ± 155.72 µg GAE / mL) to (915.42 ± 32.45 µg GAE / mL). The vegetable waters of the Abani variety for storage time t12 have the highest total polyphenol content with a value equal to (961.11 ± 65.96 µg GAE / mL). On the other hand, for time t6 (595.30 ± 42.69 µg GAE / mL) represents the lowest content of phenolic compounds.

It is also observed that for the two varieties, the most significant content of total polyphenols was obtained after 12 months of storage (961.11 ± 65.96 µg GAE / mL for Abani, and 915.42 ± 32.45 µg GAE / mL for Zlitni). While the lowest content was obtained after 6 months of storage (595.31 ± 42.69 µg GAE / mL for Abani and 630.94 ± 155.72 µg GAE / mL for Zlitni).

The content of total phenolic compounds in OMW of Abani variety was as follows from the highest to the lowest values: t12 (961.11 ± 65.96 µg GAE / mL), t0 (931.94 ± 74.98 µg GAE / mL), t1 (769.06 ± 41.28 µg GAE / mL), t2 (721.36 ± 43.75 µg GAE / mL) and t6 (595.31 ± 42.69 µg GAE / mL). For the Zlitni variety, the recorded values were as follows: t12 (915.42 ± 32.45 µg GAE / mL), t0 (915.08 ± 3.03 µg GAE / mL), t2 (714.17 ± 132.55 µg GAE / mL), t1 (641.17 ± 118.91 µg GAE / mL) and t6 (630.94 ± 155.72 µg GAE / mL).

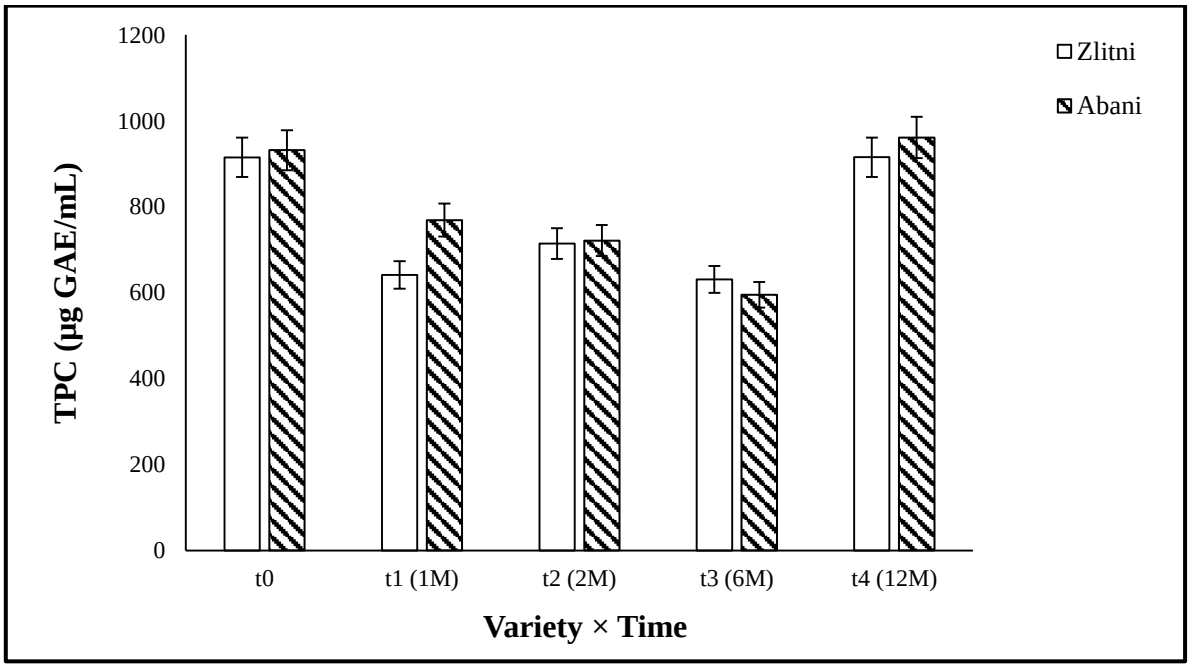

Figure 41: Total polyphenol content of OMW during storage

In comparison with other studies, the concentration of polyphenols in the olive mill waters studied is close to that found by **Gueboudji et al. (2021),** which is between (950 ± 14.2 µg GAE/mg of extract) and (778.1 ± 12.92 µg GAE/mg of extract). On the other hand, it was lower than the results found by **El Abbassi et al. (2012)**, which found (9.82 ± 0.3 g TYE/L - 6.11 ± 0.2 g TYE/L) for OMW from Marrakech, Morocco. **Degirmenbasi and Takac (2018)** found a (0.5 - 24 g/L) range for OMW from a three-phase centrifugation Taylieli olive oil company Burhaniye, Turkey. A range of (3.02 ± 0.18 to 5.20 ± 0.21 g/L gallic acid) is recorded by **Di Mauro et al. (2017)** for OMW from three-phase olive oil mill processing system located in Menfi and Mascalucia, Italy. Recent studies are found a range of (788.96 ± 1.41 mg / 100mL) recorded by **Romeo et al. (2020)** for OMW from a three-phase centrifugation process from Ottobratica olive cultivar, Calabrian region, Italy, and (0.65 ± 0.36 g / L) found by **Bombino et al. (2021)** for OMW from Calabria, Southern Italy.

2.2.1.4.2. Total flavonoid content (TFC)

Figure 42 showes the total flavonoid content of OMW during the storage. The flavonoid content in the vegetable waters studied varies from (13.85 ± 0.1 µg QE / mL) to (27.96 ± 3.71 µg QE / mL). The results show that the content of total flavonoids in the vegetable waters of the Abani variety varies from (13.85 ± 0.1 to 26.16 ± 4.99 µg QE / mL), and for the variety Zlitni varies from (15.36 ± 1.7 to 27.96 ± 3.71 µg QE / mL). Zlitni at t0 had the highest total polyphenol content with a value equal to (27.96 ± 3.71 µg QE / mL). Abani at t6M had a low content of total flavonoids (13.85 ± 0.1 µg QE / mL).

It is also observed that for the two varieties, the highest content of total polyphenols was obtained after 0 months of storage (26.16 ± 4.99 µg QE / mL) for Abani, and (27.96 ± 3.71 µg QE / mL) for Zlitni. On the contrary, the lowest content was obtained after 6 months of storage (13.85 ± 0.1 µg QE / mL) for Abani and after 2 months for Zlitni (15.36 ± 1.7 µg QE / mL).

The total flavonoid content of olive oil mill wastewaters of the Zlitni variety was as follows from the highest to the lowest values: t1 (27.96 ± 3.71 µg QE / mL), t12 (21.42 ± 1.22 µg QE / mL), t1 (20.52 ± 1.6 µg QE / mL), t6 (17.77 ± 1.02 µg QE / mL) and t2 (15.36 ± 1.7 µg QE / mL). For the Abani variety, the values recorded were as follows: t0 (26.16 ± 4.99 µg QE / mL), t1 (20.15 ± 1.37 µg QE / mL), t12 (15.89 ± 1.05 µg QE / mL), t2 (15.81 ± 1.25 µg QE / mL) and t6 (13.85 ± 0.1 µg QE / mL).

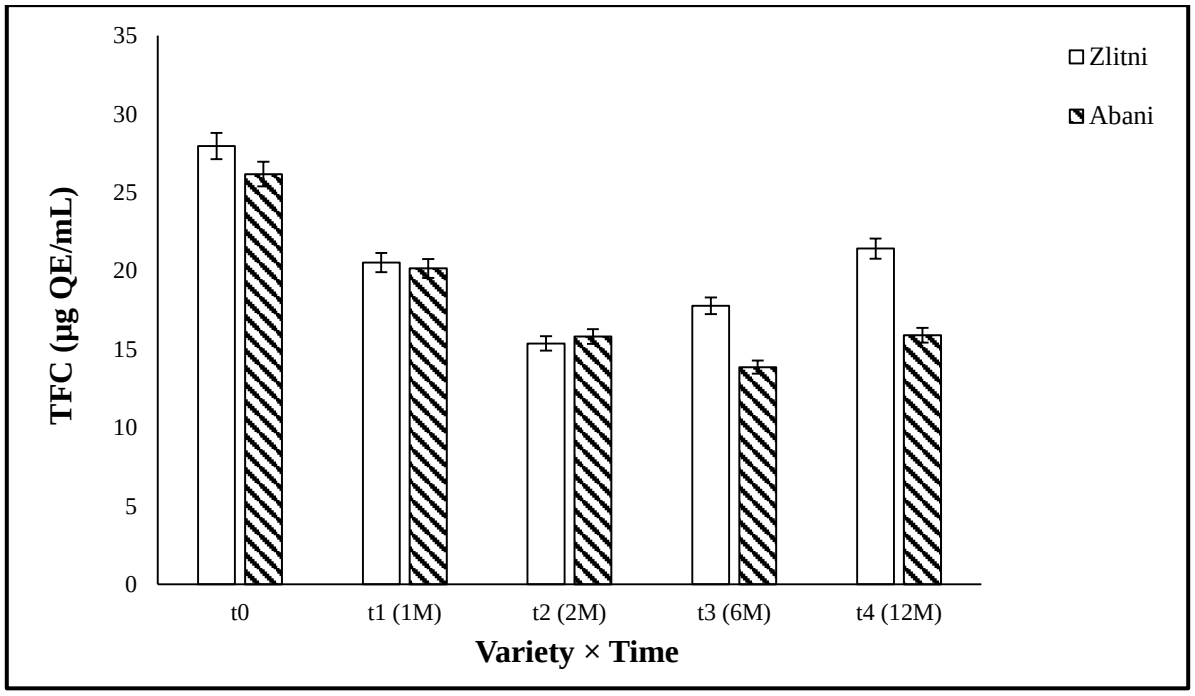

Figure 42: Total flavonoid content of OMW during storage

In this study, the flavonoid content is lower than that found by **El Abbassi et al. (2012)**, which was in a range of (6.56 ± 0.21 - 2.71 ± 0.14 CAE g /L), for OMW from Marrakech, Morocco. In addition, the range (0.95 ± 0.17 - 2.28 ± 0.23 g/L catechin) recorded by **Di Mauro et al. (2017)** for OMW from a three-phase olive oil mill processing system located in Menfi and Mascalucia, Italy. **Gueboudji et al. (2021)** found a range of (80.6 ± 17.27 - 43.9 ± 9.77 µg QE / mg of extract) for OMW from a modern olive oil mill located in Khenchela, Algeria.

2.2.1.4.3. Total tannin content (TTC)

Figure 43 represents to total tannin content of OMW during storage. The total tannin content of vegetable waters varied between (21.08 ± 0.64 µg of CAE / mL) and (90.47 ± 21.24 µg of CAE / mL). The vegetable water of the Zlitni variety contains the highest content (90.47 ± 21.24 µg of CAE / mL) and the lowest content with a value of (21.08 ± 0.64 µg CAE / mL).

It was also observed that the highest total tannin content was obtained after 0 months of storage (69.07 ± 0.01 µg CAE/ mL) for Abani, and after 1 month of storage for Zlitni (90.47 ± 21.24 µg CAE/ mL). While, the lowest content was obtained after 12 months of storage for Abani (23.62 ± 4.14 µg CAE/ mL) and after 6 months for Zlitni (21.08 ± 0.64 µg CAE / mL).

The total tannin content of vegetable waters of the Zlitni variety was as follows from the highest to the lowest values: t1 (90.47 ± 21.24 CAE / mL), t0 (87.17 ± 19.7 CAE / mL), t2 (56.2 ± 2.34 CAE / mL), t12 (29.05 ± 1.65 CAE / mL) and t6 (21.08 ± 0.64 µg CAE / mL). For the Abani variety, the total tannin content decreases over time. They were as follows: t1 (69.07 ± 1.01 µg CAE / mL), t1 (66.51 ± 10.21 µg CAE / mL), t2 (62.4 ± 3.11 µg CAE / mL), t6 (27.34 ± 5.43 µg CAE / mL) and t12 (23.62 ± 4.14 µg CAE / mL). These results are in agreement with those obtained by **Gueboudji et al. (2021d)** for the same olive oil extraction process. **Aggoun (2016)** was showed that for the quantification test for total tannins via their ability to precipitate bovine serum albumin (BSA), it is not detectable in phenolic extracts of OMW from 15 units located in 4 regions (Constantine, Guelma, Skikda, and Batna) in Algeria.

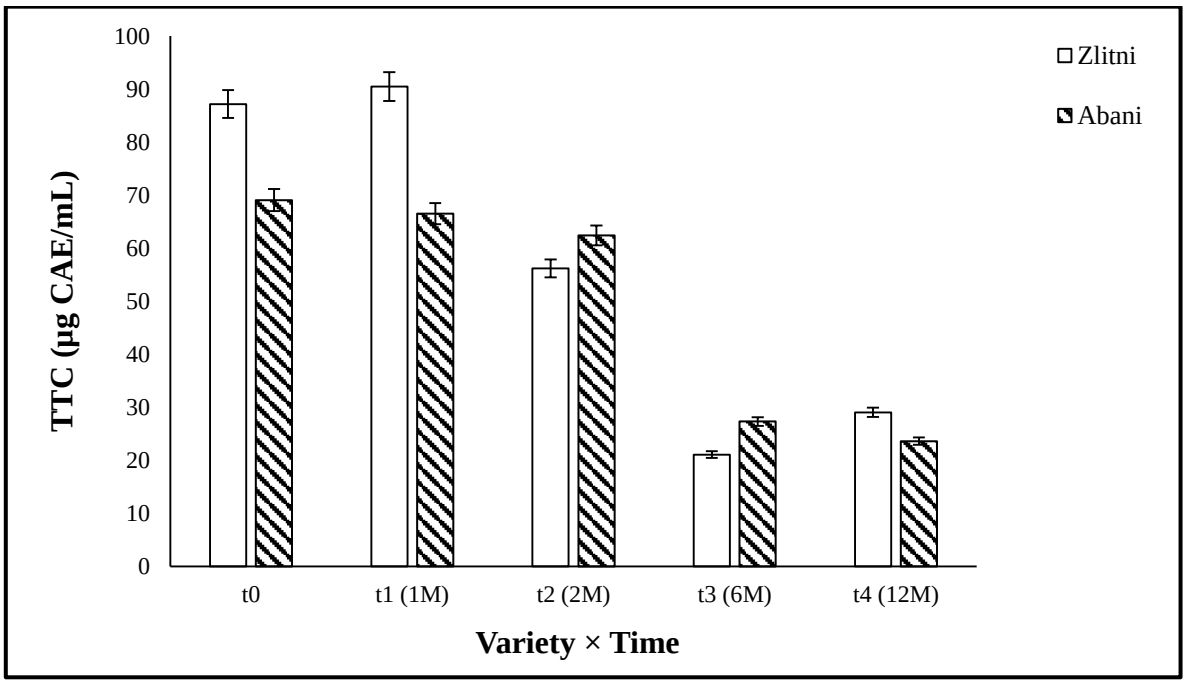

Figure 43: Total tannin content of OMW during storage

2.2.1.4.4. Condensed tannin content (CTC)

Figure 44 represents the condensed tannin content of OMW during storage. The content of condensed tannins in vegetable waters varies between (4.63 ± 0.81 µg TAE / mL) and (8.66 ± 3.22 µg of TAE / mL). The vegetable water of the variety Abani contains the highest content (8.66 ± 3.22 µg TAE / mL), and the lowest content was recorded for Zlitni with a value of (4.63 ± 0.81 µg of TAE / mL).

It is also observed that the highest content of condensed tannins was obtained after 6 months of storage (8.66 ± 3.22 µg TAE / mL) for Abani and after 2 months of storage for Zlitni

(6.24 ± 2.11 µg TAE/ mL). While, the lowest content was obtained after 12 months of storage for Abani (5.49 ± 0.62 µg TAE / mL) and after 0 months for Zlitni (4.63 ± 0.81 µg TAE/ mL).

The condensed tannin content of vegetable waters of the Abani variety was as follows from the highest to the lowest values: t6 (8.66 ± 3.22 µg TAE / mL), t0 (7.59 ± 0.66 µg TAE / mL), t2 (6.98 ± 0.8 µg TAE / mL), t1 (6.16 ± 1.65 µg TAE / mL) and t12 (5.49 ± 0.62 µg TAE / mL). For the Zlitni variety, the content of the condensed tannins were as follows: t2 (6.24 ± 2.11 µg TAE / mL), t12 (5.51 ± 0.19 µg TAE / mL), t1 (5.35 ± 0.44 µg TAE / mL), t6 (4.74 ± 0.61 µg TAE / mL) and t0 (4.63 ± 0.81 µg TAE / mL). In this study, the results obtained are corroborated their showed by **Aggoun (2016)** that found it in the range of (2.93 – 5.29 g /L) for OMW from 15 units located in 4 regions (Constantine, Guelma, Skikda and Batna) in Algeria.

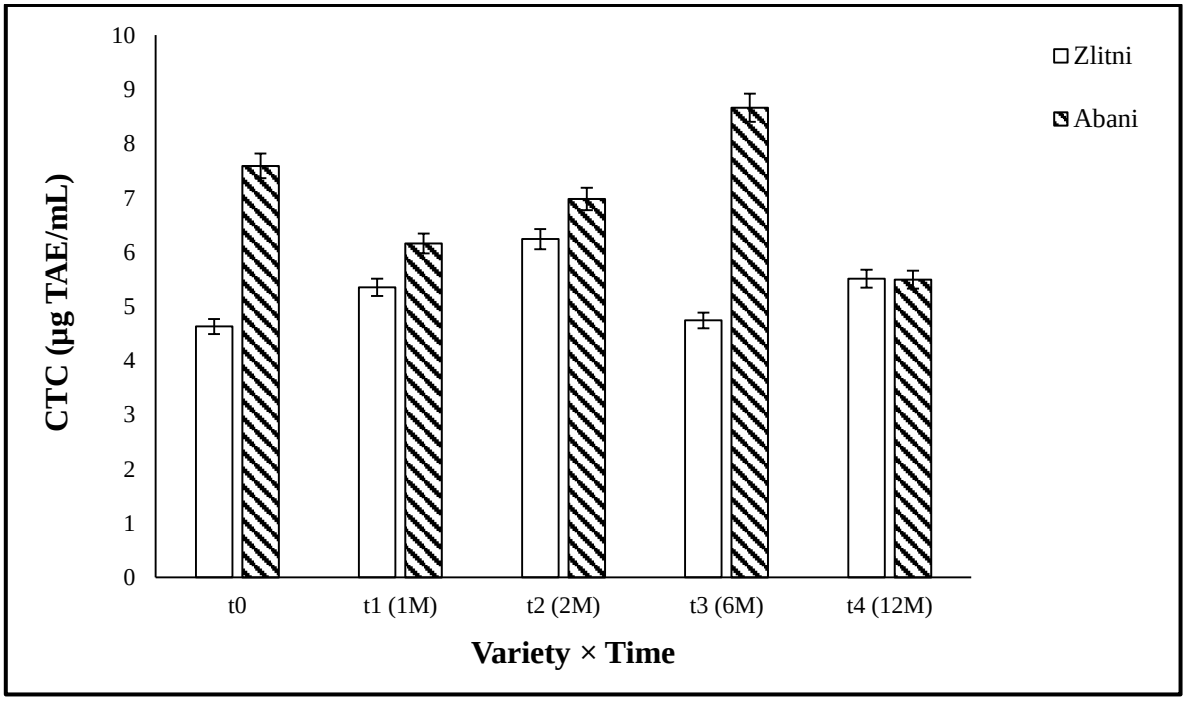

Figure 44: Condensed tannin content of OMW during storage

This variation in the phenolic content of OMW is caused by several factors, which are: climatic and geographical conditions **(Ziogas et al., 2010),** the physiological state and age of the plant **(Deleonardis et al., 2008),** the temperature of olives before and during grinding influence on the solubilization of polyphenols **(Caponio and Catalano, 2001),** the variety of the olive tree, the growing conditions and the degree of ripening of the olives **(Ziogas et al., 2010).** In general, the polyphenol content varies between (0.63% to 5.45%) **(Zbakh and EL Abbassi, 2012; Dermeche et al., 2013).**

2.2.2. Qualitative study

2.2.2.1. Storage time effect on LC-MS of the polyphenols

The composition of the retention times of the compounds found in the phenolic extract of vegetable waters with those of the different standards made it possible to highlight twenty-eight (28) phenolic compounds. The OMW samples showed different levels of phenolic compounds depending on the variety and/or the storage time **(Table 9).**

Among the thirty-one (31) phenolic compounds from OMW chosen for analysis, 3,4-di-O-caffeoyquinic acid, Luteolin and, Cirsilineol were not quantified since their respective concentrations in the samples were less than the limit of quantification or detection of the assay conditions. 28 phenolic compounds were detected in extracts of Zlitni, and 26 phenolic compounds in extracts of the variety Abani. Based on the mean values of the concentration of the twenty-eight (28) phenolic compounds quantified in all OMW samples, kampherol was the major phenolic compound. These 28 quantified phenolic compounds are: kampherol, quinic acid, gallic acid, protocatechuic acid, catechin, caffeic acid, syringic acid, 1,3-di-o-caffeoyquinic acid, epicatechin, p-coumaric acid, rutin, trans frulic acid, hyperoside (quercetin-3-o-galactoside), luteolin-7-o-glucoside, 4,5-di-o-caffeoyquinic acid, naringin, rosmarinic acid, quercetrin (quercetin-3-o-rhamnoside), apegenin-7- o-glucoside, o-coumaric acid, salviolinic acid, quercetin, trans cinnamic, naringenin, apegenin, cirsiliol, acacetin, and chlorogenic acid. The chemical structure of the phenolic compounds detected in the OMW studied was shown in **(Annex 3)**.

The highest value obtained for kampherol was recorded for storage time t12 for the OMWs of the two varieties Abani (2361.58 ± 767.86 ppm) and Zlitni (2088.7 ± 419.01 ppm), respectively. It is also observed that there is the appearance of four phenolic acids in all the samples in addition to kempherol, which are quercetin, apegenin, rutin, and cirsiliol. In addition, it was detected rosmarinic acid in extracts of the Abani variety during times t0 and t12 with successively the values (28.42 ± 49.22 ppm and 112.03 ± 9.33 ppm). Rosmarinic acid is a phenolic acid that was first detected in olive oil mill wastewater in this study. In contrast, catechin (+) and syringic acid were only found in extracts from Zlitni. Catechin + (0.98 ± 1.69 ppm) was detected for storage time t2, and syringinic acid was detected for times t1 (18.7 ± 32.38 ppm) and t6 (36.5 ± 63.22 ppm). Note that the lowest value was quantified during the time t6 for the two varieties. This value was apegenin (0.36 ± 0.62 ppm) for the Zlitni extracts and acetin (0.02 ± 0.03 ppm) for the Abani extracts.

Two-way statistical analysis of variance (ANOVA) **(Table 9)** showed that there is a very highly significant difference between the times with respect to; quinic acid, caffeic acid, 1,3-di-o-caffeoyquinic acid, rutin, hyperoside (quercetin-3-o-galactoside), rosmarinic acid 4,5-di-o-caffeoyquinic acid, quercetrin (quercetin-3-o-rhamnoside), apegenin-7-o-glucoside, naragenin, cirsiliol and chlorogenic acid. On the other hand, there is a non-significant difference regarding; gallic acid, protocatechuic acid, catechin (+), syringic acid, trans frulic acid, luteolin-7-o-glucoside. In addition, it showed that there is no significant difference for epicatechin, naringin, o-coumaric acid, salviolinic acid, kampherol, quercetin, trans-cinnamic, and apegenin.

For the variety effect, statistical analysis of variance showed a very highly significant difference for quinic acid, 1,3-di-o-caffeoyquinic acid, and rosmarinic acid. In addition, there is a highly significant difference between caffeic acid and naringenin. While it showed a non-significant difference with regard to Gallic acid, protocatechuic acid, catechin (+), syringic acid, trans-frulic acid, and luteolin-7-o-glucoside. For the rest of the phenolic compounds which are: epicatechin, p-coumaric acid, rutin, hyperoside (quercetin-3-o-galactoside), naringin, 4,5-di-o-caffeoyquinic acid, quercetrin (quercetin-3-o- rhamnoside), apegenin-7-o-glucoside, o-coumaric acid, salviolinic acid, kampherol, quercetin, trans cinnamic, apegenin, cirsiliol, acacetin, and chlorogenic acid.

The variance analysis of the variety time interaction showed a very highly significant difference with regard to quinic acid, caffeic acid, 1,3-di-o-caffeoyquinic acid, hyperoside (quercetin -3-o-galactoside) rosmarinic acid, 4,5-di-o-caffeoyquinic acid, quercetrin (quercetin-3-o-rhamnoside), quercetin, naringenin, and cirsiliol. In addition, there is a highly significant difference for apegenin-7-o-glucoside, and a non-significant difference for Gallic acid, protocatechuic acid, catechin (+), syringic acid, trans frulic acid. On the other hand, there is no significant difference with regard to epicatechin, p-coumaric acid, rutin, luteolin-7-o-glucoside, naringin, o-coumaric acid, salviolinic acid, kampherol, Trans cinnamic, apegenin, acacetin, and chlorogenic acid.

Table 9: ANOVA tow ways of storage effect on LC-MS of the phenolic extract of OMW

Source	Time effect	Variety effect	Variety × Time	Error
DF	4	1	4	20
Quinic acid	309892.44***	27895.17***	103074.935***	939.365
Gallic acid	2001.87 ^{ns}	617.63 ^{ns}	1049.64 ^{ns}	1979.56
Protocatechuic acid	833772.36 ^{ns}	1040297.05 ^{ns}	1445991.33 ^{ns}	1550088.67
Catechin (+)	0.28 ^{ns}	0.28 ^{ns}	0.28 ^{ns}	0.28
Caffeic acid	11227.03***	1001.88**	1284.93***	75.94
Syringic acid	402.13 ^{ns}	913.93 ^{ns}	402.13 ^{ns}	504.49
1,3-di-O-caffeoyquinic acid	95.84***	107.29***	100.30***	1.09
Epicatechin	255.08	48.39	275.14	209.98
p-coumaric acid	9054.73**	1177.97	3093.03	1528.87
Rutin	9.89***	1.11	0.35	0.28

(*Continued*)

Trans frulic acid	10.07 ^{ns}	22.36 ^{ns}	10.48 ^{ns}	8.65
Hyperoside (quercetin-3-o-galactoside)	32.73***	1.52	9.25***	0.31
Luteolin-7-o-glucoside	238.90 ^{ns}	0.88 ^{ns}	9.88	1.67
3,4-di-O-caffeoyquinic acid	N.D	N.D	N.D	N.D
Naringin	1130.00	69.01	190.15	6.21
Rosmarinic acid	3529.85***	5917.52***	3529.85***	250.97
4,5-di-O-caffeoyquinic acid	77372.30***	39759.28	96585.47***	6842.63
Quercetrin (quercetin-3-o-rhamnoside)	90.11***	1.71	96.98***	0.67
Apegenin-7-o-glucoside	13.31***	0.21	0.87**	0.09
O-coumaric acid	35.29	2.13	68.62	55.32
Salviolinic acid	2210.55	211.51	430.14	10.86
Kampherol	1447430.58	108322.44	192353.43	85434.57

(*Continued*)

Quercetin	4.97	0.67	17.73***	1.83
Trans cinnamic	94334.37	111821.01	71037.90	91457.30
Naringenin	51.23***	81.11**	75.55***	29.63
Apegenin	21216.51	465.76	1716.31	468.13
Luteolin	N.D	N.D	N.D	N.D
Cirsiliol	4744.99***	1859.47	4235.22***	233.34
Cirsilineol	N.D	N.D	N.D	N.D
Acacetin	0.52	0.51	0.53	0.52
Chlorogenic acid	40.57***	1.62	14.88	4.59
Total (ppm)	2369563.21	806790.78	1339724.21	1590058.84

ns, ** and ***: effect not significant, highly significant and very highly significant at 5%, 1% and 1 ‰ respectively

2.2.2.2.Time effect on LC-MS analysis of polyphenols

The comparison of the averages of the phenolic compounds for the five storage times was distributed in the homogeneous groups, as shown in **(Table 10).**

Table 10: Time effect on LC-MS analysis of polyphenols

Time / Polyphenols	t 0	t (1M)	t (2M)	t (6M)	t (12M)	LSD
Quinic acid	601.72 ± 27.62 [a]	270.41 ± 17.98 [b]	246.94 ± 43.40 [b]	17.85 ± 27.69 [d]	80.35 ± 18.28 [c]	36.91
Gallic acid	11.69 ± 20.24	4.05 ± 7.02	46.27 ± 75.70	N.D	16.92 ± 29.31	53.58
Protocatechuic acid	371.2 ± 41.39	1075.4 ± 1862.60	128.75 ± 26.84	396.98 ± 49.05	178.2 ± 178.88	1425.5
Catechin	N.D	0.48 ± 0.84	N.D	N.D	N.D	0.64
Caffeic acid	105.90 ± 7.59 [a]	40.83 ± 5.21 [c]	62.33 ± 11.44 [b]	6.93 ± 6.03 [d]	N.D	10.49
Syringic acid	N.D	9.35 ± 16.19	N.D	18.25 ± 31.61	N.D	27.05
3-Di-O-caffeoyquinic acid	N.D	N.D	N.D	2.99 ± 0.28 [b]	9.20 ± 1.64 [a]	1.26
Epicatechin	13.20 ± 14.16	N.D	N.D	1.95 ± 3.38	11.63 ± 20.15	17.45
p-Coumaric acid	98.76 ± 11.37 [a]	29.12 ± 5.52 [b]	N.D	42.10 ± 72.92 [b]	9.21 ± 15.95 [b]	47.09
Rutin	3.70 ± 0.41 [b]	3.36 ± 0.72 [b]	3.74 ± 0.49 [b]	4.88 ± 0.59 [a]	1.35 ± 0.28 [c]	0.64
Trans frolic acid	3.157 ± 4.05	2.45 ± 0.45	N.D	1.98 ± 3.44	0.66 ± 1.16	3.54
Hyperoside-quercetin-3-o-galactoside	3.89 ± 0.19 [b]	5.25 ± 0.94 [a]	2.29 ± 0.09 [c]	N.D	N.D	0.67

(*Continued*)

Luteolin-7-o-glucoside	15.61 ± 1.09 [a]	13.27 ± 1.20 [b]	10.41 ± 1.84 [c]	1.34 ± 0.10 [d]	2.90 ± 0.37 [d]	1.56
4-Di-O-caffeoyquinic-acid	N.D	N.D	N.D	N.D	N.D	N.D
Naringin	36.66 ± 1.13 [a]	22.12 ± 1.92 [b]	13.23 ± 1.37 [c]	N.D	10.83 ± 3.46 [c]	3.00
Rosmarinic acid	14.20 ± 24.61 [b]	N.D	N.D	N.D	56.01 ± 4.67 [a]	19.07
5-Di-O-caffeoyquinic acid	N.D	258.58 ± 70.95 [a]	62.99 ± 109.10 [b]	171.23 ± 18.39 [a]	N.D	99.62
Quercetin-3-o-rhamnoside	3.98 ± 0.43 [b]	10.85 ± 1.49 [a]	10.11 ± 0.54 [a]	3.51 ± 0.42 [bc]	2.89 ± 0.34 [c]	0.98
Apegenin-7-o-glucoside	3.31 ± 0.24 [a]	2.28 ± 0.21 [b]	0.32 ± 0.28 [b]	0.17 ± 0.31 [c]	N.D	0.36
O-Coumaric acid	1.16 ± 2.02	N.D	4.64 ± 8.05	4.81 ± 8.34	N.D	8.95
Salviolinic acid	45.5 ± 3.40 [a]	25.88 ± 6.34 [b]	15.7 ± 1.06 [b]	N.D	N.D	3.96
Kampherol	1137.8 ± 106.55 [c]	1186.2 ± 103.08 [c]	1543.2 ± 136.70 [b]	1011.1 ± 29.89 [c]	2225.1 ± 593.43 [a]	352.02
Quercetin	5.79 ± 0.87 [a]	3.41 ± 1.44 [b]	3.75 ± 0.56 [b]	4.22 ± 1.57 [ab]	4.18 ± 1.01 [ab]	1.63
Trans cinnamic	N.D	289.0 ± 500.51	N.D	N.D	54.1 ± 93.76	364.21
Naringenin	1.96 ± 0.87 [ab]	2.98 ± 2.00 [ab]	2.38 ± 2.16 [ab]	7.87 ± 8.20 [a]	N.D	6.55
Apegenin	99.56 ± 18.03 [b]	96.06 ± 7.98 [b]	113.24 ± 9.18 [b]	105.11 ± 11.06 [b]	235.67 ± 35.88 [a]	26.05
Luteolin	N.D	N.D	N.D	N.D	N.D	N.D
Cirsiliol	94.48 ± 4.02 [b]	62.68 ± 11.63 [c]	50.49 ± 7.55 [c]	53.14 ± 5.74 [c]	114.41 ± 24.82 [a]	18.39
Cirsilineol	N.D	N.D	N.D	N.D	N.D	N.D

(*Continued*)

Acacetin	N.D	N.D	N.D	0.007 ± 0.01	0.66 ± 1.15	0.87
Chlorogenic acid	6.53 ± 3.87 [a]	2.47 ± 0.47 [bc]	4.13 ± 1.80 [ab]	0.96 ± 0.84 [c]	N.D	2.58
Total (ppm)	2679.8 ± 88.38 [ab]	3416.0 ± 2324.76 [a]	2301.4 ± 316.48 [ab]	1790.9 ± 200.67 [b]	3014.4 ± 560.50 [ab]	1518.6

[a, b, c, d]: homogeneous groups

The comparison of the averages of the total means of the phenolic compounds of OMW studied was divided into 3 homogeneous groups (LSD = 1518.6). The first group of which is characterized by the highest content recorded after 1 month of storage of OMW (3416.0 ± 2324.76 ppm), followed by the second group recorded after 6 months of storage (1790.9 ± 200.67 ppm). Finally, the third group includes the lowest content, which was recorded in the times t0 (2679.8 ± 88.38 ppm), t2 (2301.4 ± 316.48 ppm), and t12 (3014.4 ± 560.50 ppm).

The comparison of the mean of kampherol, which was the most abundant phenolic compound in the extracts of the vegetable waters studied, is divided into three groups (LSD = 352.02). The first group is characterized by the highest content (2225.1 ± 593.43 ppm) obtained after 12 months of storage, followed by the second group obtained after 2 months of storage with a value of (1543.2 ± 136.70 ppm). The third group represented the lowest content, found during times t0 (1137.8 ± 106.55 ppm), t1 (1186.2 ± 103.08 ppm), and finally t6 (1011.1 ± 29.89 ppm).

2.2.2.3.Variety effect on LC-MS analysis of polyphenols

Comparing the means from olive oil mill wastewetr (OMW) of the two studied varieties revealed the homogeneous groups shown in **(Table 11).**

Table 11: Variety effect on LC-MS analysis of polyphenols

Polyphenols	Zlitni	Abani	LSD
Quinic acid	212.96 ± 31.78 [b]	273.95 ± 22.21 [a]	23.34
Gallic acid	11.25 ± 17.70	20.32 ± 35.20	33.88
Protocatechuic acid	234.79 ± 63.29	625.40 ± 800.21	901.59
Catechin	0.19 ± 0.34	N.D	0.40
Caffeic acid	48.98 ± 7.35 [a]	37.42 ± 4.76 [b]	6.63
Syringic acid	11.03 ± 19.12	N.D	17.10
3-di-O-caffeoyquinic acid	0.54 ± 0.05 [b]	4.33 ± 0.72 [a]	0.79
Epicatechin	4.08 ± 3.59	6.62 ± 11.48	11.03
p-coumaric acid	29.57 ± 10.09	42.10 ± 32.21	29.78
Rutin	3.21 ± 0.41	3.60 ± 0.59	0.40
Trans frulic acid	2.51 ± 2.25	0.79 ± 1.37	2.24
Hyperoside-quercetin-3-o-galactoside	2.51 ± 0.39 [a]	2.06 ± 0.11 [b]	0.42
Luteolin-7-o-glucoside	8.54 ± 0.49	8.88 ± 1.35	0.98
4-di-O-caffeoyquinic acid	N.D	N.D	N.D
Naringin	15.05 ± 0.80 [b]	18.08 ± 2.35 [a]	1.89
Rosmarinic acid	N.D	28.08 ± 11.71 [a]	12.06
5-di-O-caffeoyquinic acid	134.96 ± 32.34 [a]	62.16 ± 47.03 [b]	63.00
Quercetin-3-o-rhamnoside	6.51 ± 0.59	6.03 ± 0.71	0.62
Apegenin-7-o-glucoside	1.30 ± 0.23	1.13 ± 0.19	0.22
o-coumaric acid	1.85 ± 3.22	2.39 ± 4.14	5.66
Salviolinic acid	20.08 ± 1.77 [a]	14.77 ± 2.55 [b]	2.51
Kampherol	1360.6 ± 160.49	1480.8 ± 227.37	222.63
Quercetin	4.12 ± 0.94	4.42 ± 1.24	1.03
Trans cinnamic	129.7 ± 224.60	7.6 ± 13.11	230.35
Naringenin	1.39 ± 1.13	4.68 ± 4.63	4.14
Apegenin	125.98 ± 10.45	133.86 ± 22.40	16.48
Luteolin	N.D	N.D	N.D
Cirsiliol	67.17 ± 9.16 [b]	82.91 ± 12.35 [a]	11.63
Cirsilineol	N.D	N.D	N.D

(*Continued*)

Acacetin	0.003 ± 0.01	0.26 ± 0.46	0.55
Chlorogenic acid	2.58 ± 0.73	3.05 ± 2.07	1.63
Total (ppm)	2476.5 ± 359.24	2804.5 ± 1037.08	960.47

[a, b]: homogeneous groups

Comparison of the means of OMW of the varieties studied revealed 2 homogeneous groups in: quinic acid (LSD = 23.34), caffeic acid (LSD = 6.63), 1,3-di-o-caffeoyquinic acid (LSD = 0.79), hyperoside quercetin 3-o-galactoside (LSD = 0.42), naringin (LSD = 1.89), 4,5-di-o-caffeoyquinic acid (LSD = 63.00), salviolinic acid (LSD = 2.51), and cirsiliol (LSD = 11.63). It revealed only one group in rosmarinic acid (LSD = 12.06), which was detected in extracts of the Abani variety only. The rest of the phenolic compounds detected in the extracts of the studied vegetable waters are in the same group, which shows the non-significant difference or the absence of significance between the two varieties studied for these phenolic compounds.

2.2.2.4. Interaction effect of time and variety on the chromatographic profile of the phenolic extract of OMW

The interaction effect of time and varieties on the chromatographic profile of the phenolic extract of OMW is illustrated in **Table 12.**

Table 12: Interaction effect of time and varieties on the chromatographic profile of the phenolic extract of OMW

Variety	**Zlitni**					**Abani**				
Time	**t 0**	**t (1M)**	**t (2M)**	**t (6M)**	**t (12M)**	**t 0**	**t (1M)**	**t (2M)**	**t (6M)**	**t (12M)**
Quinic acid	854.54 ± 39.31^{a}	225.61 ± 14.46^{c}	277.71 ± 41.36^{c}	11.87 ± 15.90^{f}	N.D	348.90 ± 15.93^{b}	315.21 ± 21.49^{b}	216.16 ± 45.43^{d}	23.82 ± 39.47^{f}	160.71 ± 36.57^{e}
Gallic acid	16.65 ± 28.84	N.D	18.93 ± 23.89	N.D	20.67 ± 35.79	6.72 ± 11.64	8.10 ± 14.03	73.62 ± 127.51	N.D	13.18 ± 22.83
Protocatechuic acid	487.35 ± 58.47	N.D	112.90 ± 30.69	399.25 ± 72.66	174.46 ± 154.64	254.96 ± 24.30	2150.75 ± 3725.20	144.61 ± 22.99	394.71 ± 25.44	181.98 ± 203.12
Catechin (+)	N.D	N.D	0.98 ± 1.69	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Caffeic acid	135.21 ± 12.66^{a}	43.84 ± 8.13^{c}	51.98 ± 3.88^{c}	13.87 ± 12.06^{f}	N.D	76.59 ± 2.53^{b}	37.83 ± 2.29^{b}	72.70 ± 19.00^{b}	N.D	N.D
Syringic acid	N.D	18.70 ± 32.38	N.D	36.5± 63.22	N.D	N.D	N.D	N.D	N.D	N.D
1,3-di-O-caffeoyquinic acid	N.D	N.D	N.D	2.74 ± 0.27^{b}	N.D	N.D	N.D	N.D	3.24 ± 0.29^{b}	18.41 ± 3.29^{a}

(*Continued*)

Epicatechin	20.44 ± 17.97	N.D	N.D	N.D	N.D	5.97 ± 10.35	N.D	N.D	3.90 ± 6.76	23.27 ± 40.30
p-coumaric acid	70.64 ± 6.58	58.24 ± 11.03	N.D	18.97 ± 32.85	N.D	126.87 ± 16.15	N.D	N.D	65.23 ± 112.99	18.42 ± 31.90
Rutin	3.47 ± 0.23	3.21 ± 0.59	3.75 ± 0.30	4.88 ± 0.66	0.77 ± 0.24	3.94 ± 0.59	3.52 ± 0.84	3.73 ± 0.69	4.89 ± 0.51	1.94 ± 0.32
Trans frulic acid	3.70 ± 3.57	4.91 ± 0.81	N.D	3.97 ± 6.88	N.D	2.61 ± 4.52	N.D	N.D	N.D	1.34 ± 2.32
Hyperoside (quercetin-3-o-galactoside)	2.53 ± 0.06^{c}	7.35 ± 1.72^{a}	2.69 ± 0.14^{c}	N.D	N.D	5.25 ± 0.32^{b}	3.15 ± 0.17^{c}	1.91 ± 0.04^{d}	N.D	N.D
Luteolin-7-o-glucoside	14.19 ± 0.99^{b}	14.61 ± 2.34^{b}	10.92 ± 2.80^{c}	2.70 ± 0.20^{d}	2.00 ± 0.40^{d}	17.05 ± 1.18^{a}	11.93 ± 0.06^{c}	9.91 ± 0.87^{c}	N.D	3.82 ± 0.35^{d}
3,4-di-O-caffeoyquinic acid	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Naringin	35.37 ± 0.61^{a}	26.51 ± 2.01^{b}	13.4 ± 1.37^{d}	N.D	N.D	37.97 ± 1.64^{a}	17.74 ± 1.83^{c}	13.07 ± 1.38^{d}	N.D	21.66 ± 6.92^{c}
Rosmarinic acid	N.D	N.D	N.D	N.D	N.D	28.42 ± 49.22^{b}	N.D	N.D	N.D	112.03 ± 9.33^{a}

(*Continued*)

4,5-di-O-caffeoyquinic acid	N.D	517.17 ± 141.90[a]	N.D	157.66 ± 19.82[b]	N.D	N.D	N.D	125.98 ± 218.20[b]	184.80 ± 16.96[b]	N.D
Quercetrin (quercetin-3-o-rhamnoside)	7.97 ± 0.86[c]	5.33 ± 1.21[d]	14.29 ± 0.28[b]	2.77 ± 0.22[f]	2.20 ± 0.35[f]	N.D	16.39 ± 1.78[a]	5.94 ± 0.80[d]	4.26 ± 0.62[e]	3.58 ± 0.34[e]
Apegenin-7-o-glucoside	3.20 ± 0.27[a]	2.97 ± 0.26[a]	N.D	0.36 ± 0.62[c]	N.D	3.42 ± 0.21[a]	1.60 ± 0.15[b]	0.65 ± 0.57[c]	N.D	N.D
o-coumaric acid	N.D	N.D	9.29 ± 16.09	N.D	N.D	2.33 ± 4.04	N.D	N.D	9.62 ± 16.67	N.D
Salviolinic acid	41.86 ± 2.75[b]	43.44 ± 5.41[b]	15.14 ± 0.68[c]	N.D	N.D	49.27 ± 4.06[a]	8.34 ± 7.26[d]	16.27 ± 1.44[c]	N.D	N.D
Kampherol	1348.35 ± 63.70	938.97 ± 134.60	1563.52 ± 142.18	863.48 ± 42.93	2088.70 ± 419.01	927.21 ± 149.39	1433.51 ± 71.56	1522.96 ± 131.22	1158.66 ± 16.85	2361.58 ± 767.86
Quercetin	8.63 ± 1.15[a]	2.75 ± 0.26[c]	2.79 ± 0.08[c]	2.71 ± 2.44[c]	3.74 ± 0.77[b]	2.95 ± 0.60[c]	4.09 ± 2.63[b]	4.72 ± 1.04[b]	5.73 ± 0.70[b]	4.64 ± 1.26[b]
Trans cinnamic	N.D	540.10 ± 935.47	N.D	N.D	108.27 ± 187.52	N.D	37.84 ± 65.54	N.D	N.D	N.D
Naringenin	0.74 ± 1.28	3.58 ± 1.89	2.67 ± 2.50	N.D	N.D	3.20 ± 2.82	2.39 ± 2.12	2.10 ± 1.82	15.74 ± 16.40	N.D

(*Continued*)

Apegenin	112.24 ± 14.59[b]	108.92 ± 11.41[b]	91.47 ± 3.61[c]	84.81 ± 6.30[c]	232.49 ± 16.36[a]	86.88 ± 21.47[c]	83.20 ± 4.56[c]	135.00 ± 14.75[b]	125.41 ± 15.81[b]	238.84 ± 55.39[a]
Luteolin	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Cirsiliol	54.85 ± 5.41[c]	96.53 ± 20.32[b]	43.85 ± 6.99[c]	41.94 ± 3.27[c]	98.69 ± 9.79[b]	134.11 ± 2.64[a]	28.84 ± 2.93[d]	57.14 ± 8.11[c]	64.34 ± 8.21[c]	130.15 ± 39.84[a]
Cirsilineol	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Acacetin	N.D	N.D	N.D	N.D	1.3 3 ± 2.30	N.D	N.D	N.D	0.02 ± 0.03	N.D
Chlorogenic acid	7.16 ± 2.21[a]	N.D	5.79 ± 1.42[a]	N.D	N.D	5.91 ± 5.53[a]	4.95 ± 0.95[a]	2.48 ± 2.19[b]	1.93 ± 1.67[b]	N.D
Total (ppm)	3229.08 ± 16.59	2662.71 ± 906.06	2242.08 ± 184.15	1515.39 ± 315.45	2733.33 ± 373.95	2130.55 ± 160.18	4169.37 ± 3743.47	2360.73 ± 448.81	2066.32 ± 85.89	3295.53 ± 747.04

[a, b, c, d, e, f]: homogeneous groups

The reported phenolic composition of OMW in the literature is highly variable in comparison with the various studies. Indeed, the phenolic fraction of vegetable waters is characterized by significant complexity, as demonstrated by several researchers as **El Abbassi et al. (2012).** They identified 07 phenolic compounds by HPLC-MS for OMW from Marrakech, Morocco. The phenolic compounds detected are gallic acid (0.58 ± 0.04 - 0.33 ± 0.02 g of TYE / L), hydroxytyrosol-4-ß-glucoside (0.17 ± 0.01 - 0.23 ± 0.02 g of TYE / L), hydroxytyrosol (3.77 ± 0.24 - 2.13 ± 0.15 g of TYE / L), tyrosol (2.49 ± 0.02 - 0.25 ± 0.01 g of TYE / L), cafeic acid (0.09 ± 0.01 - 0.06 ± 0.01 g of TYE / L), para-coumaric acid (0.55 ± 0.04 - 0.79 ± 0.06 g of TYE / L), and oleuropein aglycone (0 - 0.12 ± 0.01 g of TYE / L).

Aggoun (2016) detected 12 phenolic compounds for OMW from 15 units located in 4 regions (Constantine, Guelma, Skikda and Batna) in Algeria. They are tyrosol (196.87 ± 41.52 µg / g of DM), oleuropein (512.97 ± 693.43 µg / g of DM), luteolin (96.57 ± 12.43 µg / g of DM), caffeic acid (68.70 ± 9.53 µg / g of DM), luteolin-7-o-glucoside (51.38 ± 8.56 µg / g of DM), vanillic acid (43.05 ± 6.52 µg / g of DM), 3,4-dihydroxyphenylacetic acid (38.87 ± 5.62 µg / g of DM), 4-hydroxyphenylacetic acid (5.62 ± 0.96 µg / g of DM), gallic acid (4.06 ± 0.47 µg / g of DM), apigenin (3.45 ± 0.55 µg / g of DM), hydroxytyrosol (1.55 ± 0.96 µg / g of DM) and chlorogenic acid (0.19 ± 0.03 µg / g of DM).

Di Mauro et al. (2017) detected 10 phenolic compounds for OMW from three-phase olive oil mill processing system located in Menfi and Mascalucia, Italy. They are hydroxytyrosol (267.17-821.86 mg / L), pyrocatechol (6.59 mg / L), tyrosol (37.49-105.93 mg / L), p-hydroxyphenylacetic acid (traces), caffeic acid (9.12-10.42 mg / L), floretic acid (21.17-33.87 mg / L), verbascoside (traces), p-coumaric acid (4.27-14.44 mg / L), trans-ferulic acid (2.35 mg / L), and oleuropein (14.32 mg / L).

Senani-Oularbi (2018) determined 08 phenolic compounds for OMW from a modern oil mill using the three-phase extraction system, located in Tizi Ouzou, Algeria. They are gallic acid (0.34 ± 0.04 g in GAE / 100 g OMW), hydroxytyrosol (2.87 ± 0.01 g in GAE / 100 g OMW), tyrosol (0.25 ± 0.05 g in GAE / 100 g OMW), p-coumaric acid (0.51 ± 0.01 g in GAE / 100 g OMW), protocatechuic acid (0.76 ± 0.04 g in GAE / 100 g OMW), oleuropein (0.05 ± 0.02 g in GAE / 100 g OMW), vanillic acid (0.08 ± 0.02 g in GAE / 100 g OMW), and caffeic acid (0.06 ± 0.01 g in GAE / 100 g OMW).

Rajhi et al. (2018) that identified twelve (12) phenolic compounds by HPLC-MS system at 280 nm for OMW from Tunisia. They are quinic acid (0.56 mg / mL), protocatechuic

acid (7.86 mg / mL), caffeic acid (39.91 mg / mL), p-coumaric acid (38.62 mg / mL), naringin (0.91 mg / mL), hyperoside quercetin-3-o (1.60 mg / mL), salviolinic acid (5.06 mg / mL), naringenin (0.31 mg / mL), luteolin (14.92 mg / mL), cirsiliol (5.66 mg / mL), apegenin (0.55 mg / mL), and acacetin (14.45 mg / mL).

Genethliou et al. (2020) detected 11 phenolic compounds by reversed Phase-HPLC (RP-HPLC) for OMW from a continuous three-phase olive processing mill located in Patras, Western Greece. There were gallic acid (11.41 ± 0.98 µg/mL), hydroxytyrosol (116.14 ± 1.43 µg/mL), 3,4-dihydroxybenzoic acid (15.09 ± 0.20 µg/mL), tyrosol (25.73 ± 0.34 µg/mL), 4-hydroxybenzoic acid (17.99 ± 0.24 µg/mL), vanillic acid (26.06 ± 0.36 µg/mL), caffeic acid (52.50 ± 0.59 µg/mL), p-coumaric acid (69.88 ± 0.85 µg/mL), ferulic acid (50.98 ± 0.61 µg/mL), o-coumaric acid (64.38 ± 0.72 µg/mL) and oleuropein (33.20 ± 0.18 µg/mL).

Gueboudji et al. (2021) detected 10 phenolic compounds by LC-MS for OMW from a modern olive oil mill located in Khenchela, Algeria. They were quinic acid (23.94 ppm), p-coumaric acid (1.43 ppm), rutin (0.26 ppm), luteolin-7-o-glucoside (0.20 ppm), naringin (0.15 ppm), quercetrin (quercetin-3-o-rhamnoside) (0.08 ppm), kampherol (3.64 ppm), quercetin (0.47 ppm), apegenin (0.84 ppm) and cirsiliol (2.35 ppm).

In this study, Kampherol (kaempferol) was the most phenolic compound detected in OMW. It was named after a German naturalist called Engelbert Kaempfer. Kaempferol ($C_{15}H_{10}O_6$, molecular weight: 286.23), 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, is a natural flavonol extracted from a broad variety of plants, including the root of *Kaempferia galanga L.*, grapefruit, cabbage, endive, kale, beans, tomato, strawberries, tea, and broccoli. The structure of kampherol is identical to that of quercetin. Due to its numerous biological and therapeutic properties, such as antioxidant, anti-inflammatory, antiobesity, cardiovascular protection, and antiplatelet aggregation **(Choi et al., 2015; Ashrafizadeh et al., 2019),** it is widely used in traditional medicine **(Jiang et al., 2019).** Moreover, kaempferol has a lipophilic property that allows it to be absorbed by the small intestine **(Imran et al., 2019b).** However, despite its strong pharmacological activity, certain issues have restricted its use, particularly in clinical studies. The fact that it has a low bioavailability is one of the most critical considerations. Furthermore, it has a large particle size, limiting its water solubility. In addition to water, the solubility of kampherol in various solvents such as ethanol, DMSO, and dimethyl formamide has been investigated **(Zhao et al., 2013; Da et al., 2019).**

Biological Activities

2.3. Effect of storage time of OMW on biological activities

2.3.1. Antioxidant activity *in vitro* of the phenolic compounds of OMW

Polyphenols have significant antioxidant activity due to their ability to donate hydrogen and form stable intermediate radicals.

Four antioxidant tests (DPPH, ABTS, FRAP, and H_2O_2 tests) were applied to evaluate the antioxidant activity of the phenolic compounds of olive oil mill wastewater of the two varieties, Zlitni and Abani. In addition, the results obtained were compared with those obtained from tests carried out with phenolic acids (ascorbic acid, Trolox, and BHT) used as reference antioxidants.

The mean squares of the analysis of variance of the storage effect of OMW on antioxidant activity are shown in **(Table 13).**

Table 13: Mean squares of the analysis of variance of the storage effect of vegetable water on antioxidant activity

Source	DF	DPPH	ABTS	FRAP	H_2O_2
Time effect	4	235.03^{***}	32251.55^{***}	14382.06^{***}	45642.98^{***}
Variety effect	1	9.78^{ns}	5242.36^{ns}	2930.99^{*}	0.04^{ns}
Variety × Time	4	25.92^{***}	13715.73^{*}	3065.81^{***}	10168.54^{***}
Error	20	2.60	2190.49	357.27	14.23

ns: not significant, *: significant, ***: very highly significant

Two-way analysis of variance over time showed a very highly significant difference for the four tests for antioxidant activity: DPPH, ABTS, FRAP and H_2O_2. For the variety effect, there is a significant difference for FRAP. On the other hand, for the three other tests, DPPH, ABTS, and H_2O_2, there is a non-significant difference. The (variety × time) interaction showed a very highly significant difference for DPPH, FRAP, and H_2O_2; on the other hand, there is a significant difference for the ABTS test.

2.3.1.1.DPPH free radical-scavenging activity

The profiles of the anti-radical activity shown in **(Figure 45)** revealed that the polyphenolic extracts of vegetable waters of the two varieties have antioxidant activity, thus causing the reduction of the DPPH • radical to its non-radical form DPPH-H. The values recorded during t12 represent the lowest IC_{50} for the extracts of the two varieties. The best value was recorded in the vegetable waters of the Abani variety (151.12 ± 0.22 µg / mL), while the

Zlitni extracts showed a value of (153.9 ± 0.19 µg / mL). These values show a potent antioxidant activity, which is lower than that of the standards used, ascorbic acid, Trolox, and BHT with IC_{50} of (174.69 ± 4.84 µg / mL, 190.5 ± 4.36 µg / mL and 265.58 ± 10.2 µg / mL) respectively. The anti-free radical activity of the Abani extracts is classified as follows: t12 > t1 > t2 > t6 > t0 > As Ac > BHT > Trolox, with the values (151.12 ± 0.22 µg / mL, 162.26 ± 3.72 µg / mL, 163.57 ± 2.15 µg / mL, 166.98 ± 0.17 µg / mL, 169.24 ± 0.99 µg / mL, 174.69 ± 4.84 µg / mL, 190.5 ± 4.36 µg / mL and 265.58 ± 10.2 µg / mL). For extracts of the Zlitni variety, the anti-free radical activity is classified as follows: t12 > t2 > t0 > t1 > t6 > As Ac > BHT > Trolox, with the values (153.9 ± 0.19 µg / mL, 162.67 ± 2.00 µg / mL, 164.52 ± 0.99 µg / mL, 168.57 ± 0.82 µg / mL, 169.23 ± 0.86 µg / mL, 174.69 ± 4.84 µg / mL, 190.5 ± 4.36 µg / mL and 265.58 ± 10.2 µg / mL). DPPH test of OMW was discussed in the literature; **El Abbassi et al. (2012)** found the values of (123.0 ± 3.7 - 263 ± 2.5 µg / mL) for OMW from Marrakech, Morocco. **Senani-Oularbi (2018)** found (25 ± 0.01 µg / mL) for the phenolic extract of OMW and (83 ± 0.07 µg / mL) for OMW powder that is obtained from a modern oil mill using the three-phase extraction system, located in Tizi Ouzou, Algeria. **Evci et al. (2019)** found a value of (118 µg / mL) for OMW from olive oil mill located in Mersin, Turkey. A recent study of **Romeo et al. (2020)** showed the result (114.37 ± 151.87 mmol TE / 100 mL) for OMW from a three-phase centrifugation process from Ottobratica olive cultivar, Calabrian region, Italy.

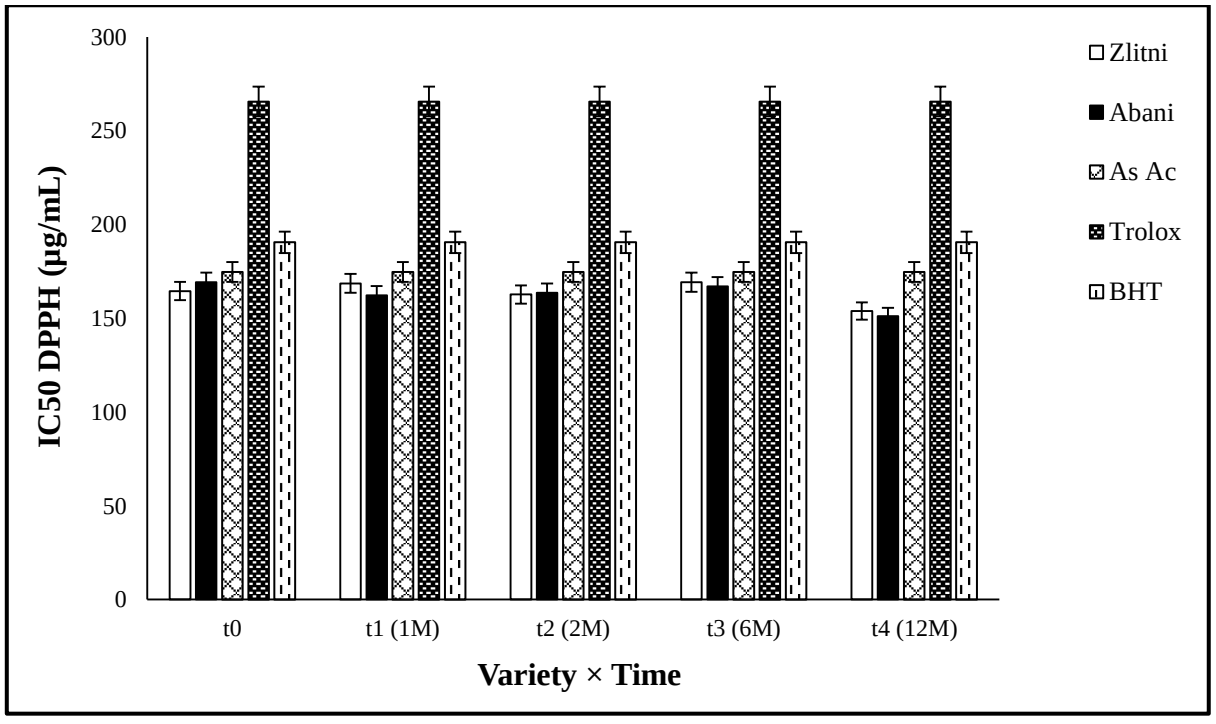

Figure 45: IC_{50} of DPPH in the presence of standards (As Ac, Trolox and BHT) and phenolic extracts of OMW during storage

2.3.1.2.$ABTS^{+}$ free radical scavenging activity

The antioxidant activity of the polyphenols in OMW is deduced from their capacity to inhibit the $ABTS^{\bullet+}$ radical compared to the reference antioxidants: ascorbic acid, Trolox, and BHT. **Figure (46)** illustrates the representative curves of this activity. This activity results in the reduction of the initial optical density (OD) of the $ABTS^{\bullet+}$ radical in the reaction medium, which is 0.700 in the presence of the various samples tested.

The values recorded during t12 represent the lowest IC_{50} for the extracts of the two varieties. The best value was recorded in the vegetable waters of the Abani variety (72.42 ± 26.09 µg / mL), while the Zlitni extracts showed a value of (75.93 ± 25.53 µg / mL). These values show a powerful antioxidant activity which is much lower than that of the standards used, ascorbic acid, BHT and Trolox with IC_{50} of (154.76 ± 51.16 µg / mL, 195.77 ± 36.8 µg / mL and 273.87 ± 59.94 µg / mL) respectively. The anti-free radical activity of the Abani extracts is classified as follows: t12 > t2 > t1 > As Ac > t6 > BHT > t0 > Trolox, with the values (72.42 ± 26.09µg / mL, 124.23 ± 39.26 µg / mL, 150.8 ± 29.25 µg / mL, 154.76 ± 51.16 µg / mL, 177.95 ± 54.46 µg / mL, 195.77 ± 36.8 µg / mL, 270.19 ± 64.26 µg / mL, and 273.87 ± 59.94 µg / mL). For extracts of the Zlitni variety, the anti-free radical activity is classified as follows: t12 > t2 > As Ac > t0 > BHT > t1 > Trolox > t6, with successively the values (75.93 ± 25.53 µg / mL, 118.38 ± 57.49 µg / mL, 154.76 ± 51.16 µg / mL, 169.57 ± 35.31 µg / mL, 195.77 ± 36.8 µg / mL, 235.17 ± 63.77 µg / mL, 273.87 ± 59.94 µg / mL, and 328.83 ± 49.62 µg / mL). A recent study of **Romeo et al. (2020)** showed the result (2569.19 ± 399.90 mmol TE / 100 mL) for OMW from a three-phase centrifugation process from Ottobratica olive cultivar, Calabrian region, Italy.

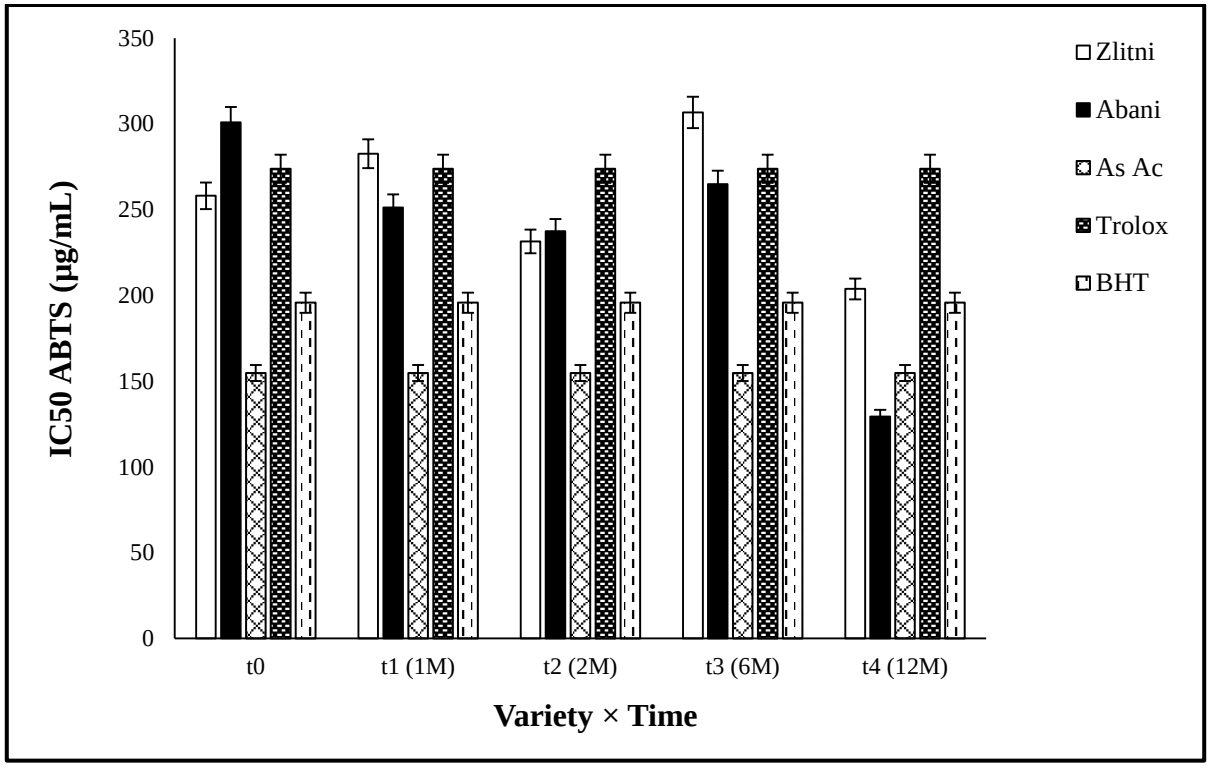

Figure 46: IC_{50} of ABTS in the presence of standards (As Ac, Trolox and BHT) and phenolic extracts of OMW during storage

2.3.1.3.Ferric reducing antioxidant power FRAP

By this test, the ability of the phenolic extract of olive oil mill wastewater to transform ferric iron into ferrous iron was evaluated. This mechanism is known as an indicator of electron-donating activity, characteristic of anti-oxidant capacity. Potassium Ferricyanide is reduced to Ferro-cyanide by polyphenols. In fact, the combination of ferric ions with Ferro-cyanide leads to forming a complex of a green color, which absorbs UV at 700 nm. Furthermore, there is a proportionality relationship between the measured optical density (OD) and the antioxidant activity **(Alam et al., 2013).** The reducing power of iron by the phenolic extract of OMW is illustrated in **Figure 47.**

The values recorded during t12 represent the lowest IC_{50} for the extracts of the two varieties. The best value was recorded in the vegetable waters of the Abani variety (129.32 ± 8.59 µg / mL), while the Zlitni extracts showed a value of (203.75 ± 21.61 µg / mL). These values show a powerful antioxidant activity which is much lower than that of the standards used, ascorbic acid, BHT and Trolox with IC_{50} of (232.15 ± 28.95 µg / mL, 298.92 ± 7.46 µg / mL and 416.67 ± 14.31 µg / mL) respectively. The anti-free radical activity of the Abani extracts is classified as follows: t12 > As Ac > t2 > t1 > t6 > BHT > t0 > Trolox, with the values

(129.32 ± 8.59 µg / mL, 232.15 ± 28.95 µg / mL, 237.4 ± 3.11 µg / mL , 251.34 ± 29.27 µg / mL, 264.82 ± 17.4 µg / mL 9, 298.92 ± 7.46 µg / mL, 300.88 ± 31.18 µg / mL and 416.67 ± 14.31 µg / mL). For the extracts of the Zlitni variety, the anti-radical activity is classified as follows: t12 > t2 > As Ac > t0 > t1 > BHT > t6 > Trolox, with successively the values (203.75 ± 21.61 µg / mL, 231.47 ± 8.66 µg / mL, 232.15 ± 28.95 µg / mL, 258.14 ± 0 µg / mL, 282.6 ± 1.41 µg / mL, 298.92 ± 7.46 µg / mL, 306.66 ± 28.47 µg / mL and 416.67 ± 14.31 µg / mL). **Senani-Oularbi (2018)** found a result of (11.90 ± 0.01 µg / mL) for phenolic extract of OMW and (19.23 ± 0.04 µg / mL) for OMW powder obtained from a modern oil mill using the three-phase extraction system, located in Tizi Ouzou, Algeria.

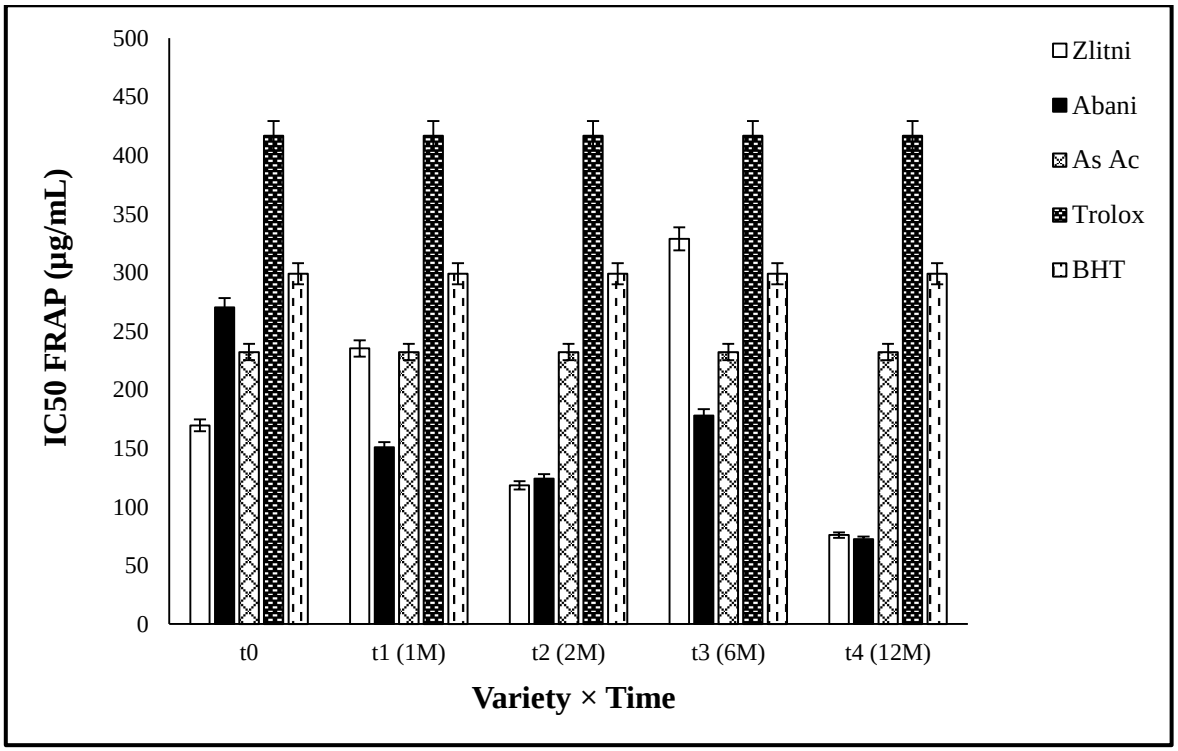

Figure 47: IC_{50} of FRAP in the presence of standards (As Ac, Trolox and BHT) and phenolic extracts of OMW during storage

2.3.1.4.Scavenger activity on hydrogen peroxide H_2O_2

Figure 48 showed the hydrogen peroxide inhibition rates of the different phenolic extracts of olive oil vegetable waters. Based on the scavenger activity results obtained on hydrogen pyroxide H_2O_2, the values recorded during t12 represent the lowest IC_{50} for extracts of both varieties. The best value was recorded in OMW of the Abani variety (75.52 ± 0.85 µg / mL), while the Zlitni extracts showed a value of (78.02 ± 1.1 µg / mL). These values show a powerful antioxidant activity which is much lower than that of the standards used, ascorbic

acid, BHT and Trolox with IC_{50} of (173.66 ± 22.64 µg / mL, 223.62 ± 10.27 µg / mL and 311.75 ± 17.27 µg / mL) respectively. The scavenger activity of the Abani extracts is classified as follows: t12 > t2 > As Ac > t1 > BHT > t6 > t0 > Trolox, with the values (75.52 ± 0.85 µg / mL, 144.98 ± 6.04, 173.66 ± 22.46, 186.76 ± 7.17, 223.62 ± 10.27, 240.99 ± 0.85, 301.48 ± 3.23 and 311.75 ± 17.27 µg / mL). For the extracts of the Zlitni variety, the anti-free radical activity is classified as follows: t12 > t2 > As Ac > t0 > BHT > t1 > Trolox > t6, with successively the values (78.02 ± 1.1, 92.73 ± 2.2, 173.66 ± 22.46, 193.04 ± 4.49, 223.62 ± 10.27, 271.26 ± 1.4, 311.75 ± 17.27 and 315.03 ± 3.78 µg / mL). In literature, antioxidant activity using H_2O_2 test was studied by **Nadour (2015)** and found a result of (400 ± 0 µg / mL) for a soluble fraction extracted from OMW obtained from an olive oil mill using the three-phase extraction system located in the region of Tizi Ouzou, Algeria.

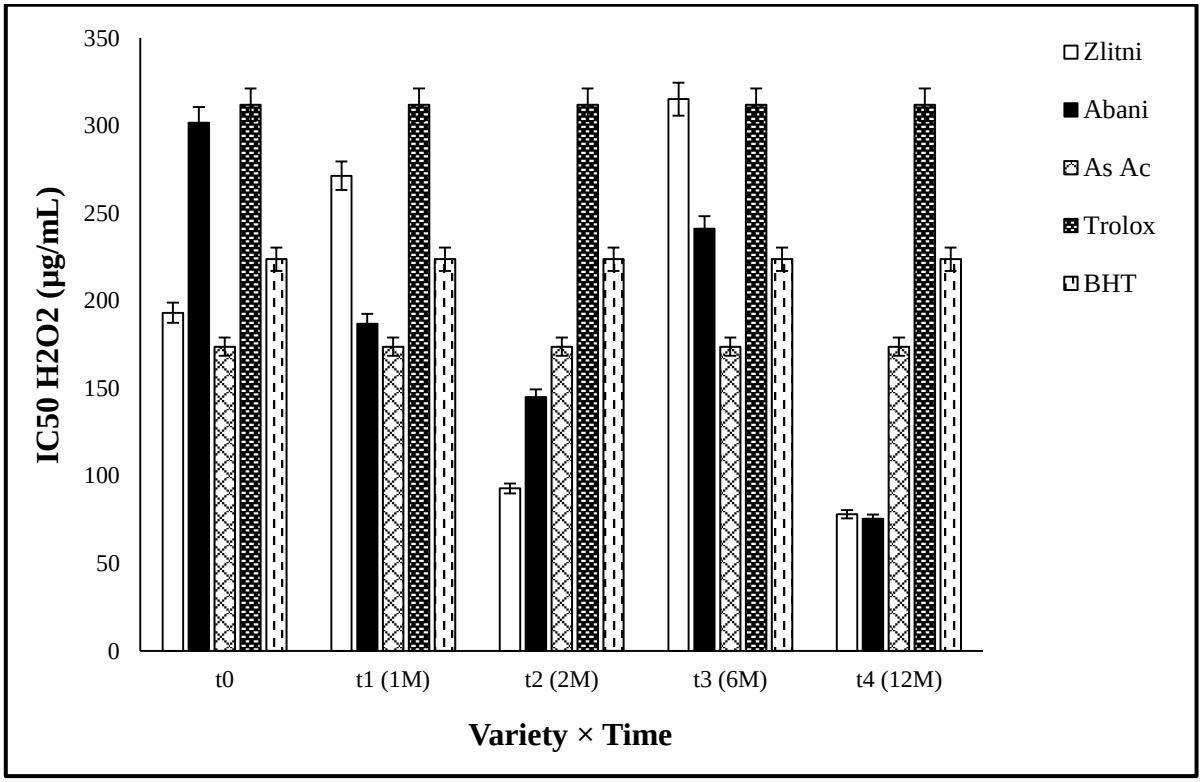

Figure 48: IC_{50} of H_2O_2 in the presence of standards (As Ac, Trolox and BHT) and phenolic extracts of OMW during storage

2.3.2. Time effect of OMW storage on the antioxidant tests

The averages of the evaluation of antioxidant activity by four methods (DPPH, ABTS, FRAP, and H_2O_2) as a function of storage time were illustrated in **(Table 14).**

Table 14: The averages of the evaluation of antioxidant activity by four methods as a function of storage time

Time	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	FRAP (IC_{50} µg/mL)	H_2O_2 (IC_{50} µg/mL)
t 0	166.87 ± 0.99 [ab]	219.92 ± 49.78 [ab]	279.51 ± 15.59 [a]	247.26 ± 3.86 [b]
t (1M)	165.41 ± 2.27 [b]	192.98 ± 46.51 [b]	266.97 ± 15.34 [a]	229.01 ± 4.29 [c]
t (2M)	163.11 ± 2.08 [c]	121.30 ± 48.38 [c]	234.43 ± 5.88 [b]	118.85 ± 4.12 [d]
t (6M)	168.10 ± 0.52 [a]	253.39 ± 52.04 [a]	285.73 ± 22.98 [a]	278.00 ± 2.32 [a]
t (12M)	152.51 ± 0.21 [d]	74.17 ± 25.81 [c]	166.53 ± 15.10 [c]	76.76 ± 0.98 [e]

[a, b, c, d]: homogeneous groups

Results showed that for the four tests: DPPH, ABTS, FRAP, and H_2O_2, the IC_{50} content dropped significantly to t2, then increased to t6, and finally steadily reduced until t12. It is worth noting that the IC_{50} content of DPPH remained approximately constant during the period of storage.

Comparison of the means of DPPH revealed that it is divided into 5 homogeneous groups (LSD = 1.94). The first group is characterized by the highest value (168.10 ± 052 µg / mL), which was recorded after 6 months of storage of the studied vegetable waters, followed by the second group, which was obtained a value of (165.41 ± 2.27 µg / mL) recorded after 1 month of storage. Finally, the last group is characterized by the lowest value (152.51 ± 0.21 µg / mL), which was recorded after 12 months of storage of the studied vegetable waters.

Comparison of ABTS averages revealed that it is divided into 4 homogeneous groups (LSD = 56.36). The first group is characterized by the highest value (253.39 ± 52.04 µg / mL) recorded after 6 months of storage. The second group which revealed a value of (192.98 ± 46.51 µg / mL), recorded after 1 month of storage, followed by the third group with a value of (219.92 ± 49.78 µg / mL). Finally, the fourth group was characterized by the lowest value (121.30 ± 48.38 µg / mL) recorded after 2 months of storage and (74.17 ± 25.81 µg / mL) recorded after 12 months of storage.

Regarding the FRAP test, the comparison of means revealed that it is divided into 3 homogeneous groups (LSD = 22.76). The first group is characterized by the highest value (279.51 ± 15.59 µg / mL), which was recorded before storage, (266.97 ± 15.34 µg / mL), which was recorded after 1 month of storage, and (285.73 ± 22.98 µg / mL), which was recorded after

6 months of storage. The value (234.43 ± 5.88 µg / mL) recorded after 2 months of storage reveals the second group. The lowest value (166.53 ± 15.10 µg / mL), which was recorded after 12 months of storage represents the third group.

Regarding the H_2O_2 test, the comparison of the means revealed that it is divided into 5 homogeneous groups (LSD = 4.54). The value (278.00 ± 2.32 µg / mL) recorded after 6 months of storage, was the highest value that revealed the first group, followed by the value (247.26 ± 3.86 µg / mL) that reveals the second group that was recorded before the storage of the studied vegetable waters. Finally, the last group was characterized by the lowest content with a value of (76.76 ± 0.98 µg / mL) which was recorded after 12 months of storage of OMW.

2.3.3. Variety effect of OMW storage on antioxidant activity

The averages of the evaluation of the antioxidant activity by four methods according to the varieties used were presented in **(Table 15).**

Table 15: The averages of the evaluation of the antioxidant activity by four methods according to the varieties used

Variety	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	FRAP (IC_{50} µg/mL)	H_2O_2 (IC_{50} µg/mL)
Abani	162.63 ± 1.45	159.13 ± 42.66	236.75 ± 17.93 [b]	189.94 ± 3.63
Zlitni	163.77 ± 0.97	185.57 ± 46.34	256.52 ± 12.03 [a]	190.01 ± 2.59

[a, b]: homogeneous groups

For the FRAP test, there were 2 homogeneous groups (LSD = 14.39). The first group represented the highest value (256.52 ± 12.03 µg / mL) recorded in vegetable waters of the Zlitni variety, and the second group represented a value of (236.75 ± 17.93 µg / mL). On the other hand, the three other tests of the antioxidant activity, which are DPPH (LSD = 1.22), ABTS (LSD = 35.64), and H_2O_2 (LSD = 2.87) are located in the same group, which shows that there are differences, and not significant between the two varieties studied for these three tests.

2.3.4. Effect of (time × variety) interaction on the evaluation of antioxidant activity

The averages of the evaluation of the antioxidant activity by four methods according to the varieties studied and the storage time was presented in **(Table 16).**

Table 16: The averages of the evaluation of the antioxidant activity by four methods according to the varieties studied and the storage time

Variety	Time	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	FRAP (IC_{50} µg/mL)	H_2O_2 (IC_{50} µg/mL)
Abani	**t 0**	169.24 ± 0.99^{a}	300.88 ± 64.26^{a}	270.19 ± 31.18^{a}	301.48 ± 3.23^{b}
	t (1M)	162.26 ± 3.72^{b}	251.34 ± 29.25^{b}	150.80 ± 29.27^{c}	186.76 ± 7.17^{e}
	t (2M)	163.57 ± 2.15^{b}	237.40 ± 39.26^{b}	124.23 ± 3.11^{c}	144.98 ± 6.04^{f}
	t (6M)	166.98 ± 0.17^{a}	264.82 ± 54.46^{b}	177.95 ± 17.49^{b}	240.99 ± 0.85^{d}
	t (12M)	151.12 ± 0.22^{d}	129.32 ± 26.09^{d}	72.42 ± 8.59^{c}	75.52 ± 0.85^{h}
Zlitni	**t 0**	164.52 ± 0.99^{b}	258.14 ± 35.31^{b}	169.57 ± 0^{a}	193.04 ± 4.49^{e}
	t (1M)	168.57 ± 0.82^{a}	282.60 ± 63.77^{a}	235.17 ± 1.41^{b}	271.26 ± 1.40^{c}
	t (2M)	162.67 ± 2.00^{b}	231.47 ± 57.49^{c}	118.38 ± 8.66^{c}	92.73 ± 2.20^{g}
	t (6M)	169.23 ± 0.86^{a}	306.66 ± 49.62^{a}	328.83 ± 28.47^{a}	315.03 ± 3.78^{a}
	t (12M)	153.90 ± 0.19^{c}	203.75 ± 25.53^{c}	75.93 ± 21.61^{c}	78.02 ± 1.10^{h}

$^{a, b, c, d, e, f, g, h}$: homogeneous groups

Findings demonstrated that the IC_{50} value of DPPH in the two varieties is nearly constant during storage. It also noted that the IC_{50} of H_2O_2, FRAP, ABTS, and DPPH dropped somewhat until t2, then increased until t6, and then gradually decreased to t12.

Regarding the DPPH test, the comparison of means revealed that it is divided into 4 homogeneous groups. The first group is characterized by the highest value, which was found respectively before storage of vegetable waters of the Abani variety (169.24 ± 0.99 µg / mL) and after 6 months of their storage (166.98 ± 0.17 µg / mL). The value (168.57 ± 0.82 µg / mL) was recorded after 1 month of storage of Zlitni vegetable waters and the value (151.12 ± 0.22 µg / mL) after 12 months of their storage.

For ABTS test, it is divided into 4 homogeneous groups. The high values (300.88 ± 64.26 µg / mL, 282.60 ± 63.77 µg / mL, and 306.66 ± 49.62 µg / mL) were successively for the Abani vegetable waters before storage, Zlitni after 1 month of storage and after 6 months included the first group. On the other hand, the vegetable waters of Abani after 12 months of storage represented the lowest value (129.32 ± 26.09 µg / mL) encompasses the last group.

For FRAP test, it is divided into 3 homogeneous groups, the first of which included Abani vegetable water before storage (270.19 ± 31.18 µg / mL), Zlitni vegetable water before storage (169.57 ± 0 µg / mL) and after 6 months of storage (328.83 ± 28.47 µg / mL). On the other hand, the values recorded after 1 month, 12 months for the OMW of Abani, as well as those recorded after 2 months and 12 months for Zlitni are in the last group.

Concerning the H_2O_2 test, it is divided into 8 homogeneous groups, the first of which included Zlitni vegetable water after 6 months of storage, which was the highest value (315.03 ± 3.78 µg / mL). On the other hand, the values recorded after 12 months of storage of vegetable waters of the two varieties Abani (75.52 ± 0.85 µg / mL) and Zlitni (78.02 ± 1.10 µg / mL) included the lowest values that are in the last group.

Although there are several research projects focused on the antioxidant activity of various plant extracts, this activity has not been studied with the 4 tests together (DPPH, FRAP, ABTS, and H_2O_2) for the polyphenolic extracts of OMW from Zlitni and Abani varieties during storage. The subject of this thesis is considered the first of its kind that is part of the search for a possible antioxidant activity of polyphenols in OMW.

The antioxidant activity of OMW might be due to kampherol. In the literature, the antioxidant effect of kampherol was determined in recent studies **(Du et al., 2018; Wu et al., 2018; Jiang et al., 2019).**

2.3.5. Anti-inflammatory activity *in vitro*

Tow tests are used to evaluate the anti-inflammatory activity *in vitro* of phenolic extracts of OMW; inhibition of denaturation of proteins (IDP) and membrane stabilizing potential (MSP).

2.3.5.1.ANOVA two ways of the effect of OMW storage on anti-inflammatory activity

Table 17 represents the mean squares of the analysis of variance of the storage effect of vegetable water on anti-inflammatory activity.

Two-way variance analysis showed a very highly significant difference between the two tests, IPD and MSP, for the time effect, variety, and (variety × time) interaction.

Table 17: Mean squares of the analysis of variance of the storage effect of vegetable water on anti-inflammatory activity

Source	DF	MSP (IC_{50} (µg/mL)	IPD (IC_{50} µg/mL)
Time effect	4	1511.61^{***}	597.28^{***}
Variety effect	1	8.41^{***}	808.24^{***}
Variety × Time	4	62.26^{***}	900.81^{***}
Error	20	0.062	5.08

***: very highly significant

2.3.5.2.Inhibition of protein denaturation (IPD)

The inhibition rates obtained were shown in **(Figure 49).** According to the results obtained, the extract of the polyphenols of the two varieties of vegetable water exerted inhibition of the denaturation of proteins with an IC_{50} lower than the diclofenac sodium standard. The lowest value of IC_{50} (25.47 ± 1.5 µg / mL) was recorded for the variety Abani after 1 month of storage, while the highest value of IC_{50} (78.75 ± 1.48 µg / mL) was recorded for the variety Zlitni. After 1 month of storage by comparing with diclofenac sodium (83.00 ± 5.75µg / mL). For the Zlitni variety, the IC_{50} values recorded were in ascending order as follows: (27.95 ± 1.69 µg / mL, 30.74 ± 0.31 µg / mL, 44.43 ± 0.59 µg / mL, 54.6 ± 1.85 µg / mL, 78.75 ± 1.48 µg / mL, and diclofenac sodium (83 ± 5.75 µg / mL)), respectively for times (t0, t6, t12, t2, t1). For the Abani variety, the IC_{50} values were in ascending order as follows: (25.47 ± 1.5 µg / mL, 33.99 ± 0.53 µg / mL, 34.64 ± 6.2 µg / mL, 42.46 ± 0.41 µg / mL, 48 ± 0.87 µg / mL, diclofenac sodium (83 ± 5.75 µg / mL), respectively for the times (t1, t6, t0, t12, t2).

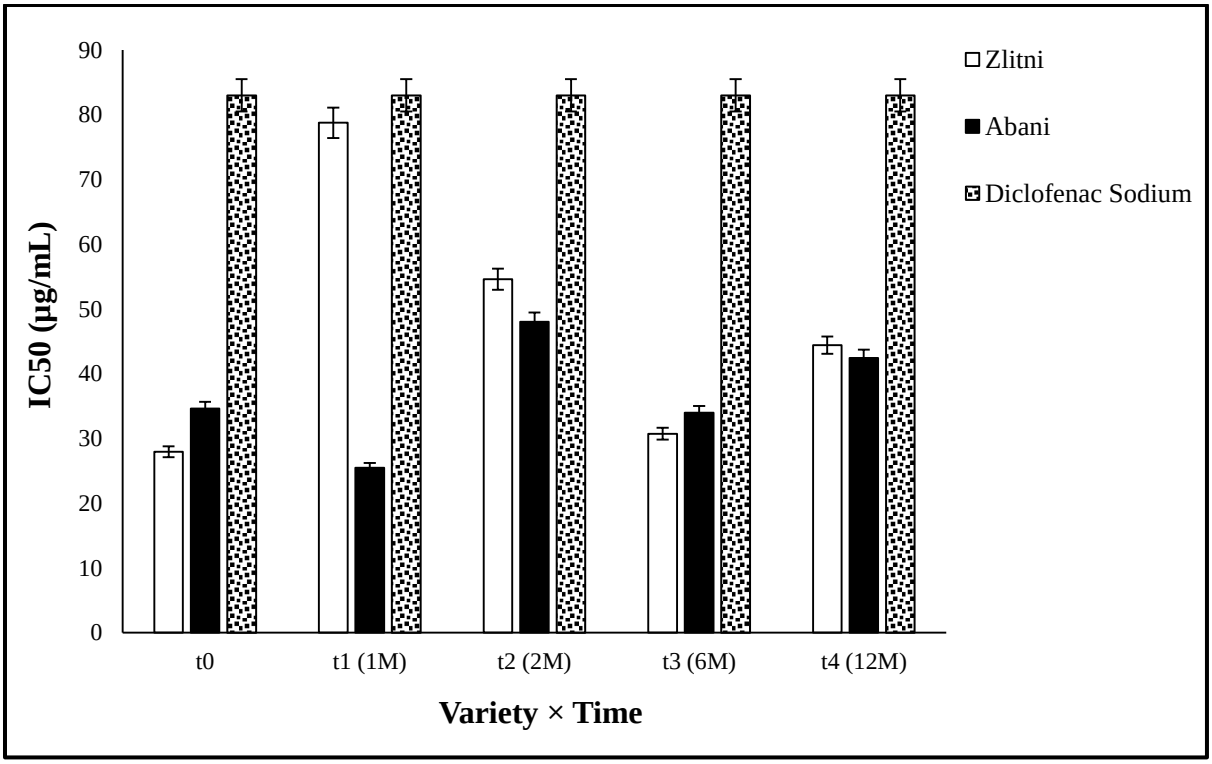

Figure 49: IDP of phenolic extracts of OMW from the two varieties Abani and Zlitni compared to positive control

Denaturation of proteins is caused by applying external stress to a compound such as a strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. It is a process in which proteins lose their secondary and tertiary structures without fragmentation of the peptide chain following the breakdown of low-energy stabilization interactions (electrostatic, hydrogen, hydrophobic, and disulfide bridges), therefore fragile. This denaturation modifies proteins' properties and biological functions **(Karthik et al., 2013; Sivaraj et al., 2017).** Denatured proteins presenting a bad folding during the dysfunction of the organism constitutes one of the causes of induction of the inflammatory reaction **(Chatterjee et al., 2012).** Reports from the literature suggest that the anti-denaturing activity of extracts may be due to the interaction of certain components with two sites (present in certain proteins such as albumin) of bonds rich in Tyrosine, Threonine, and Lysine. They also reported that therapeutic molecules could reactivate the pattern-rich receptor of Tyrosine with Threonine, which regulates biological signal transduction pathways for their overall biological action **(Duganath et al., 2010).**

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation of the mechanisms of anti-inflammation activity, the ability of the extract to

inhibit the denaturation of proteins was investigated. The extract of the polyphenols from the vegetable waters tested exhibits inhibitory effectiveness against thermal denaturation, as well as the stabilizing power of ovalbumin. Protein stabilization by extracts from olive oil vegetable waters probably involves polyphenols and their metabolites that act as modulators of inflammation signaling pathways **(Cappelli et al., 2021).**

2.3.5.3.Membrane stabilizing potential (MSP)

The protective effect of the phenolic extract of vegetable water from olive oil against heat-induced hemolysis of red blood cells is illustrated in **(Figure 50).** According to the results shown in the histogram of the percentage of stabilization of the membrane of red blood cells, it is observed that the extract of polyphenols from vegetable waters exhibits a very high inhibition of hemolysis of red blood cells at different times compared to the standard anti-inflammatory drug diclofenac sodium.

Indeed, the inhibition rates obtained are shown in the following figure **(Figure 50).** According to the results obtained, the extract of the polyphenols of the two varieties of vegetable water exert an inhibition of membrane sensitization with a lower IC_{50} than diclofenac sodium. The lowest value of IC_{50} (16.11 ± 0.1 µg / mL) was recorded for the variety Abani after 12 months of storage, while the highest value of IC_{50} (59.34 ± 0.22 µg / mL) was recorded for the variety Zlitni. After 6 months of storage by comparing with diclofenac sodium (77.66 ± 2.84 µg / mL). For the Zlitni variety, the IC_{50} values recorded were in ascending order as follows: (17.67 ± 0.14 µg / mL, 42.17 ± 0.2 µg / mL, 49.49 ± 0.42 µg / mL, 55.2 ± 0.48 µg / mL, 59.34 ± 0.22 µg / mL, and diclofenac sodium (77.66 ± 2.84 µg / mL)), respectively for times (t12, t2, t0, t1, t6). For the Abani variety, the IC_{50} values were in ascending order as follows: (16.11 ± 0.1 µg / mL, 45.98 ± 0.19 µg / mL, 48.43 ± 0.25 µg / mL, 51.59 ± 0.27 µg / mL, 56.46 ± 0.2 µg / mL, diclofenac sodium (77.66 ± 2.84 µg / mL)), respectively for the times (t12, t2, t1, t6, t0).

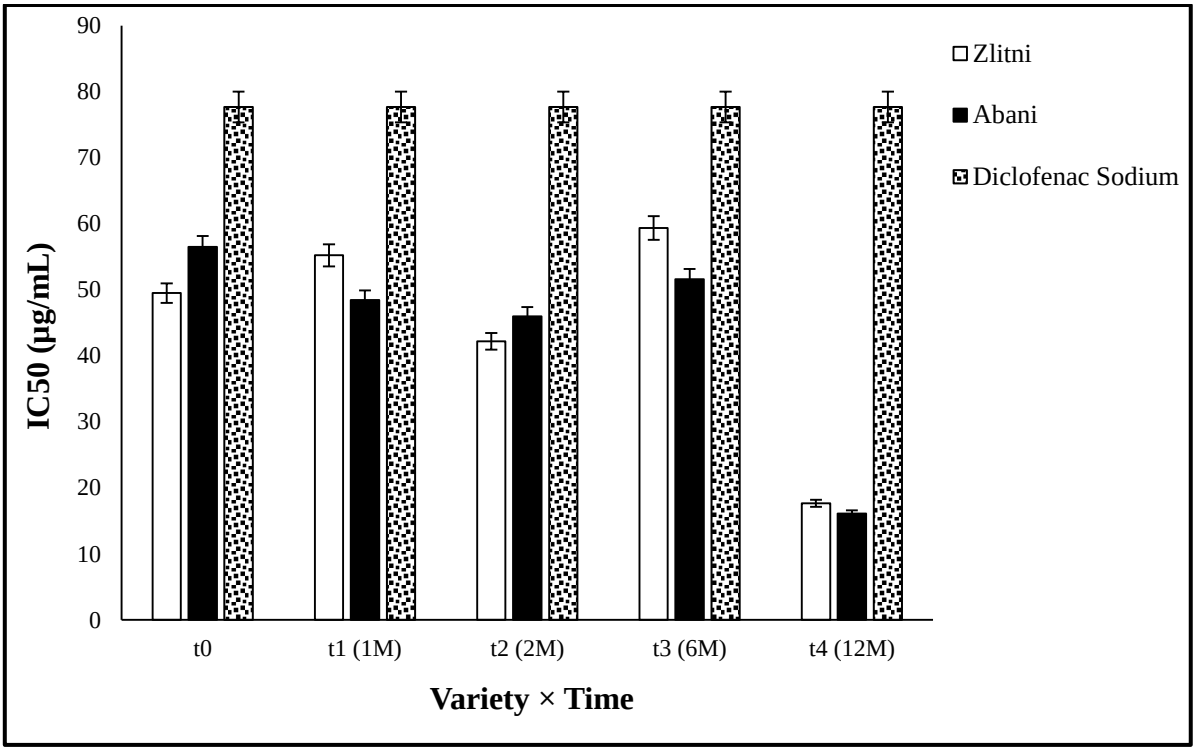

Figure 50: MSP of phenolic extracts of OMW from the two varieties Abani and Zlitni compared to positive control

Because the erythrocyte membrane is similar to the lysosomal membrane, red blood cell membrane stabilization has been used to examine anti-inflammatory effects *in vitro* **(Marliyah and Ananthi, 2015).** According to the results of this investigation, polyphenol extracts from vegetable waters significantly stabilized the red blood cell membrane at different concentrations compared to different indomethacin doses.

Lysosome stabilization is crucial in reducing the inflammatory response by inhibiting the release of lysosomal components from active neutrophils, such as bacterial enzymes and protease. The lysosomal enzyme secreted during inflammation causes a variety of diseases. These enzymes' extracellular activity is thought to be connected to acute and chronic inflammation. Nonsteroidal medications, such as indomethacin, act by either blocking lysosomal enzymes or stabilizing lysosomal membranes **(Kumari et al., 2015).**

2.3.5.4.Time effect of OMW storage on anti-inflammatory activity

The averages of the evaluation of anti-inflammatory activity by two methods as a function of storage time were presented in **(Table 18).**

Table 18: The means of evaluation of anti-inflammatory activity by two methods as a function of storage time

Time	IPD (IC_{50} µg/mL)	MSP (IC_{50} µg/mL)
t 0	31.29 ± 3.95 [c]	52.97 ± 0.23 [b]
t (1M)	52.10 ± 1.49 [a]	51.81 ± 0.36 [c]
t (2M)	51.30 ± 1.36 [a]	44.07 ± 0.20 [d]
t (6M)	32.36 ± 0.42 [c]	55.46 ± 0.25 [a]
t (12M)	43.44 ± 0.50 [b]	16.88 ± 0.12 [e]

[a, b, c, d, e]: homogeneous groups

According to the results obtained, the content of IC_{50} increased until t1 and subsequently decreased slightly at t2 for both IPD and MSP tests. Then, for IPD IC_{50}, it slowly decreased at t6 before increasing to t12, but for MSI, it increased to t6 before progressively decreasing to t12.

The comparison of IPD test means was divided into 3 homogeneous groups (LSD = 2.71). The first group represented the highest value (52.10 ± 1.49 µg / mL and 51.30 ± 1.36 µg / mL) obtained respectively after 1 month and 2 months of storage. The second group represented the value (43.44 ± 0.50 µg / mL) of vegetable waters after 12 months of storage. Finally, the last group contained the lowest value obtained before storage (31.29 ± 3.95 µg / mL) and after 6 months of storage (32.36 ± 0.42 µg / mL).

The comparison of MSP test means was divided into 5 homogeneous groups (LSD = 0.30); the first group had the highest value (55.46 ± 0.25 µg / mL) recorded after 6 months of storage, the second group represented a value of (52.97 ± 0.23 µg / mL) recorded before storage, the third group represented a value of (51.81 ± 0.36 µg / mL) recorded after 1 month of storage, the fourth group represented a value of (44.07 ± 0.20 µg / mL) recorded after 2 months of storage. Finally, the last group had the lowest value (16.88 ± 0.12 µg / mL) recorded after 12 months of storage of vegetable waters.

2.3.5.5.Variety effect of OMW storage on anti-inflammatory activity

The averages of the evaluation of anti-inflammatory activity by two methods depending on the varieties studied are shown in **(Table 19).**

Table 19: The averages of the evaluation of the anti-inflammatory activity by two methods according to the varieties used

Variety	IPD (IC_{50} µg/mL)	MSP (IC_{50} µg/mL)
Abani	36.91 ± 1.90 [b]	43.71 ± 0.21 [b]
Zlitni	47.29 ± 1.19 [a]	44.77 ± 0.26 [a]

[a, b]: homogeneous groups

The comparison of IPD test means is divided into 2 homogeneous groups (LSD = 1.71). The first group was revealed the high value (47.29 ± 1.19 µg / mL) recorded for vegetable waters of the Zlitni variety, and the second group represented the low value (36.91 ± 1.90 µg / mL). For the test MSP, it was divided into 2 homogeneous groups (LSD = 0.19). The first group was revealed the high value (44.77 ± 0.26 µg / mL) recorded for Zlitni, and the second group represents the low value (43.71 ± 0.21 µg / mL) recorded for Abani.

2.3.5.6.Effect of (time × variety) interaction on the evaluation of the anti-inflammatory activity

The averages of the evaluation of anti-inflammatory activity by two methods according to the two varieties studied and the storage time are shown in **(Table 20).**

Table 20: The averages of the evaluation of anti-inflammatory activity by two methods depending on the varieties used and the storage time

Variety	Time	IPD (IC_{50} µg/mL)	MSP (IC_{50} µg/mL)
Abani	**t 0**	34.64 ± 6.20 [e]	56.46 ± 0.20 [b]
	t (1M)	25.47 ± 1.50 [f]	48.43 ± 0.25 [f]
	t (2M)	48.00 ± 0.87 [c]	45.98 ± 0.19 [g]
	t (6M)	33.99 ± 0.53 [e]	51.59 ± 0.27 [d]
	t (12M)	42.46 ± 0.41 [d]	16.11 ± 0.10 [j]
Zlitni	**t 0**	27.95 ± 1.69 [f]	49.49 ± 0.42 [e]
	t (1M)	78.75 ± 1.48 [a]	55.20 ± 0.48 [c]
	t (2M)	54.60 ± 1.85 [b]	42.17 ± 0.20 [h]
	t (6M)	30.74 ± 0.31 [e]	59.34 ± 0.22 [a]
	t (12M)	44.43 ± 0.59 [c]	17.67 ± 0.14 [i]

[a, b, c, d, e, f, g, h, i]: homogeneous groups

According to the results obtained, the IC_{50} of Abani's IPD decreased to (t1M), and then increased to (t2M), then decreased to (t6M), and then increased again to (t12M). For Zlitni, on the other hand, it increased up to the threshold at (t1M), then decreased until (t6M), and then increased again.

For MSP, the IC_{50} in Abani decreased up to 2 months of storage, then increased gradually until (t6M), then progressively decreased until (t12M).

Regarding the IPD test, the comparison of means was divided into 6 homogeneous groups. The first group had the highest value (78.75 ± 1.48 µg / mL) for vegetable waters of the Zlitni variety after 1 month of storage, followed by the second group, which had a value of (54.60 ± 1.85 µg / mL) recorded after 2 months of storage of Zlitni vegetable waters. The last group had the lowest value (25.47 ± 1.50 µg / mL) recorded after 1 month of storage of vegetable water from Abani and before storage (27.95 ± 1.69 µg / mL) of OMW from Zlitni.

Regarding the MSP test, the comparison of means was divided into 10 homogeneous groups. The first group was characterized by the highest value (59.34 ± 0.22 µg / mL), which was recorded after 6 months of storage of vegetable waters of the Zlitni variety, followed by the second group with a value of (56.46 ± 0.20 µg / mL) which was recorded before the storage of the Abani vegetable water. The last group was characterized by the lowest value (16.11 ± 0.10 µg / mL) which was recorded after 12 months of storage of vegetable waters of the Abani variety.

Although there are several research projects focused on the anti-inflammatory activity of various plant extracts, this activity has not been studied for polyphenolic extracts of OMW from Zlitni and Abani varieties with the two tests together during 12 months of storage time. Therefore, the subject of this thesis is considered to be the first one of its kind, which is part of studies interested in the valorization of bioactive compounds like polyphenols, and this by looking for a possible anti-inflammatory activity for OMW polyphenols.

The anti-inflammatory effect of OMW might be due to kampherol. Many researchers studied the anti-inflammatory effect of kampherol recently **(Sun et al., 2019; Yao et al., 2019; Jiang et al., 2019).**

2.3.6. Anticoagulant activity

Table 21 illustrates the two-way analysis of variance. For the time effect, it showed a very highly significant difference, as regards activated partial thromboplastin time (APTT) and prothrombin time (PT). For the variety effect, there is a non-significant difference for the two tests APTT and PT.

Table 21: Mean squares of the analysis of variance of the effect of storage of OMW on the anticoagulant activity

Source	DF	APTT (second)	PT (second)
Time effect	4	2366.93^{***}	427.56^{***}
Variety effect	1	1.54^{ns}	0.48^{ns}
Variety × Time	4	70.61^{**}	16.61^{***}
Error	20	15.03	0.11

ns: not significant, **: highly significant, ***: very highly significant

Analysis of variance of (variety × time) interaction showed a highly significant difference for the APTT test. For the second test, the PT showed a very highly significant difference.

All the anticoagulant activities of extracts from olive oil mill wastewaters of different cultivars were evaluated *in vitro* with respect to the exogenous route and the endogenous route of coagulation using two general chronometric tests that explore coagulation in a non-specific way, PT and APTT, respectively.

2.3.6.1. Prothrombin time test (PT)

This classic test is used commonly known as the prothrombin time (PT), which explores the extrinsic pathway (VII) and the common pathway (X, V, II, fibrinogen) of blood clotting where tissue factor (thromboplastin) is the trigger for this path **(Tripodi, 2009).** The reagent contains the polybrene (heparin inhibitor), which is why PT is not prolonged by treatment with heparin; therefore, it has not been used as a standard anticoagulant. With the aim of seeking an extension in the level of the coagulation time, which is defined by an anticoagulant activity of the phenolic extracts of olive oil mill wastewaters versus the cascade of this route. A normal PT is between 12 and 14 seconds depending on the reagents used **(Caquet, 2004).**

In the light of the results obtained **(Figure 51),** it emerged that the extracts of the two varieties are able to prolong the coagulation time, and the Zlitni extracts during the storage time t0 were represented the long prolongation induced with a value of (37.13 ± 0.31 s) and also the short time represented it during t12 with a value of (16.23 ± 0.15 s) compared to (C-) the negative control (13 ± 0.1 s). The values recorded for Zlitni were in descending order as follows: (37.13 ± 0.31 s, 34.17 ± 0.4 s, 29.1 ± 0.2 s, 22.07 ± 0.5 s, 16.23 ± 0.15 s, 14.17 ± 0.06 s, 13 ± 0.1 s) for respectively (t0 > t2 > t1 > t6 > t12 > C + > C -). For the Abani extracts, the large value was recorded during the time t1 with a value of the order of (37 ± 0.1 s). The recorded values were in descending order as follows: (37 ± 0.1 s, 36.3 ± 0.53 s, 27.53 ± 0.25 s, 20.97 ± 0.5 s, 18.17 ± 0.06 s, 14.17 ± 0.06 s, 13 ± 0.1 s) respectively for (t0 > t2 > t1 > t12 > t6 > C + > C -).

According to findings, it is assumed that the anticoagulant activity of the polyphenolic extracts of OMW may be due to the synergistic effect of the different classes of polyphenols and other compounds present in these extracts.

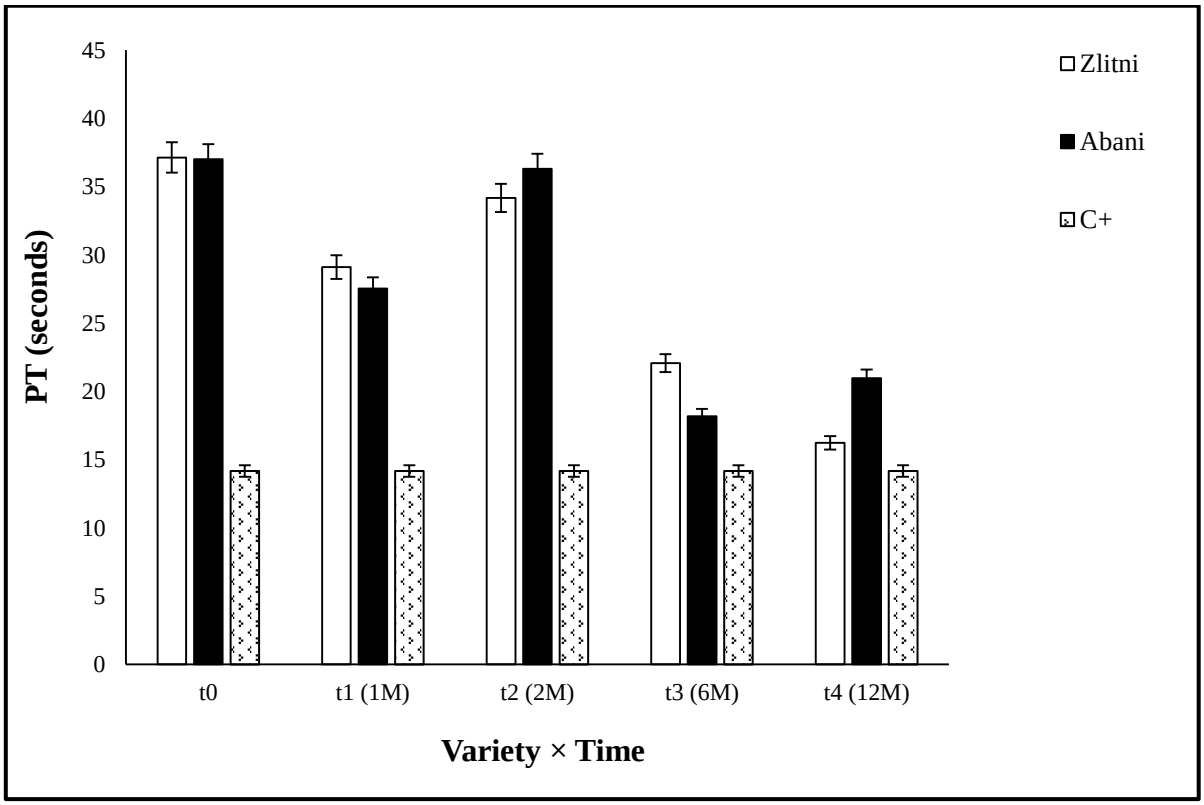

Figure 51: PT of phenolic extracts of OMW from the two varieties Abani and Zlitni compared to positive and negative controls.

2.3.6.2.Activating partial thromboplastin time (APTT)

The evaluation of the anticoagulant capacity of extracts from olive oil mill wastewaters with respect to the endogenous coagulation pathway was carried out using activated partial thromboplastin time test (APTT).

In this test, this coagulation pathway is activated by the contact between factor XII and the electronegative surface of the activator, which is kaolin (substitute for collagen and connective tissue *in vivo*). This interaction induces the activation of factor XII and consequently the sequential activation of factors XI, IX, X, and thrombin (factor II) **(Athukorala et al., 2007).**

A longer clotting time compared to the negative control where the sample is replaced by physiological water reflects the anticoagulant activity of the material tested.

The heparin that was used as a reference in this test to compare its anticoagulant capacity to that exerted by olive oil mill wastewater extracts, is low molecular weight heparin (LMWH) in the form of a ready-to-use injectable solution characterized by high anti-Xa activity and low anti-IIa or anti-thrombin activity.

In the light of the results obtained **(Figure 52),** it emerges that the extracts of the two varieties are able to lengthen the coagulation time significantly and the Zlitni extracts during the storage time t0 were represented the long prolongation induced with a value of (80.07 ± 0.15 s) and also the short time represented it during t12 with a value of (32.2 ± 0.26 s) compared to the negative control (30.07 ± 0.06 s). The values recorded for Zlitni were in descending order as follows: (80.07 ± 0.15 s, 63.5 ± 3.16 s, 48 ± 0.4 s, 36.8 ± 0.7 s, 35.13 ± 0.06 s, 32.2 ± 0.26 s, 30.07 ± 0.06 s) for respectively (t0 > t2 > t1 > t6 > C + > t12 > C -). For the Abani extracts, the large value was recorded during the time t2 with a value of the order of (74.73 ± 11.74 s). The recorded values were in descending order as follows: (74.73 ± 11.74 s, 74.4 ± 1.01 s, 45 ± 0.6 s, 35.37 ± 0.46 s, 35.13 ± 0.06 s, 33.33 ± 0.42 s, 30.07 ± 0.06 s) respectively for (t2 > t0 > t1 > t12 > C + > t6 > C -).

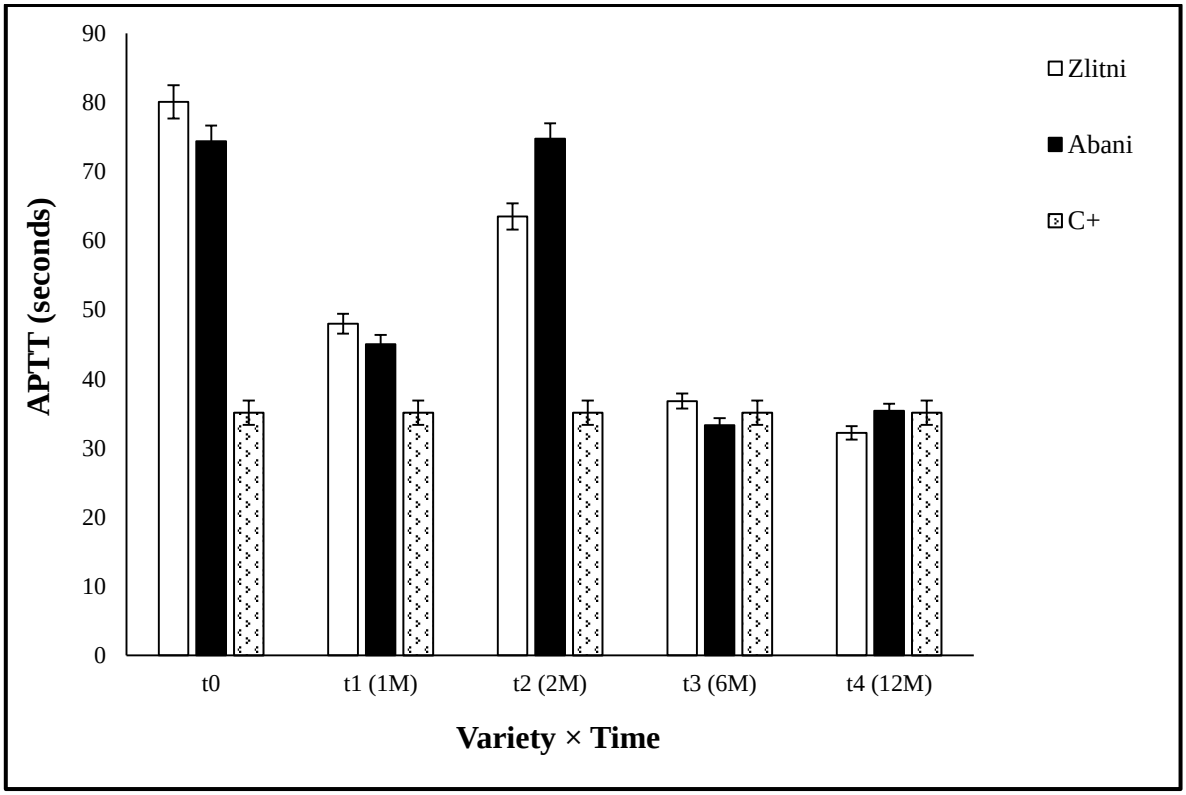

Figure 52: APTT of phenolic extracts of OMW from the two varieties Abani and Zlitni compared to positive and negative controls.

Therefore, in order to have effective anticoagulant activity, it is probably necessary for OMW's polyphenols to act synergistically with each other and/or with other compounds present in extracts from vegetable water.

Although there are several research projects focused on the anticoagulant activity of various plant extracts, this activity has not been studied for polyphenolic extracts of OMW during storage time. Therefore, the subject of this thesis is considered the first one of its kind, which falls within the framework of studies interested in the valorization of polyphenols from the liquid by-product of olive oil mill, and that by the search for possible anticoagulant activity.

2.3.6.3.Time effect of OMW storage on anticoagulant activity

The comparison of the means of the two APTT and PT tests of anticoagulant activity as a function of time has been shown in **(Table 22).**

It is revealed that the APTT test is divided into 4 homogeneous groups (LSD = 4.66). The first group represented the highest value (77.23 ± 0.58 s) which was recorded before storage (t0), followed by the second group, which recorded after 2 months of storage (69.11 ± 7.45 s),

and finally, the third group obtained after 1 month of storage with a value of (46.50 ± 0.50 s). The fourth group represented the lowest value (35.06 ± 0.56 s and 33.78 ± 0.36 s) which was recorded successively after 6 months and 12 months of storage.

For the PT test, it is divided into 5 homogeneous groups (LSD = 0.41). The largest value (37.06 ± 0.20 s) represented the first group, which was obtained before the storage of OMW (t0). The second group was recorded after 2 months of storage with a value of (35.23 ± 0.47 s). The third group was recorded after 1 month of storage (28.31 ± 0.23 s). The fourth group recorded a value of (20.11 ± 0.28 s) obtained after 6 months of storage. Finally, the last group represented the lowest value (18.60 ± 0.33 s) which was obtained after 12 months of storage.

Table 22: The means of the evaluation of the anticoagulant activity by two methods according to the storage time

Time	APTT (second)	PT (second)
t 0	77.23 ± 0.58 [a]	37.06 ± 0.20 [a]
t (1M)	46.50 ± 0.50 [c]	28.31 ± 0.23 [c]
t (2M)	69.11 ± 7.45 [b]	35.23 ± 0.47 [b]
t (6M)	35.06 ± 0.56 [d]	20.11 ± 0.28 [d]
t (12M)	33.78 ± 0.36 [d]	18.60 ± 0.33 [e]

[a, b, c, d, e]: homogeneous groups

2.3.6.4.Effect variety of OMW storage on anticoagulant activity

The comparison of the means of the two tests APTT and PT of the anticoagulant activity according to variety was shown in **(Table 23).** It revealed that the two tests, APTT (LSD = 2.95) and PT (LSD = 0.26), are in the same group, showing no significant differences between the two varieties studied for these two tests.

Table 23: The means of the evaluation of the anticoagulant activity by two methods according to the varieties used

Variety	APTT (second)	PT (second)
Abani	52.56 ± 2.85	27.99 ± 0.29
Zlitni	52.11 ± 0.94	27.74 ± 0.31

2.3.6.5.Effect of (time × variety) interaction on the evaluation of the anticoagulant activity

The homogeneous groups from the comparison of the means of the two tests APTT and PT of anticoagulant activity as a function of the time × variety interaction were shown in **(Table 24).**

Table 24: The averages of the evaluation of the anticoagulant activity by two methods according to the varieties used and the storage time.

Variety	Time	APTT (second)	PT (second)
Abani	**t(0M)**	74.40 ± 1.01^{a}	37.00 ± 0.10^{a}
	t(1M)	45.00 ± 0.60^{c}	27.53 ± 0.25^{e}
	t(2M)	74.73 ± 11.74^{a}	36.30 ± 0.53^{b}
	t(6M)	33.33 ± 0.42^{d}	18.17 ± 0.06^{h}
	t(12M)	35.37 ± 0.46^{d}	20.97 ± 0.50^{g}
Zlitni	**t(0M)**	80.07 ± 0.15^{a}	37.13 ± 0.31^{a}
	t(1M)	48.00 ± 0.40^{c}	29.10 ± 0.20^{d}
	t(2M)	63.50 ± 3.16^{b}	34.17 ± 0.40^{c}
	t(6M)	36.80 ± 0.70^{d}	22.07 ± 0.50^{f}
	t(12M)	32.20 ± 0.26^{d}	16.23 ± 0.15^{i}

a, b, c, d, e, f, g, h, i: homogeneous groups

According to the results, it was demonstrated that APTT and PT decreased to (t1M), then increased to (t2M), then decreased to (t6M), and finally decreased to (t12M). Furthermore, it continued to decrease for Zlitni until t12, but it increased again for Abani.

For the test APTT, it was divided into 4 homogeneous groups. The first group represented the highest values (74.40 ± 1.01 s, 74.73 ± 11.74 s, and 80.07 ± 0.15 s), respectively for the times (t0, t2 of the variety Abani and t0 for the variety Zlitni. The second group (63.50 ± 3.16 s) was obtained after 2 months of storage of vegetable waters of the Zlitni variety. The third group included vegetable waters after 1 month of storage in the two varieties Abani (45.00 ± 0.60 s) and Zlitni (48.00 ± 0.40 s). The last group represented the lowest value found after respectively 6 months and 12 months of storage of the vegetable waters of Abani (33.33 ± 0.42 s and 35.37 ± 0.46 s) and of Zlitni (36.80 ± 0.70 s and 32.20 ± 0.26 s).

Regarding the PT test, the comparison of means was divided into 9 homogeneous groups. The first group represented the highest value recorded before storage of vegetable

waters of the Zlitni variety (37.13 ± 0.31 s) and Abani (37.00 ± 0.10 s). The second group represented the vegetable waters of the Abani variety (36.30 ± 0.53 s) obtained after 2 months of storage and followed by the third group represented the vegetable waters of Zlitni (34.17 ± 0.40 s) also after 2 months of storage. The last group represented the lowest value (16.23 ± 0.15 s) obtained after 12 months of storage of Zlitni.

The anticoagulant effect of OMW might be due to kampherol, which had antiplatelet aggregation **(Choi et al., 2015),** thus cardiovascular protection **(Suchal et al., 2017).**

2.4. Principal component analysis (PCA) of the measured parameters

The projection of the variables and the individuals was carried out on 04 axes, representing 58.85% of the total variance of which all the measured parameters have been taken into account.

Axis I expressed 41.43% of the total variance, axis II expressed 17.42% of the total variance. The configuration of the parameters studied by the PCA **(Figure 53)** made it possible to define four groups. The first, of which grouped together almost all the physicochemical parameters measured in addition to the contents of total flavonoids and tannins and the anticoagulant activity (the two methods of evaluation) and the following compounds: caffeic acid, trans frulic acid, quercetin 3-0-galactoside, luteolin-7-o-glucoside, naringin, quercetrin (quercetin-3-o-rhamnoside), quercetin, chlorogenic acid, which are positively correlated with axis I and axis II unlike gallic acid, syringic acid, o-coumaric acid, apegenin and the percentage of water in the vegetable waters which formed the second group and that are negatively correlated with l 'axis I and with axis II. The F2 axis is positively correlated with the total polyphenol content defined by the following compounds: Epicatechin, acacetin, cirsiliol, apegenin, kampherol, rosmarinic acid, epicatechin, 1,3-di-o-caffeoyquinic acid and which are correlated positively with axis II and negatively with axis I. The two phenolic compounds kampherol and Apegenin presented the most responded polyphenols (with a significant quantity) in the vegetable waters of the two varieties studied after 12 months of storage.

The fourth group encompassed the content of condensed tannins and the following phenolic compounds: Rutin, trans frulic acid, 4,5-di-O-caffeoyquinic acid, trans cinnamic in addition to the methods used in the evaluation of antioxidant activity (DPPH, ABTS, FRAP, and H_2O_2) and anti-inflammatory (IPD and MSP) and which are positively correlated with axis I and negatively with axis II. This indicated that these metabolites (the condensed tannins) gave very important antioxidant and anti-inflammatory activities.

The projection of the observations on the F1 × F2 factorial plane showed that the F1 axis was the major axis that grouped together the physicochemical parameters and the evaluated biological activities that are related.

The observation of the factorial plane F1 × F2 showed that the positive pole grouped together with the total flavonoids and the total tannins with the physicochemical parameters of the studied vegetable waters having relation and influence on the anticoagulant activity such as pH, BOD_5, COD, MM, OM, and EC. It is also noted that the two varieties studied had a high anticoagulant activity evaluated with the two methods (APTT and PT). It was after 0, 1, and 2 months of storage, these parameters of which recorded very high values compared to the other months (after 6 and 12 months); therefore, the efficacy of the vegetable waters studied decreased with the duration of storage and its use as an anticoagulant is in the fresh state. The negative pole grouped the vegetable waters after 6 months of storage, which are characterized by the lowest values of almost all the parameters measured and gave the lowest values of the biological activities evaluated. After 12 months of storage, the highest total polyphenol content was recorded after 12 months of storage of which kampherol, apegenin were the most important, and which had low antioxidant activity.

In conclusion, the use of olive oil mill wastewater is more effective and beneficial when it is fresh, especially for its phenolic content.

In the light of the results obtained, it can be said that stored OMW can undergo various reactions (hydrolysis, oxidation, and biotransformation), which continually modify its composition. Therfore, storage of olive oil mill wastewaters at room temperature does not prevent the rapid decrease in the concentrations of polyphenols, which occurs during the storage period.

In addition, the antioxidant activity of OMW cannot be attributed exclusively to the presence of phenolic compounds. The capacity of phenolic compounds to scavenge free radicals, donate electrons or hydrogen atoms, or bind metal ions may describe their antioxidant activity. The structure of phenolic compounds has an important role in their radical scavenging and metal chelating actions. The amount and location of the hydroxyl groups in relation to the carboxyl or keto functional groups in the case of phenolic acids and flavonoids, respectively, determine antioxidant activity **(González-Laredo et al., 2018).**

The results showed that a high content of phenolic compounds does not necessarily correlate positively with the chelating activity of ferrous ions (Fe^{2+}). The effect of antioxidant

concentration on auto-oxidation rates depends on many factors, including the structure of the antioxidant, the oxidation conditions, and the nature of the oxidized sample **(Pradal, 2016).** The chemical structure of phenolic compounds gives them a high oxidative capacity. As a result, they are excellent antioxidants. They can self-oxidize in the air or in the presence of metals, and they can be very quickly oxidized by enzymatic reaction as soon as the integrity of the plants is disturbed. The degradation of antioxidant polyphenols generally occurs during an oxidation process and results in many products, particularly antioxidant dimers. These dimers can be produced by the formation of phenoxyl radicals followed by radical rearrangement and a coupling reaction with another radical **(Pradal, 2016).** Flavonoid glycosides that are present in acylated form have good prospects to be developed into therapeutic agents due to their improved biological properties, stability, and physicochemical properties compared to their maternal compounds **(Chear et al., 2019).**

The antioxidant activity of flavonoids is determined by the structural skeletons and substitution patterns in the B and C rings. A catechol group (e.g., ortho-dihydroxylation of the B-ring) or a pyrogallol group (e.g., hydroxylation at 3,4,5 in the B-ring) has been demonstrated to have higher antioxidant activity than mono B ring hydroxylated flavonoids. In addition to a double bond between C-2 and C-3, conjugation with a keto group (e.g., 4-oxo in a C-ring) increases their radical scavenging ability. In addition, an unsaturation (e.g., a double bond between C-2 and C-3) connected to a hydroxyl at C-3, as in kaempferol, boosts flavonoids' active radical scavenging capacity **(González-Laredo et al., 2018).**

Chlorogenic acids are a family of esters formed between certain phenolic acids (trans-cinnamic acids) and quinic acid. The chlorogenic acids group includes caffeoylquinic acids, dicaffeoylquinic acids, and other caffeic acid derivatives **(Jaiswal et al., 2014; Craig et al., 2016).** The group of caffeoylquinic acids includes 5-o-caffeoylquinic acid, 4-o-caffeoylquinic acid, and 3-o-caffeoylquinic acid **(Craig et al., 2016; Liang et al., 2016).** Caffeoylquinic acids are found in almost every existing plant, but the major dietary source for humans is coffee **(Craig et al., 2016).** Caffeic acid, a cinnamic acid derivative, is a nine-carbon phenolic acid commonly found in fruits and vegetables **(Magnani et al., 2014; Salau et al., 2020).** It protects against cardiotoxicity **(Salau et al., 2020).** Caffeic acid and chlorogenic acid inhibit together inflammation **(Gamaro et al., 2011)** and have cardio-protective and antioxidant properties **(Agunloye et al., 2019).** Rutin and its aglycone quercetin had important anti-inflammatory activity **(de Silva et al., 2019; Gonzalez-Trujano et al., 2017).**

In addition, Gallic tannins (hydrolyzable) and catechiuc (condensed) tannins had antioxidant activities **(Nounagnon et al., 2017).** On the contrary, such a high concentration of polyphenols could be at the origin of the toxicity of OMW with respect to biological activity. Finally, the pharmacological properties of phenolics have been attributed to their structural feature, which enables them to donate H atom from their –OH group as well as substitution of the benzene ring to reduce the free radicals ions **(Salau et al., 2020).**

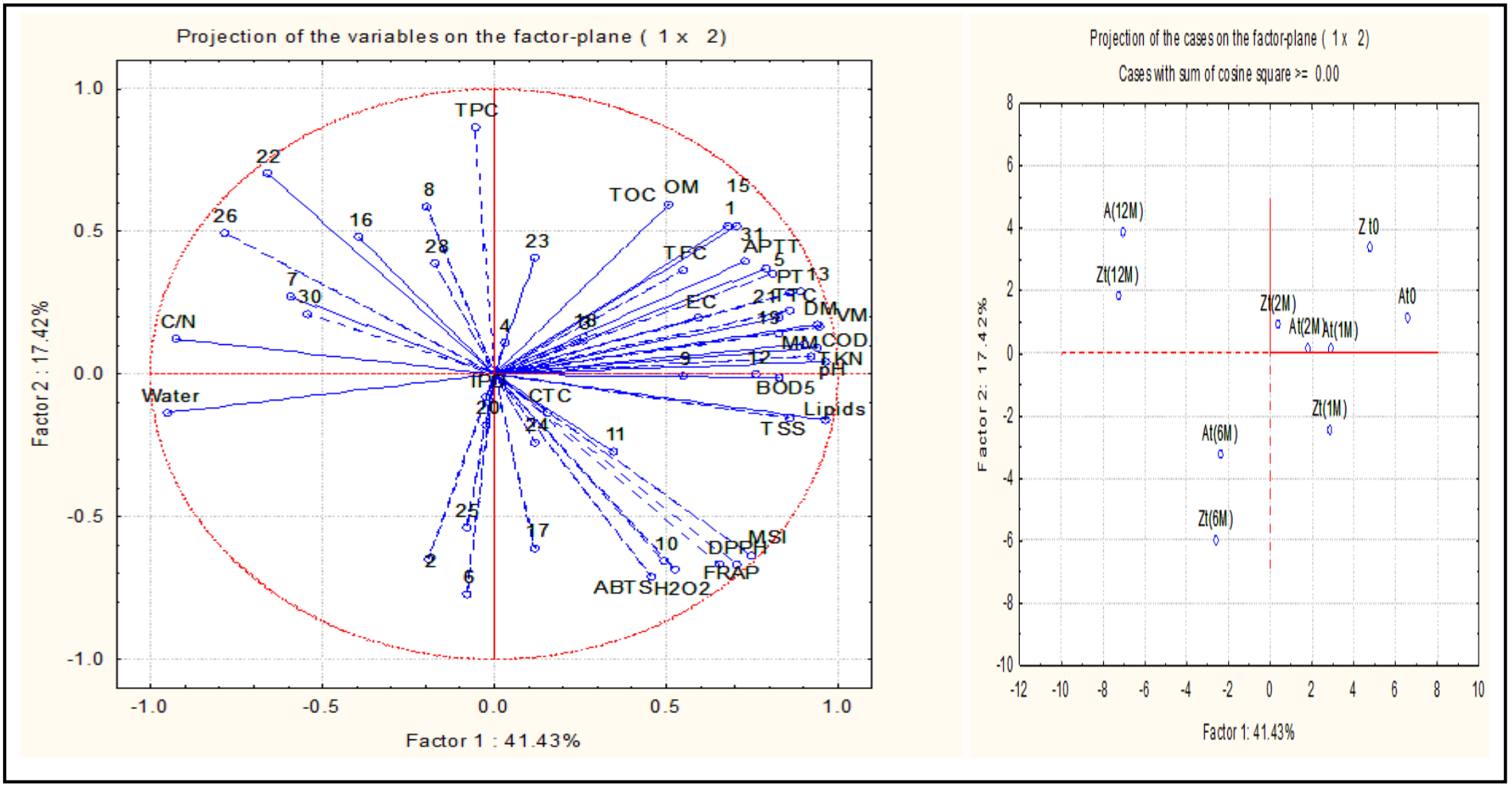

Figure 53: Principal component analysis (PCA) of the measured parameters. **1.** Quinic acid, **2.** Gallic acid, **3.** Protocatechuic acid, **4.** Catechin (+), **5.** Caffeic acid, **6.** Syringic acid, **7.** 1,3-di-o-caffeoyquinic acid, **8.** Epicatechin, **9.** P-coumaric acid, **10.** Rutin, **11.** Trans frolic acid, **12.** Hyperoside (quercetin-3-o-galactoside), **13.** Luteolin-7-o-glucoside, **15.** Naringin, **16.** Rosmarinic acid, **17.** 4,5-di-o-caffeoyquinic acid, **18.** Quercetrin (quercetin-3-o-rhamnoside), **19.** Apegenin-7-o-glucoside, **20.** O-coumaric acid, **21.** Salviolinic acid, **22.** Kampherol, **23.** Quercetin, **24.** Trans cinnamic, **25.** Naringenin, **26.** Apegenin, **28.** Cirsiliol, **30.** Acacetin, **31.** Chlorogenic acid.

General Conclusion and Perspectives

General Conclusion and Perspectives

One of the most serious problems confronting olive oil-producing countries is effluent from olive oil mills named olive oil mill wastewaters (OMW). Because they are rejected directly in ecosystems without treatment, it damages the environment because its properties are critical in the treatment and the valorization of these effluents.

This topic was interested in the physicochemical characterization of OMW, in the extraction and characterization of their content in phenolic compounds, in order to study their antioxidant and anti-inflammatory potential and their anticoagulant action during 12 months of storage in room temperature.

The physicochemical characterizations of OMW from two separate and mixed varieties showed that the storage of OMW could help decrease the pollution produced by this waste over time. During one year, the reduction rate of chemical oxygen demand (COD), biological oxygen demand (BOD_5), total oxidizable matter (TOM), and biodegradability index (BI) is correspondingly (29.4%, 54.8%, 39.16%, and 54.2%); however, C/N, BOD_5/COD, and pH continue to decrease.

The highest phenolic concentration (961.11 ± 65.95 µg GAE/mL) was recorded for OMW of Abani variety after 12 months of storage. The highest concentration of flavonoids (27.96 ± 3.71 µg QE/mL) was recorded for OMW of Zlitni just after olive oil extraction. The highest concentration of total tannin (90.47 ± 21.24 µg CAE/mL) was recorded for OMW of Zlitni after 1 month of storage. For condensed tannin, the highest concentration (8.66 ± 3.22 µg TAE/mL) was recorded after 6 months of storage of OMW of Abani.

The results obtained from this study showed that there was variation in the proportions of the bioactive elements of the two-variety samples. The storage time effect also revealed a very substantial difference in the rate components of the same variety. The diversity in the results was attributable to differences in olive type, ripening stage, time, place, and storage procedures before pressing olives, and storage temperature of olive oil mill wastewaters.

The organic load rate, microbial load, the acidity of the environment, type of olives, storage conditions, and the storage period all contribute to OMW's biodegradability. It was noticed that the acidity of OMW rose in proportion to storage duration and that its biodegradability is directly lowered with storage time, which will include the selection of the appropriate treatment procedure later.

Concerning the characterization of polyphenols by HPLC-MS, the analysis revealed that there are 28 phenolic compounds contained in the OMW studied. They are: quinic acid, gallic acid, protocatechuic acid, catechin, caffeic acid, syringic acid, 1,3-di-o-caffeoyquinic acid, epicatechin, p-coumaric acid, rutin, trans frulic acid, kampherol, hyperoside (quercetin-3-o-galactoside), luteolin-7-o-glucoside, 4,5-di-o-caffeoyquinic acid, naringin, rosmarinic acid, quercetrin (quercetin-3-o-rhamnoside), apegenin-7- o-glucoside, o-coumaric acid, salviolinic acid, quercetin, trans cinnamic, naringenin, apegenin, cirsiliol, acacetin, and chlorogenic acid.

Kampherol represents the highest peak that is the major phenolic compound in OMW followed by protocatechuic acid, and it is noted that rosmarinic acid is detected for the first time in OMW in this study. The latter two stand out as compounds with high added values due to their various biological activities, particularly their antioxidant activity, which is beneficial for human health in addition to the anti-inflammatory and anticoagulant activity.

The antioxidant potentials of these products compared to standard antioxidants, namely ascorbic acid, Trolox and BHT were evaluated by various methods; direct scavenging of free radicals by the method of DPPH, ABTS, FRAP and H_2O_2. It showed a very highly significant difference for DPPH, ABTS, FRAP and H_2O_2. For the variety effect, there was a significant difference for FRAP. On the other hand, for the three other tests DPPH, ABTS and H_2O_2, there was a non-significant difference. The (varieties × time) interaction showed that there was a very highly significant difference for DPPH, FRAP and H_2O_2; on the other hand, there was a significant difference for the ABTS test.

Concerning the best result of the antioxidant activity, it was obtained in the vegetable waters of Abani after 12 months of storage for the four tests DPPH (151.12 ± 0.22 µg/mL), ABTS (129.32 ± 26.09 µg/mL), FRAP (72.42 ± 8.59 µg/mL), and H_2O_2 (75.52 ±0.85 µg/mL).

Concerning the anti-inflammatory activity of the phenolic compounds, the results of the two tests, inhibition of denaturation of proteins (IDP) and membrane stabilizing potential (MSP), compared to standard diclofenac sodium, that there was a very highly significant difference between the two tests IPD and MSP for the time effect, variety, and (variety × time) interaction. The best value obtained for the IPD test (25.47 ± 1.50 µg/mL) was recorded after 1 month of storage of OMW from the Abani variety. The best value obtained for the MSP test (16.11 ± 0.10 µg/mL) was recorded in OMW from Abani after 12 months of storage.

For the anticoagulant activity, the finding of the two tests prothrombin time (PT) and activated partial thromboplastin time (APTT) showed, for the time effect, a very highly

significant difference, as regards APTT and PT. For the variety effect, there was a non-significant difference for APTT and PT, and the (variety × time) interaction showed a highly significant difference for the APTT test and a very highly significant difference from PT. The best values obtained for APTT (80.07 ± 0.15 s) and PT (37.13 ± 0.31 s) were recorded in Zlitni OMW just after olive oil extraction.

It concluded that the physicochemical and biological properties of OMW are affected by the storage time. OMW is biodegradable effluents that the degree of pollution decreases with storage time. It is easily biodegradable after one month of storage and treatment feasibility using selected microbial strains.

Olive oil mill wastewater is an important source of phenolic compounds. The phenolic compounds of OMW have innumerable biological properties, among which the antioxidant, anti-inflammatory, and anticoagulant activities studied in this thesis give preliminary results and require further in-depth studies.

Thus, many perspectives can be considered:

- Application of biological treatment on stored OMW during all times of storage.
- *In vivo* evaluation of the cytotoxicity effect of the phenolic extract of OMW.
- *In vivo* evaluation of the antioxidant, anti-inflammatory and anticoagulant activities of the phenolic extract of OMW.
- Application of specific tests for the estimation of anticoagulant activity by targeting key coagulation factors: FII (thrombin) and FX (prothrombin).
- Purification of polyphenols and determination of their antioxidant, anti-inflammatory and anticoagulant activities separately and combined to bring out the synergistic effect between these molecules.

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ANNEXES

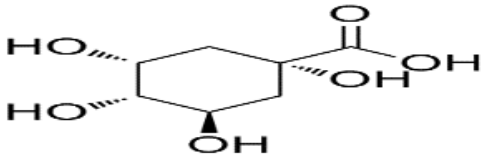
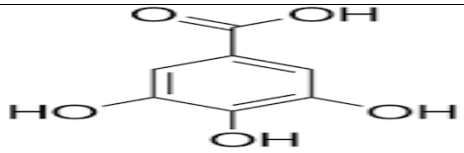
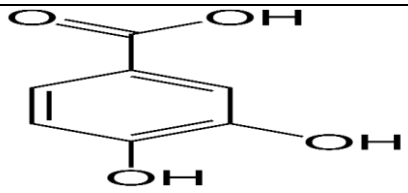
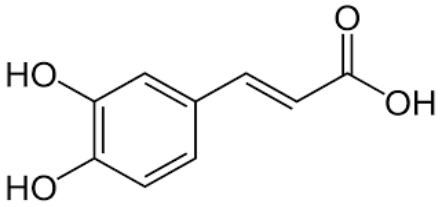
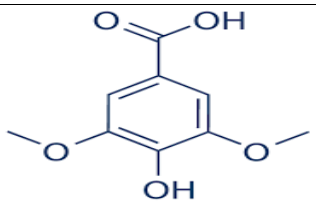
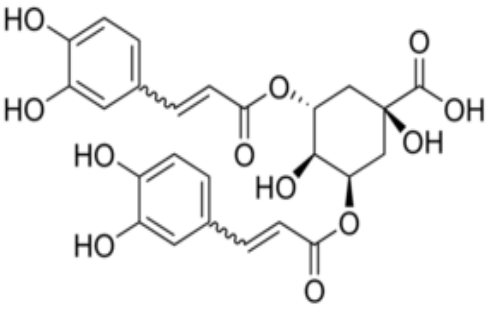
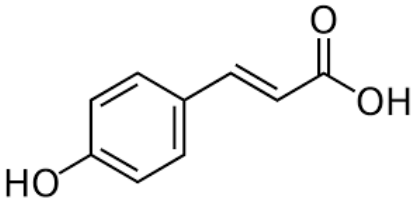
(a) **(b)**

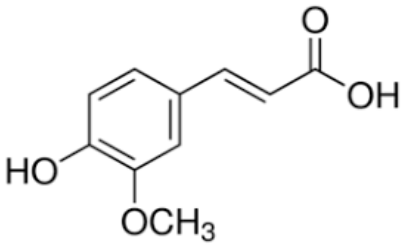
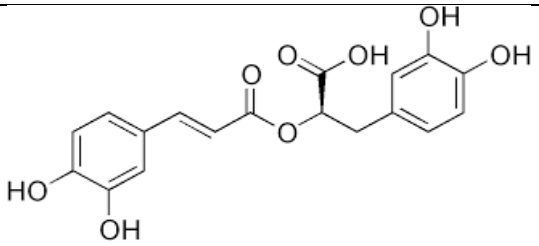
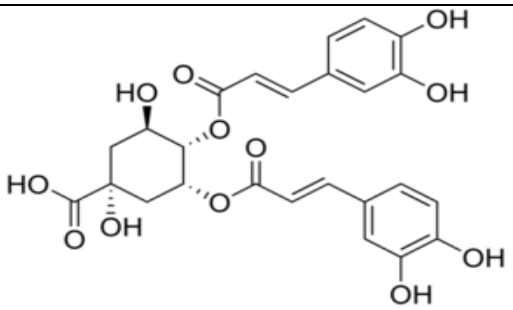
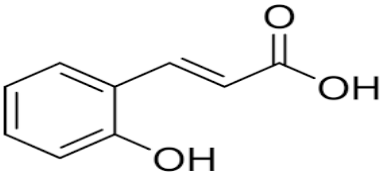
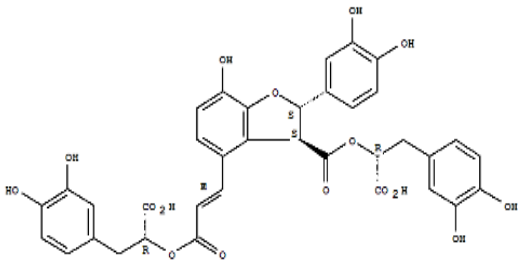
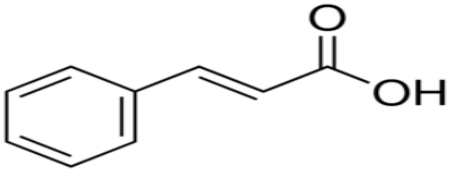
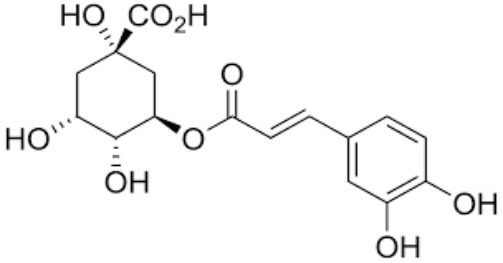
Annex 1: The two varieties of pressed olives. **(a):** Abani, **(b):** Zlitni

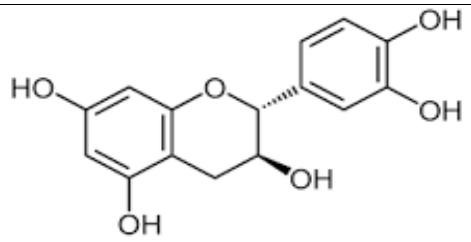
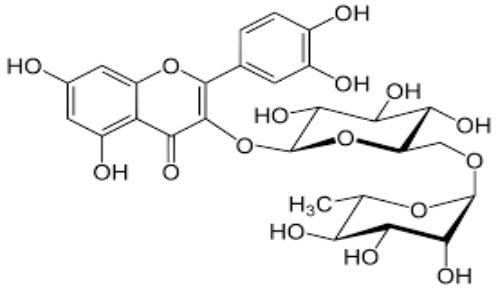
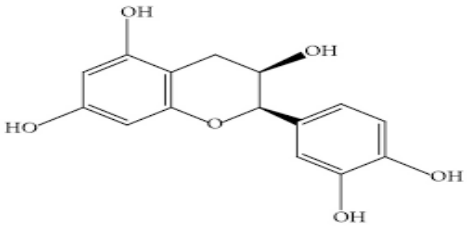
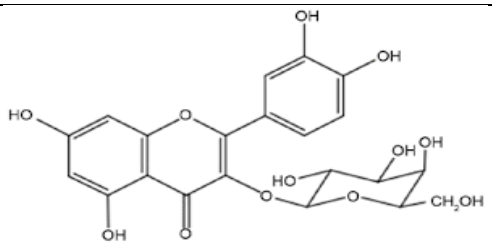
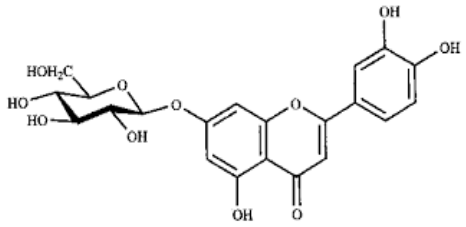
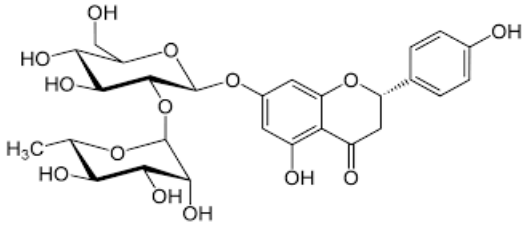
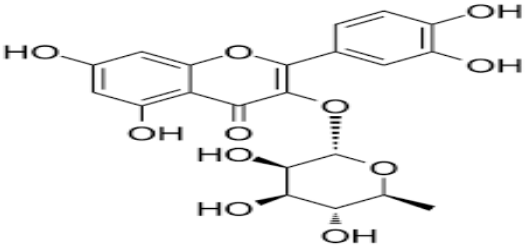
(a) **(b)** **(c)**

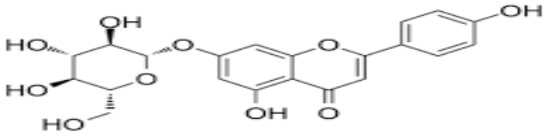
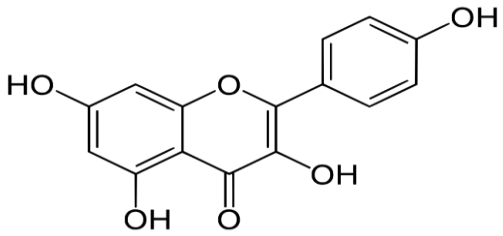
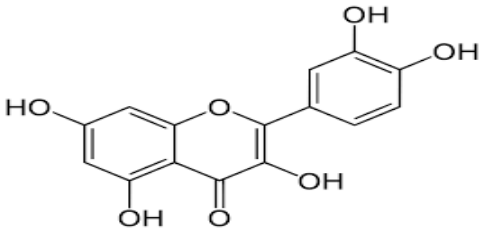
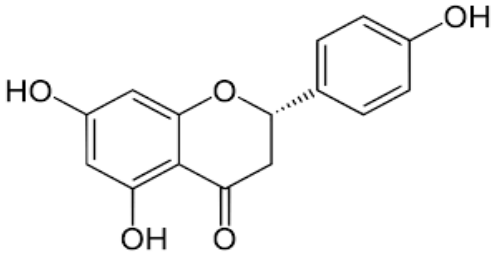
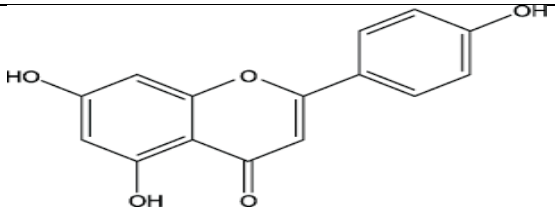
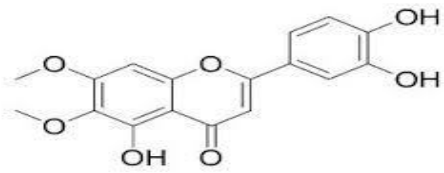
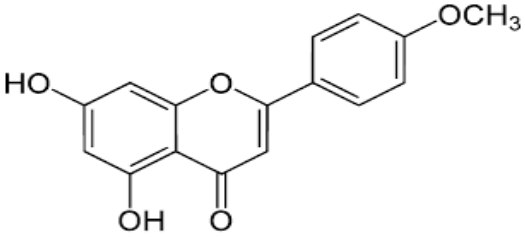
Annex 2: The olive oil extraction system used. **(a):** Mixer; **(b):** Crusher, **(c):** Centrifuge

Annex 3: Chemical structure of the phenolic compounds detected in the OMW studied

Number	**Compound**	**Molecular Formula**	**Chemical Structure**
01	Quinic acid	$C_7H_{12}O_6$	
02	Gallic acid	$C_7H_6O_5$	
03	Protocatechuic acid	$C_7H_6O_4$	
04	Caffeic acid	$C_9H_8O_4$	
05	Syringic acid	$C_9H_{10}O_5$	
06	1,3-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	
07	p-coumaric acid	$C_9H_8O_3$	

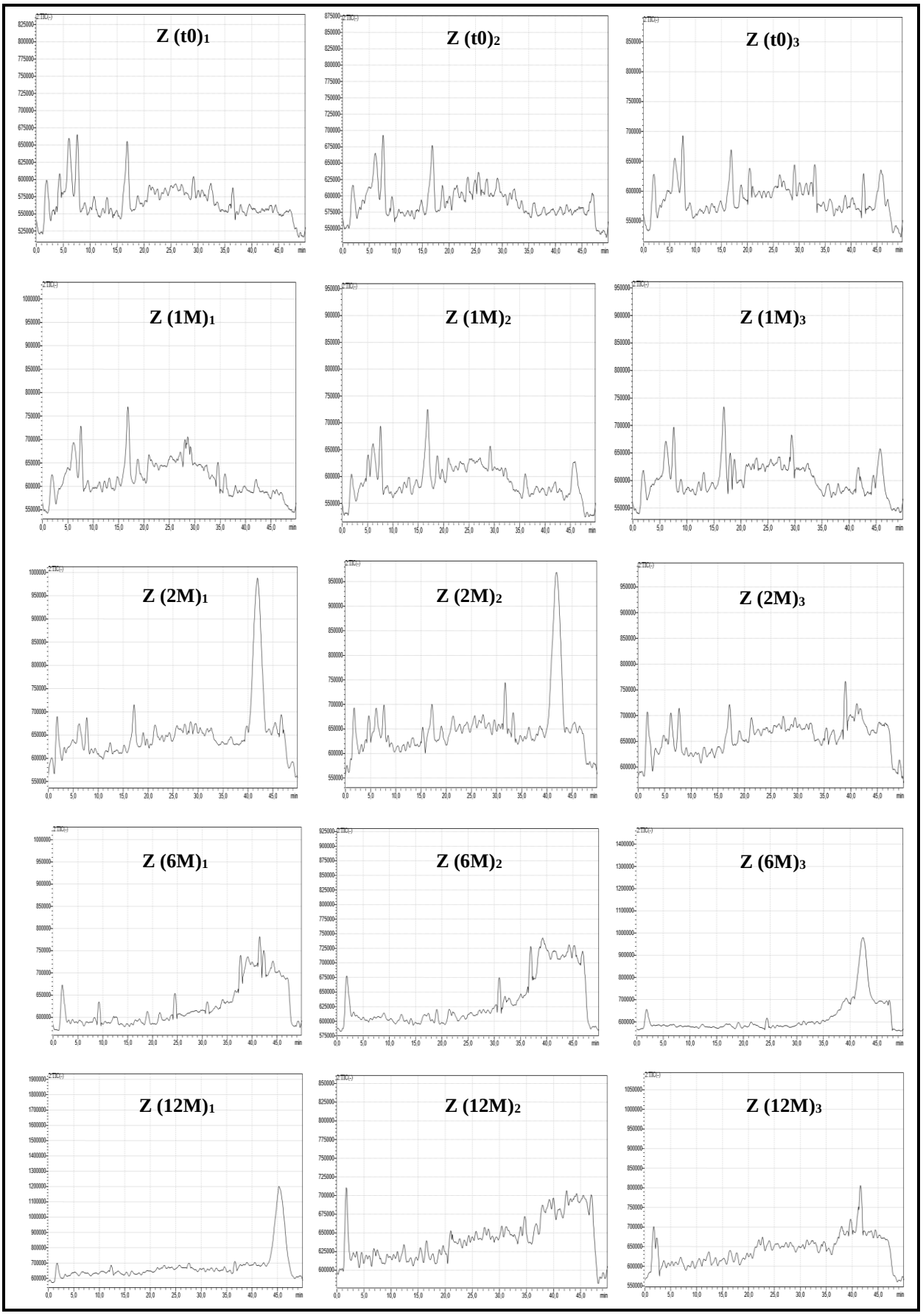
08	Trans frulic acid	$C_{10}H_{10}O_4$	
09	Rosmarinic acid	$C_{18}H_{16}O_8$	
10	4,5-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	
11	O-Coumaric acid	$C_9H_8O_3$	
12	Salviolinic acid	$C_{36}H_{30}O_{16}$	
13	Trans cinnamic	$C_9H_8O_2$	
14	Chlorogenic acid	$C_{16}H_{18}O_9$	

15	Catechin (+)	$C_{15}H_{14}O_6$	
16	Rutin	$C_{27}H_{30}O_{16}$	
17	Epicatechin	$C_{15}H_{14}O_6$	
18	Hyperoside (quercetin-3-o-galactoside)	$C_{21}H_{20}O_{12}$	
19	Luteolin-7-o-glucoside	$C_{21}H_{20}O_{11}$	
20	Naringin	$C_{27}H_{32}O_{14}$	
21	Quercetrin (quercetin-3-o-rhamnoside)	$C_{21}H_{20}O_{11}$	

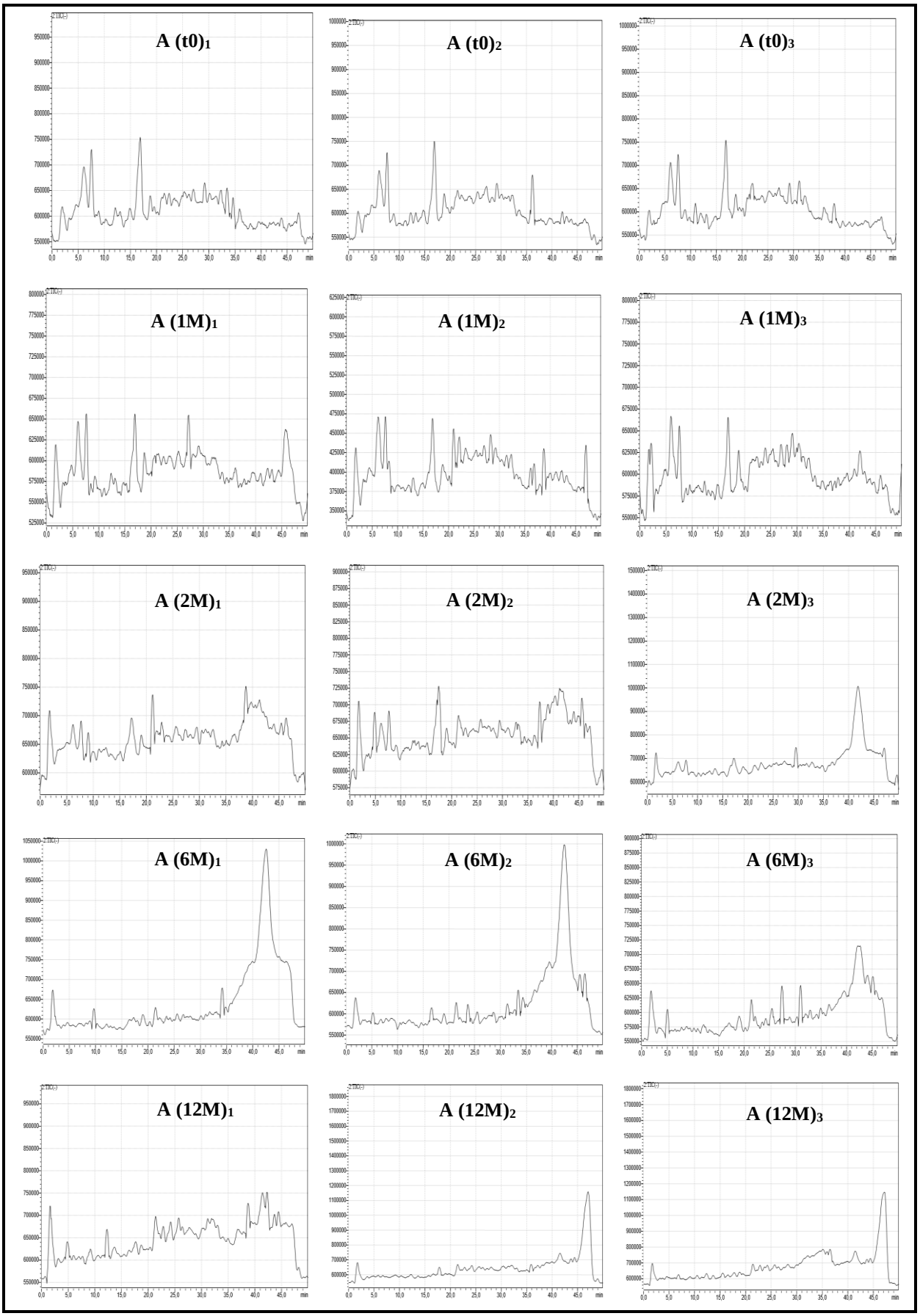
22	Apegenin-7-o-glucoside	$C_{21}H_{20}O_{10}$	
23	Kampherol	$C_{15}H_{10}O_6$	
24	Quercetin	$C_{15}H_{10}O_7$	
25	Naringenin	$C_{15}H_{12}O_5$	
26	Apegenin	$C_{15}H_{10}O_5$	
27	Cirsiliol	$C_{17}H_{14}O_7$	
28	Acacetin	$C_{16}H_{12}O_5$	

Annex 4: Treatment technologies applied to OMW **(Senani-Oularbi, 2018)**

Type of treatment	**Process**	**Principle**
Physical treatments	Natural evaporation	Natural auto purification of vegetable waters in deep ponds (0.7 to 1.5m) by aerobic and anaerobic fermentation processes
	Forced evaporation	Facilitate the evaporation of vegetable waters due to the evaporator panels.
	Thermal concentration	Raising of the dry matter content of vegetable waters to around 70%.
	Drying	Provided by the fumes from the combustion of crushed stones or dried pomace.
	Incineration	Evaporation of the aqueous phase of the vegetable waters in an oven; and converting the solid residue to ash.
Physico-chemical treatments	Coagulation-flocculation	Agglomeration and settling of suspended particles from vegetable waters by the injection of surfactants or certain coagulants.
	Electrocoagulation	Elimination of certain pollutions of vegetable waters by using iron or aluminum electrodes with the addition of H_2O_2 and polyaluminum chloride (PAC) as a coagulant aid.
	Adsorption on activated carbon	Eliminate a large proportion of the residual chemical demand.
Biological treatments	Anaerobic treatments	Transformation of organic substances present in OMW to methane by anaerobic bacteria by limiting release of bad odors.
	Aerobic treatments	Degradation of organic matter in vegetable waters by microorganisms in the presence of oxygen, transforming it into CO_2, H_2O and in cell mass.



Annex 5: Chromatograms of OMW samples of the Zlitni variety



Annex 6: Chromatograms of OMW samples of the Abani variety

Annex 7: Principal component analysis (PCA) of the measured parameters

Factor coordinates of cases, based on correlations (Spreadsheet1)(Annexe)									
	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**	**Factor 5**	**Factor 6**	**Factor 7**	**Factor 8**	**Factor 9**
Z t0	4.76173	3.40086	-1.14269	1.47660	-2.04812	2.03657	0.27965	-2.17646	0.26660
Zt(1M)	2.85334	-2.44420	-4.25278	-2.89442	1.06730	-1.53356	0.92148	-1.08774	-0.22453
Zt(2M)	0.39417	0.95426	2.75016	-3.44999	0.46236	2.72614	1.00987	0.94686	-0.74693
Zt(6M)	-2.60671	-5.95854	-1.34178	1.58669	-2.40790	1.34154	0.41928	1.21997	-0.05815
Zt(12M)	-7.29312	1.86729	-1.13090	-1.49492	-0.74647	-0.07638	-3.24256	-0.24200	0.07861
At0	6.59787	1.15857	-1.45458	1.88965	2.23924	0.28247	-1.62768	1.99960	-0.40790
At(1M)	2.92535	0.14441	3.17432	0.19973	-2.13315	-3.06477	-0.17157	-0.05298	-1.38256
At(2M)	1.78904	0.17166	1.77982	-0.68878	-0.26595	-1.14093	0.33384	0.74274	2.74987
At(6M)	-2.39529	-3.22278	2.86005	1.66663	2.95306	0.36148	-0.36103	-2.01750	-0.00623
A(12M)	-7.02637	3.92847	-1.24161	1.70881	0.87963	-0.93255	2.43871	0.66750	-0.26878

Annex 8: Correlations between the parameters studied

Correlations (Spreadsheet1)

	pH	EC	Lipids	TSS	Water	DM	MM	VM	OM	TOC	TKN	C/N	BOD5	COD	TPC	TFC	TTC	CTC	DPPH	ABTS	FRAP	H2O2	IPD	MSI	APTT	PT	1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17	18	19	20	21	22	23	24	25	26	28	30	31
pH	1.00000000	0.74928709	0.95999509	0.93326060	-0.93606052	0.93136464	0.97762943	0.92666043	0.42819939	0.42753946	0.96766746	-0.95408383	0.91873886	0.91403838	-0.00091554	0.45847070	0.76769999	0.25412828	0.61428181	0.37531414	0.56309797	0.45125656	-0.13616193	0.68174949	0.78111616	0.84775151	0.60720404	-0.18414138	0.37363030	-0.02121228	0.75000078	-0.16724040	-0.52019525	-0.21545757	0.45428989	0.48761818	0.17808080	0.66945050	0.84545476	0.66137979	-0.30897676	-0.00737376	0.28833131	0.73198787	-0.06797958	0.71282872	-0.56823726	0.08989826	-0.02932332	-0.10546868	-0.74000038	-0.17827883	-0.56284848	0.72857272
EC	0.74928709	1.00000000	0.64873838	0.83936464	-0.59474715	0.59956969	0.67922626	0.59408181	0.22318899	0.22202021	0.72226464	-0.68333939	0.86090909	0.72104242	0.09334747	0.06623131	0.38597979	0.58494752	0.22723232	0.02417972	0.10117272	0.18654790	-0.23376363	0.31066666	0.45183939	0.50567575	0.29995555	-0.40076868	0.47529797	-0.18939377	0.33859595	-0.46229393	0.01047373	0.00758121	0.34512121	0.34100105	-0.20868634	0.35629595	0.48972626	0.50595858	0.16919090	-0.13262020	0.19054949	0.37984040	0.03000009	0.33000040	-0.18381811	0.13661010	-0.21677090	0.11703033	-0.33417676	0.07362692	-0.58094040	0.47530000
Lipids	0.95999509	0.64873838	1.00000000	0.93488773	-0.91414199	0.90000040	0.90950808	0.89721111	0.37425252	0.37341313	0.92107575	-0.96542020	0.85856060	0.86170404	-0.24262659	0.35477474	0.74384848	0.27353532	0.77239494	0.49535359	0.69848383	0.56536161	-0.09090951	0.83121213	0.71730909	0.80034242	0.56012028	-0.09130986	0.31969595	0.07291616	0.70946363	-0.02853591	-0.58384845	-0.28280808	0.46168181	0.66770101	0.24054141	0.66195757	0.77651010	0.54884242	-0.44664141	0.13363939	0.33867070	0.68316060	0.09918181	0.67968484	-0.72250250	0.07751414	0.02389999	0.08717171	-0.87793232	-0.32507676	-0.64827272	0.70569999
TSS	0.93326060	0.83936464	0.93488773	1.00000000	-0.79425555	0.78181810	0.85930101	0.77595968	0.22006666	0.21947171	0.88909008	-0.92189595	0.95427272	0.86613030	-0.20520201	0.22056521	0.56091515	0.45761515	0.66854747	0.42616383	0.58425353	0.54150202	-0.26318181	0.74852525	0.58678282	0.66200000	0.41531010	-0.10613178	0.45573131	-0.09726969	0.54200666	-0.12565653	-0.40166767	-0.24721919	0.43379595	0.67716161	0.06032121	0.50316868	0.62606088	0.46585875	-0.28092929	0.03320404	0.28020256	0.55786363	0.08141432	0.49666651	-0.62555593	0.09259596	-0.13570606	0.18611010	-0.77141441	-0.26283838	-0.67605353	0.62291515
Water	-0.93606052	-0.59474715	-0.91414199	-0.79425555	1.00000000	-0.99191778	-0.94848686	-0.99908008	-0.51538787	-0.51504949	-0.94760303	0.93393933	-0.75763636	-0.82272020	0.03303058	-0.44926969	-0.90969595	-0.10351010	-0.53256957	-0.22745740	-0.47402021	-0.28165080	-0.11448080	-0.59157557	-0.81211200	-0.89879696	-0.67758080	0.31106096	-0.31696363	-0.17503737	-0.80131315	0.20030318	0.59272835	0.21503535	-0.32345353	-0.34114040	-0.19319562	-0.78606062	-0.92860404	-0.68674001	0.38183939	-0.08076615	-0.44919393	-0.73180909	0.03979787	-0.77476666	0.47471313	-0.06991414	-0.16673535	0.17741715	0.70006464	0.22701001	0.48987272	-0.73283383
DM	0.93136464	0.59956969	0.90000040	0.78181810	-0.99191778	1.00000000	0.94828209	0.99980880	0.52722929	0.52693535	0.94393953	-0.92292919	0.75181897	0.82100909	0.00002005	0.45948383	0.91487272	0.09272752	0.50164141	0.20132727	0.44381212	0.25559090	0.12128080	0.56136262	0.81449191	0.90151111	0.68627676	-0.33312020	0.31986161	0.17245050	0.80343408	-0.22916229	-0.57506813	-0.19595913	0.31856666	0.30738989	0.18728484	0.79010202	0.93762626	0.70569292	-0.35536161	0.07322682	0.44851851	0.73587777	-0.05763030	0.77947171	-0.44454538	0.07222242	0.16960909	-0.20434350	-0.67362828	-0.20292920	-0.47804242	0.73268888

MM	0.977629	0.679226	0.909508	0.859301	-0.948486	0.948209	1.000000	0.943168	0.392261	0.391950	0.961797	-0.931749	0.864150	0.879625	0.053241	0.507908	0.809220	0.106753	0.535266	0.333616	0.514974	0.381995	-0.062823	0.607352	0.779994	0.859033	0.606950
VM	0.926643	0.594081	0.897211	0.775968	-0.999008	0.999880	0.943168	1.000000	0.532394	0.532102	0.940583	-0.920059	0.744428	0.815973	-0.002435	0.455894	0.917617	0.091818	0.498716	0.194371	0.439200	0.248783	0.129957	0.557674	0.814024	0.901201	0.688336
OM	0.428199	0.223188	0.374252	0.220666	-0.515387	0.527229	0.392261	0.532394	1.000000	0.999997	0.402527	-0.287310	0.237960	0.389233	0.477641	0.510186	0.511404	0.163895	0.080278	-0.121577	0.033771	-0.123052	0.065359	0.031125	0.713401	0.655327	0.636382
TOC	0.427539	0.222021	0.373413	0.219471	-0.515049	0.526935	0.391950	0.532102	0.999997	1.000000	0.401844	-0.286467	0.237033	0.388825	0.478476	0.511157	0.511705	0.162384	0.079723	-0.121418	0.033523	-0.123123	0.066394	0.030431	0.712944	0.654968	0.636287
TKN	0.966746	0.722264	0.921075	0.889008	-0.947603	0.943953	0.961797	0.940583	0.402527	0.401844	1.000000	-0.972880	0.889635	0.862663	-0.058008	0.388523	0.787291	0.308727	0.545210	0.230936	0.514249	0.323528	-0.040998	0.605431	0.754215	0.814995	0.535082
C/N	-0.954083	-0.683339	-0.965420	-0.921895	0.933933	-0.922919	-0.931749	-0.920059	-0.287310	-0.286467	-0.972880	1.000000	-0.870096	-0.834541	0.258520	-0.285358	-0.766821	-0.296282	-0.675288	-0.350581	-0.616286	-0.439850	0.018813	-0.742371	-0.697162	-0.781681	-0.493743
BOD5	0.918786	0.860909	0.858560	0.954272	-0.757636	0.751897	0.864150	0.744428	0.237960	0.237033	0.889635	-0.870096	1.000000	0.930262	-0.001242	0.389182	0.572113	0.449526	0.547644	0.347069	0.520424	0.496815	-0.331827	0.621213	0.561995	0.600672	0.435241

MM	-0.159412	0.380509	-0.025707	0.756110	-0.126187	-0.547383	-0.243706	0.348904	0.353140	0.208562	0.712159	0.891818	0.675803	-0.309759	-0.002694	0.313286	0.746680	-0.219602	0.722527	-0.498673	0.016754	0.050979	-0.271633	-0.685070	-0.167415	-0.471417	0.676840
VM	-0.340687	0.316026	0.181638	0.803606	-0.225661	-0.574911	-0.193059	0.316254	0.304384	0.185787	0.791813	0.937368	0.705269	-0.356679	0.076849	0.453948	0.733410	-0.049574	0.780196	-0.440761	0.074790	0.174971	-0.200486	-0.671307	-0.204144	-0.477125	0.733454
OM	-0.554455	-0.167434	0.440100	0.646978	-0.554284	-0.230078	0.184157	0.521520	-0.059892	0.030741	0.412547	0.618249	0.665339	0.022881	-0.341566	0.057504	0.476757	0.314627	0.668414	-0.012619	0.210620	-0.128905	-0.263865	-0.155087	0.294574	-0.082861	0.739904
TOC	-0.554364	-0.168037	0.439763	0.646745	-0.553522	-0.229963	0.184241	0.521568	-0.061340	0.031940	0.413292	0.618629	0.665845	0.023302	-0.341017	0.056811	0.477391	0.313319	0.668993	-0.012291	0.209787	-0.127503	-0.265317	-0.154322	0.295701	-0.081819	0.739030
TKN	-0.285678	0.414043	0.029910	0.704377	-0.264102	-0.631760	-0.345739	0.316888	0.388356	0.019949	0.672219	0.818630	0.556100	-0.427955	0.015024	0.372555	0.650440	-0.036105	0.653888	-0.484910	0.054709	0.033337	-0.071894	-0.684766	-0.238652	-0.421383	0.697148
C/N	0.163518	-0.395077	-0.042470	-0.669314	0.108843	0.637936	0.374707	-0.299675	-0.554526	-0.099598	-0.662320	-0.764052	-0.476153	0.499868	-0.153240	-0.409915	-0.616804	-0.024545	-0.613196	0.616089	-0.048151	-0.077818	-0.065548	0.799132	0.367917	0.546730	-0.642339
BOD5	-0.215883	0.526050	-0.288310	0.540007	-0.240388	-0.407025	-0.211353	0.490904	0.479514	0.032917	0.499797	0.634074	0.522283	-0.237576	-0.047420	0.167552	0.638915	-0.079603	0.527997	-0.522478	0.153260	-0.120656	0.086028	-0.618536	-0.112105	-0.482119	0.601015

COD	0.914038	0.721042	0.861704	0.866130	-0.822720	0.821009	0.879625	0.815973	0.389233	0.388825	0.862663	-0.834541	0.930262	1.000000	0.125497	0.633064	0.743995	0.219693
TPC	-0.009154	0.093347	-0.242659	-0.205201	0.033058	0.000205	0.053241	-0.002435	0.477641	0.478476	-0.058008	0.258520	-0.001242	0.125497	1.000000	0.574024	0.056776	-0.241504
TFC	0.458470	0.066231	0.354774	0.220521	-0.449269	0.459483	0.507908	0.455894	0.510186	0.511157	0.388523	-0.285358	0.389182	0.633064	0.574024	1.000000	0.587768	-0.332232
TTC	0.767699	0.385979	0.743848	0.560915	-0.909695	0.914872	0.809220	0.917617	0.511404	0.511705	0.787291	-0.766821	0.572113	0.743995	0.056776	0.587768	1.000000	-0.115419
CTC	0.254128	0.584952	0.273532	0.457615	-0.103510	0.092752	0.106753	0.091818	0.163895	0.162384	0.308727	-0.296282	0.449526	0.219693	-0.241504	-0.332232	-0.115419	1.000000
DPPH	0.614281	0.227232	0.772394	0.668547	-0.532569	0.501641	0.535266	0.498716	0.080278	0.079723	0.545210	-0.675288	0.547644	0.538220	-0.593944	0.192531	0.419652	0.243647
ABTS	0.375314	0.024179	0.495359	0.426183	-0.227457	0.201327	0.333616	0.194371	-0.121577	-0.121418	0.230936	-0.350581	0.347069	0.369987	-0.409436	0.268546	0.152637	-0.029146

COD	0.538220	0.369987	0.536057	0.507610	-0.271910	0.609406	0.601740	0.647942	0.647912	-0.301895	0.530197	-0.256986	0.672303	-0.216606	-0.444163	-0.072008	0.596591	0.364905
TPC	-0.593944	-0.409436	-0.506296	-0.426493	-0.277663	-0.585971	0.240744	0.136505	0.403756	-0.366135	0.060501	-0.164311	0.252955	-0.514663	0.305609	0.547117	0.173095	-0.663084
TFC	0.192531	0.268546	0.331552	0.288292	-0.219625	0.192521	0.496858	0.437511	0.693260	-0.163753	0.136679	-0.308030	0.662023	-0.082861	-0.380335	0.174457	0.570080	-0.176878
TTC	0.419652	0.152637	0.377146	0.228084	0.304863	0.454834	0.722819	0.791564	0.745662	-0.422337	0.200966	0.038910	0.805141	-0.164098	-0.539453	-0.045928	0.365149	0.100829
CTC	0.243647	-0.029146	0.148607	0.137357	-0.102012	0.198089	0.049971	0.016094	-0.276050	-0.373121	0.077888	0.028455	-0.154879	-0.458046	-0.088055	-0.341297	0.320543	0.376647
DPPH	1.000000	0.855210	0.953466	0.884479	0.021287	0.981225	0.357831	0.425137	0.163042	0.308494	-0.058851	-0.030361	0.376628	0.460391	-0.590145	-0.467567	0.541850	0.837223
ABTS	0.855210	1.000000	0.871146	0.957598	-0.089856	0.832911	0.096999	0.172915	-0.001616	0.596305	-0.138518	-0.226619	0.150431	0.739030	-0.320073	-0.343800	0.537368	0.683747

COD	0.245387	0.646468	0.780410	0.708546	-0.243920	-0.020281	0.213238	0.839192	-0.150581	0.711468	-0.534716	0.236992	0.035424	-0.041734	-0.627904	-0.062408	-0.439460	0.691012
TPC	-0.051270	-0.061722	0.215445	0.509243	0.551142	-0.656563	-0.205645	0.239111	-0.409143	0.201764	0.570742	0.272101	-0.283746	-0.688947	0.515463	0.572842	0.336348	0.259996
TFC	0.505590	0.451771	0.624450	0.687901	-0.141476	-0.177882	-0.155093	0.828727	-0.462353	0.719779	-0.233739	0.295177	0.109988	-0.512139	-0.177714	0.235667	0.139107	0.487783
TTC	0.390026	0.858170	0.921002	0.760153	-0.341656	0.268807	0.336723	0.838303	-0.199659	0.870564	-0.385579	0.188638	0.448264	-0.240068	-0.498223	-0.077088	-0.328382	0.573737
CTC	-0.527995	0.001852	-0.080559	-0.121928	-0.075466	-0.027119	-0.098111	-0.122670	0.630150	-0.043475	-0.179102	-0.052627	-0.250434	0.640168	-0.153316	0.112847	-0.163315	0.166048
DPPH	0.494219	0.502131	0.403493	0.172630	-0.591169	0.481339	-0.043272	0.509767	0.205034	0.512367	-0.987836	-0.122196	0.194872	0.351281	-0.878983	-0.298003	-0.521432	0.281060
ABTS	0.702355	0.352014	0.234217	0.130572	-0.314062	0.419276	-0.305035	0.459592	-0.073997	0.376537	-0.910789	-0.285677	0.177841	0.114074	-0.693080	-0.081517	-0.405058	0.014634

FRAP	0.563097	0.101172	0.698483	0.584253	-0.474021	0.443812	0.514974	0.439200	0.033771	0.033523	0.514249	-0.616286	0.520424	0.536057	-0.506296	0.331552	0.377146	0.148607
H2O2	0.451256	0.186590	0.565361	0.541502	-0.281650	0.255590	0.381995	0.248783	-0.123052	-0.123123	0.323528	-0.439850	0.496815	0.507610	-0.426493	0.288292	0.228084	0.137357
IPD	-0.136193	-0.233763	-0.090951	-0.263181	-0.114480	0.121280	-0.062823	0.129957	0.065359	0.066394	-0.040998	0.018813	-0.331827	-0.271910	-0.277663	-0.219625	0.304863	-0.102012
MSP	0.681749	0.310666	0.831213	0.748525	-0.591557	0.561362	0.607352	0.557674	0.031125	0.030431	0.605431	-0.742371	0.621213	0.609406	-0.585971	0.192521	0.454834	0.198089
APTT	0.781116	0.451839	0.717309	0.586782	-0.811200	0.814491	0.779994	0.814024	0.713401	0.712944	0.754215	-0.697162	0.561995	0.601740	0.240744	0.496858	0.722819	0.049971
PT	0.847751	0.505675	0.800342	0.662000	-0.898796	0.901511	0.859033	0.901201	0.655327	0.654968	0.814995	-0.781681	0.600672	0.647942	0.136505	0.437511	0.791564	0.016094
1	0.607204	0.299955	0.560128	0.415310	-0.677580	0.686276	0.606950	0.688336	0.636382	0.636287	0.535082	-0.493743	0.435241	0.647912	0.403756	0.693260	0.745662	-0.276050

FRAP	0.953466	0.871146	1.000000	0.883983	-0.073360	0.929308	0.284516	0.331260	0.123881	0.369664	0.019105	-0.102708	0.336611	0.495533	-0.714931	-0.569257	0.496320	0.712415
H2O2	0.884479	0.957598	0.883983	1.000000	-0.112517	0.866652	0.090487	0.154837	0.031409	0.416068	-0.024936	-0.375782	0.163066	0.600982	-0.331974	-0.321948	0.645711	0.702832
IPD	0.021287	-0.089856	-0.073360	-0.112517	1.000000	-0.069934	-0.016345	0.082020	-0.201956	-0.241957	-0.496995	0.276680	-0.094487	0.152971	-0.068257	-0.240036	-0.089774	-0.242647
MSP	0.981225	0.832911	0.929308	0.866652	-0.069934	1.000000	0.379169	0.464577	0.238087	0.320539	0.091876	-0.047783	0.419984	0.444387	-0.565270	-0.413901	0.475228	0.870137
APTT	0.357831	0.096999	0.284516	0.090487	-0.016345	0.379169	1.000000	0.966052	0.765839	-0.211562	-0.059428	0.206330	0.936236	-0.306435	-0.435440	0.062677	0.361877	0.240529
PT	0.425137	0.172915	0.331260	0.154837	0.082020	0.464577	0.966052	1.000000	0.738076	-0.189324	0.018588	0.272841	0.898906	-0.208845	-0.429581	-0.013454	0.323881	0.305515
1	0.163042	-0.001616	0.123881	0.031409	-0.201956	0.238087	0.765839	0.738076	1.000000	-0.321658	0.231953	0.048258	0.914042	-0.316410	-0.233040	0.474520	0.365881	0.072423

FRAP	0.480863	0.457089	0.366777	0.096950	-0.698337	0.392466	-0.073693	0.519001	0.087377	0.469134	-0.964051	-0.169661	0.189234	0.246293	-0.840590	-0.314476	-0.289278	0.261165
H2O2	0.648697	0.406621	0.253787	0.179505	-0.331710	0.484960	-0.311576	0.540892	-0.047408	0.424148	-0.937140	-0.156656	0.228571	0.275972	-0.684215	-0.045162	-0.430831	0.038146
IPD	0.225868	0.522532	0.206568	0.072055	-0.033807	0.695704	-0.048299	0.053474	0.036661	0.277295	-0.006331	-0.439332	0.803105	-0.065033	0.114492	0.270131	0.049470	-0.381145
MSP	0.470657	0.490279	0.439842	0.206269	-0.573477	0.423851	0.087602	0.522117	0.147648	0.483985	-0.969881	-0.062780	0.137350	0.329259	-0.928306	-0.401620	-0.609767	0.346360
APTT	0.161963	0.475616	0.784713	0.684251	-0.209540	-0.190773	0.155507	0.597733	-0.041265	0.737996	-0.262780	0.299089	-0.165901	-0.346204	-0.445081	-0.070903	-0.376971	0.764033
PT	0.222344	0.614933	0.875599	0.726237	-0.197724	-0.083530	0.278116	0.635596	-0.047033	0.766737	-0.341715	0.144985	-0.049040	-0.355731	-0.563875	-0.114498	-0.509408	0.748847
1	0.321372	0.397238	0.714368	0.782489	-0.078583	-0.242263	0.326214	0.698055	-0.175475	0.681721	-0.122793	0.633151	-0.087601	-0.328195	-0.322644	-0.127336	-0.347035	0.783839

2	-0.184138	-0.400768	-0.091386	-0.106178	0.311096	-0.333120	-0.159412	-0.340687	-0.554455	-0.554364	-0.285678	0.163518	-0.215883	-0.301895	-0.366135	-0.163753	-0.422337	-0.373121	0.308494	0.596305	0.369664	0.416068	-0.241957	0.320539	-0.211562	-0.189324	-0.321658	1.000000	-0.237232	-0.105467	-0.190223	0.842810	-0.009336	-0.199505	-0.213754	0.399077	0.350349	-0.354781	-0.343579	-0.434399	-0.156966	0.095096	-0.227889	-0.249819	-0.228733	-0.323788	-0.351649	-0.278280	-0.191206	-0.077008	-0.244762	-0.344836	-0.102506	-0.340875
3	0.373630	0.475297	0.319695	0.455731	-0.316963	0.319861	0.380509	0.316026	-0.167434	-0.168037	0.414043	-0.395077	0.526050	0.530197	0.060501	0.136679	0.200966	0.077888	-0.058851	-0.138518	0.019105	-0.024936	-0.496995	0.091876	-0.059428	0.018588	0.231953	-0.237232	1.000000	-0.153784	0.056995	-0.297774	-0.147799	-0.055637	-0.179615	0.049946	-0.328532	0.067046	0.188476	0.093339	-0.134245	-0.309339	0.655684	0.143787	-0.127668	-0.117257	0.031186	0.170872	-0.173668	0.075096	-0.263998	-0.423637	-0.119938	0.386553
4	-0.021228	-0.189377	0.072916	-0.097269	-0.175037	0.172450	-0.025707	0.181638	0.440100	0.439763	0.029910	-0.042470	-0.288310	-0.256986	-0.164311	-0.308030	0.038910	0.028455	-0.030361	-0.226619	-0.102708	-0.375782	0.276680	-0.047783	0.206330	0.272841	0.048258	-0.105467	-0.153784	1.000000	0.070954	-0.156786	-0.152738	-0.183522	-0.293288	0.096613	-0.295152	0.055656	0.127870	-0.079170	-0.138739	-0.209808	0.534647	-0.294879	0.640192	-0.040893	0.107926	-0.283726	-0.142468	-0.032351	-0.231219	-0.292978	-0.112562	0.362005
5	0.750078	0.338595	0.709463	0.542066	-0.801315	0.803408	0.756110	0.803606	0.646978	0.646745	0.704377	-0.669314	0.540007	0.672303	0.252955	0.662023	0.805141	-0.154879	0.376628	0.150431	0.336611	0.163066	-0.094487	0.419984	0.936236	0.898906	0.914042	-0.190223	0.056995	0.070954	1.000000	-0.218692	-0.463033	0.198246	0.392964	0.214817	0.333227	0.485390	0.789893	0.728042	-0.279565	-0.132595	0.209662	0.721916	-0.166502	0.776918	-0.308462	0.481984	-0.068530	-0.335335	-0.464955	-0.163621	-0.353657	0.772918
6	-0.167240	-0.462293	-0.028591	-0.125653	0.200318	-0.221629	-0.126187	-0.225661	-0.554284	-0.553522	-0.264102	0.108843	-0.240388	-0.216606	-0.514663	-0.082861	-0.164098	-0.458046	0.460391	0.739030	0.495533	0.600982	0.152971	0.444387	-0.306435	-0.208845	-0.316410	0.842810	-0.297774	-0.156786	-0.218692	1.000000	-0.059683	-0.258963	-0.041158	0.359519	0.663854	0.040151	-0.161567	-0.267421	-0.195771	0.543327	-0.248327	0.007301	-0.267831	-0.064760	-0.537880	-0.417801	0.334959	-0.012985	-0.313491	-0.193716	-0.158834	-0.486388
7	-0.520195	0.010473	-0.583845	-0.401667	0.592728	-0.575068	-0.547383	-0.574911	-0.230078	-0.229963	-0.631760	0.637936	-0.407025	-0.444163	0.305609	-0.380335	-0.539453	-0.088055	-0.590145	-0.320073	-0.714931	-0.331974	-0.068257	-0.565270	-0.435440	-0.429581	-0.233040	-0.009336	-0.147799	-0.152738	-0.463033	-0.059683	1.000000	0.685962	-0.113277	-0.266536	-0.057801	-0.441176	-0.446304	-0.022320	0.927297	-0.153733	-0.245166	-0.392407	-0.076846	-0.421198	0.549880	0.084155	-0.195842	-0.036764	0.614980	0.454610	-0.151921	-0.423274
8	-0.215457	0.007581	-0.282808	-0.247219	0.215035	-0.195913	-0.243706	-0.193059	0.184157	0.184241	-0.345739	0.374707	-0.211353	-0.072008	0.547117	0.174457	-0.045928	-0.341297	-0.467567	-0.343800	-0.569257	-0.321948	-0.240036	-0.413901	0.062677	-0.013454	0.474520	-0.199505	-0.055637	-0.183522	0.198246	-0.258963	0.685962	1.000000	0.119466	-0.266210	0.174065	-0.278496	-0.072114	0.379362	0.664183	-0.293304	-0.083227	0.061296	-0.197165	0.014426	0.459908	0.683161	-0.235314	-0.185804	0.449521	0.269660	-0.184142	0.083033

9	0.454289	0.345121	0.461681	0.433795	-0.323453	0.318566	0.348904	0.316254	0.521520	0.521568	0.316888	-0.299675	0.490904	0.596591	0.173095	0.570080	0.365149	0.320543	0.541850	0.537368	0.496320	0.645711	-0.089774	0.475228	0.361877	0.323881	0.365881	-0.213754	-0.179615	-0.293288	0.392964	-0.041158	-0.113277	0.119466	1.000000	0.325577	0.508000	0.468244	0.432518	0.610268	0.046178	0.154383	-0.480185	0.717872	0.106768	0.710423	-0.561199	0.173297	0.104993	0.126414	-0.313509	0.484805	-0.290810	0.333656
10	0.487618	0.341005	0.667701	0.677161	-0.341140	0.307389	0.353140	0.304384	-0.059892	-0.061340	0.388356	-0.554526	0.479514	0.364905	-0.663084	-0.176878	0.100829	0.376647	0.837223	0.683747	0.712415	0.702832	-0.242647	0.870137	0.240529	0.305515	0.072423	0.399077	0.049946	0.096613	0.214817	0.359519	-0.266536	-0.266210	0.325577	1.000000	0.202674	0.117674	0.119750	-0.027613	-0.374363	0.237581	0.123220	0.141141	0.422431	0.137437	-0.799149	0.008980	-0.205584	0.545617	-0.822338	-0.484038	-0.737963	0.300681
11	0.178080	-0.208634	0.240541	0.060321	-0.193195	0.187284	0.208562	0.185787	0.030741	0.031940	0.019949	-0.099598	0.032917	0.245387	-0.051270	0.505590	0.390026	-0.527995	0.494219	0.702355	0.480863	0.648697	0.225868	0.470657	0.161963	0.222344	0.321372	0.350349	-0.328532	-0.295152	0.333227	0.663854	-0.057801	0.174065	0.508000	0.202674	1.000000	0.481559	0.377179	0.462402	-0.012757	0.525578	-0.346270	0.645423	-0.416768	0.592084	-0.565799	-0.016379	0.506547	-0.246390	-0.303683	0.192207	-0.299007	-0.092300
12	0.669450	0.356295	0.661957	0.503168	-0.786062	0.790102	0.712159	0.791813	0.412547	0.413292	0.672219	-0.662320	0.499797	0.646468	-0.061722	0.451771	0.858170	0.001852	0.502131	0.352014	0.457089	0.406621	0.522532	0.490279	0.475616	0.614933	0.397238	-0.354781	0.067046	0.055656	0.485390	0.040151	-0.441176	-0.278496	0.468244	0.117674	0.481559	1.000000	0.871553	0.697726	-0.214671	0.480735	0.143531	0.802040	-0.128786	0.853031	-0.508994	-0.261715	0.660625	-0.199203	-0.505441	0.182168	-0.325131	0.315373
13	0.845476	0.489726	0.776510	0.626088	-0.928604	0.937626	0.891818	0.937368	0.618249	0.618629	0.818630	-0.764052	0.634074	0.780410	0.215445	0.624450	0.921002	-0.080559	0.403493	0.234217	0.366777	0.253787	0.206568	0.439842	0.784713	0.875599	0.714368	-0.343579	0.188476	0.127870	0.789893	-0.161567	-0.446304	-0.072114	0.432518	0.119750	0.377179	0.871553	1.000000	0.861334	-0.160285	0.071164	0.278225	0.847900	-0.204049	0.893145	-0.378767	-0.009816	0.278929	-0.428102	-0.538384	0.065072	-0.394706	0.657268
15	0.661379	0.505958	0.548842	0.465875	-0.687401	0.705692	0.675803	0.705269	0.665339	0.665845	0.556100	-0.476153	0.522283	0.708546	0.509243	0.687901	0.760153	-0.121928	0.172630	0.130572	0.096950	0.179505	0.072055	0.206269	0.684251	0.726237	0.782489	-0.434399	0.093339	-0.079170	0.728042	-0.267421	-0.022320	0.379362	0.610268	-0.027613	0.462402	0.697726	0.861334	1.000000	0.261537	-0.046752	0.030409	0.850597	-0.279032	0.859361	-0.171188	0.230894	0.168682	-0.455442	-0.234363	0.384050	-0.419061	0.570370
16	-0.308976	0.169190	-0.446641	-0.280929	0.381839	-0.355361	-0.309759	-0.356679	0.022881	0.023302	-0.427955	0.499868	-0.237576	-0.243920	0.551142	-0.141476	-0.341656	-0.075466	-0.591169	-0.314062	-0.698337	-0.331710	-0.033807	-0.573477	-0.209540	-0.197724	-0.078583	-0.156966	-0.134245	-0.138739	-0.279565	-0.195771	0.927297	0.664183	0.046178	-0.374363	-0.012757	-0.214671	-0.160285	0.261537	1.000000	-0.261977	-0.284885	-0.158966	-0.184362	-0.164334	0.551308	0.004691	-0.177893	-0.291797	0.586920	0.655701	-0.140551	-0.247967

17	-0.007376	-0.132620	0.133639	0.033204	-0.087615	0.073282	-0.002694	0.076849	-0.341566	-0.341017	0.015024	-0.153240	-0.047420	-0.020281	-0.656563	-0.177882	0.268807	-0.027119	0.481339	0.419276	0.392466	0.484960	0.695704	0.423851	-0.190773	-0.083530	-0.242263
18	0.288331	0.190549	0.338670	0.280256	-0.449193	0.448551	0.313286	0.453948	0.057504	0.056811	0.372555	-0.409915	0.167552	0.213238	-0.205645	-0.155093	0.336723	-0.098111	-0.043272	-0.305035	-0.073693	-0.311576	-0.048299	0.087602	0.155507	0.278116	0.326214
19	0.731987	0.379840	0.683160	0.557863	-0.731809	0.735877	0.746680	0.733410	0.476757	0.477391	0.650440	-0.616804	0.638915	0.839192	0.239111	0.828727	0.838303	-0.122670	0.509767	0.459592	0.519001	0.540892	0.053474	0.522117	0.597733	0.635596	0.698055
20	-0.067958	0.030009	0.099181	0.081432	0.039787	-0.057630	-0.219602	-0.049574	0.314627	0.313319	-0.036105	-0.024545	-0.079603	-0.150581	-0.409143	-0.462353	-0.199659	0.630150	0.205034	-0.073997	0.087377	-0.047408	0.036661	0.147648	-0.041265	-0.047033	-0.175475
21	0.712872	0.330040	0.679684	0.496651	-0.774766	0.779471	0.722527	0.780196	0.668414	0.668993	0.653888	-0.613196	0.527997	0.711468	0.201764	0.719779	0.870564	-0.043475	0.512367	0.376537	0.469134	0.424148	0.277295	0.483985	0.737996	0.766737	0.681721
22	-0.568237	-0.183811	-0.722550	-0.625593	0.474713	-0.444538	-0.498673	-0.440761	-0.012619	-0.012291	-0.484910	0.616089	-0.522478	-0.534716	0.570742	-0.233739	-0.385579	-0.179102	-0.987836	-0.910789	-0.964051	-0.937140	-0.006331	-0.969881	-0.262780	-0.341715	-0.122793
23	0.089826	0.136610	0.077514	0.092596	-0.069914	0.072242	0.016754	0.074790	0.210620	0.209787	0.054709	-0.048151	0.153260	0.236992	0.272101	0.295177	0.188638	-0.052627	-0.122196	-0.285677	-0.169661	-0.156656	-0.439332	-0.062780	0.299089	0.144985	0.633151

17	0.095096	-0.309339	-0.209808	-0.132595	0.543327	-0.153733	-0.293304	0.154383	0.237581	0.525578	0.480735	0.071164	-0.046752	-0.261977	1.000000	-0.185551	0.218050	-0.054037	0.252264	-0.509344	-0.259244	0.842017	0.350616	-0.209356	0.029843	-0.207900	-0.494322
18	-0.227889	0.655684	0.534647	0.209662	-0.248327	-0.245166	-0.083227	-0.480185	0.123220	-0.346270	0.143531	0.278225	0.030409	-0.284885	-0.185551	1.000000	-0.060886	0.218585	-0.084106	0.089152	0.079328	-0.071119	0.046280	-0.384946	-0.701394	-0.273421	0.489565
19	-0.249819	0.143787	-0.294879	0.721916	0.007301	-0.392407	0.061296	0.717872	0.141141	0.645423	0.802040	0.847900	0.850597	-0.158966	0.218050	-0.060886	1.000000	-0.348385	0.929533	-0.535011	0.179015	0.373530	-0.282212	-0.463120	0.212980	-0.298730	0.485608
20	-0.228733	-0.127668	0.640192	-0.166502	-0.267831	-0.076846	-0.197165	0.106768	0.422431	-0.416768	-0.128786	-0.204049	-0.279032	-0.184362	-0.054037	0.218585	-0.348385	1.000000	-0.163039	-0.129338	-0.044054	-0.243372	0.669804	-0.242289	-0.192552	-0.182127	0.257213
21	-0.323788	-0.117257	-0.040893	0.776918	-0.064760	-0.421198	0.014426	0.710423	0.137437	0.592084	0.853031	0.893145	0.859361	-0.164334	0.252264	-0.084106	0.929533	-0.163039	1.000000	-0.497718	0.101059	0.393582	-0.294821	-0.443334	0.275511	-0.310408	0.515026
22	-0.351649	0.031186	0.107926	-0.308462	-0.537880	0.549880	0.459908	-0.561199	-0.799149	-0.565799	-0.508994	-0.378767	-0.171188	0.551308	-0.509344	0.089152	-0.535011	-0.129338	-0.497718	1.000000	0.168817	-0.248313	-0.321713	0.854912	0.249348	0.487192	-0.209688
23	-0.278280	0.170872	-0.283726	0.481984	-0.417801	0.084155	0.683161	0.173297	0.008980	-0.016379	-0.261715	-0.009816	0.230894	0.004691	-0.259244	0.079328	0.179015	-0.044054	0.101059	0.168817	1.000000	-0.316283	0.168824	0.133190	-0.174614	-0.098284	0.372735

24	-0.029332	-0.216790	0.023899	-0.135706	-0.166735	0.169609	0.050979	0.174971	-0.128905	-0.127503	0.033337	-0.077818	-0.120656	0.035424	-0.283746	0.109988	0.448264	-0.250434	0.194872	0.177841	0.189234	0.228571	0.803105	0.137350	-0.165901	-0.049040	-0.087601	-0.191206	-0.173668	-0.142468	-0.068530	0.334959	-0.195842	-0.235314	0.104993	-0.205584	0.506547	0.660625	0.278929	0.168682	-0.177893	0.842017	-0.071119	0.373530	-0.243372	0.393582	-0.248313	-0.316283	1.000000	-0.010426	-0.022595	0.217895	0.080732	-0.399205
25	-0.105468	0.117033	0.087171	0.186110	0.177415	-0.204350	-0.271633	-0.200486	-0.263865	-0.265317	-0.071894	-0.065548	0.086028	-0.041734	-0.688947	-0.512139	-0.240068	0.640168	0.351281	0.114074	0.246293	0.275972	-0.065033	0.329259	-0.346204	-0.355731	-0.328195	-0.077008	0.075096	-0.032351	-0.335335	-0.012985	-0.036764	-0.185804	0.126414	0.545617	-0.246390	-0.199203	-0.428102	-0.455442	-0.291797	0.350616	0.046280	-0.282212	0.669804	-0.294821	-0.321713	0.168824	-0.010426	1.000000	-0.212136	-0.298888	-0.222711	-0.120367
26	-0.740038	-0.334176	-0.877932	-0.771441	0.700064	-0.673628	-0.685070	-0.671307	-0.155087	-0.154322	-0.684766	0.799132	-0.618536	-0.627904	0.515463	-0.177714	-0.498223	-0.153316	-0.878983	-0.693080	-0.840590	-0.684215	0.114492	-0.928306	-0.445081	-0.563875	-0.322644	-0.244762	-0.263998	-0.231219	-0.464955	-0.313491	0.614980	0.449521	-0.313509	-0.822338	-0.303683	-0.505441	-0.538384	-0.234363	0.586920	-0.209356	-0.384946	-0.463120	-0.242289	-0.443334	0.854912	0.133190	-0.022595	-0.212136	1.000000	0.538819	0.617147	-0.549431
28	-0.177883	0.073692	-0.325076	-0.262838	0.227001	-0.202920	-0.167415	-0.204144	0.294574	0.295701	-0.238652	0.367917	-0.112105	-0.062408	0.572842	0.235667	-0.077088	0.112847	-0.298003	-0.081517	-0.314476	-0.045162	0.270131	-0.401620	-0.070903	-0.114498	-0.127336	-0.344836	-0.423637	-0.292978	-0.163621	-0.193716	0.454610	0.269660	0.484805	-0.484038	0.192207	0.182168	0.065072	0.384050	0.655701	0.029843	-0.701394	0.212980	-0.192552	0.275511	0.249348	-0.174614	0.217895	-0.298888	0.538819	1.000000	0.221217	-0.278428
30	-0.562848	-0.580940	-0.648272	-0.676053	0.489872	-0.478042	-0.471417	-0.477125	-0.082861	-0.081819	-0.421383	0.546730	-0.482119	-0.439460	0.336348	0.139107	-0.328382	-0.163315	-0.521432	-0.405058	-0.289278	-0.430831	0.049470	-0.609767	-0.376971	-0.509408	-0.347035	-0.102506	-0.119938	-0.112562	-0.353657	-0.158834	-0.151921	-0.184142	-0.290810	-0.737963	-0.299007	-0.325131	-0.394706	-0.419061	-0.140551	-0.207900	-0.273421	-0.298730	-0.182127	-0.310408	0.487192	-0.098284	0.080732	-0.222711	0.617147	0.221217	1.000000	-0.346406
31	0.728572	0.475300	0.705699	0.622915	-0.732883	0.732688	0.676840	0.733454	0.739904	0.739030	0.697148	-0.642339	0.601015	0.691012	0.259996	0.487783	0.573737	0.166048	0.281060	0.014634	0.261165	0.038146	-0.381145	0.346360	0.764033	0.748847	0.783839	-0.340875	0.386553	0.362005	0.772918	-0.486388	-0.423274	0.083033	0.333656	0.300681	-0.092300	0.315373	0.657268	0.570370	-0.247967	-0.494322	0.489565	0.485608	0.257213	0.515026	-0.209688	0.372735	-0.399205	-0.120367	-0.549431	-0.278428	-0.346406	1.000000

List of Scientific Publications and Communications

Publications and Communications

Publications

- **Gueboudji Zakia,** Addad Dalila, Kadi Kenza, Nagaz Kamel, Secrafi Mansour, Ben Yahya Leila, Lachehib Belgacem, Abdelmalek Assia. **(2022)**. Biological activities and phenolic compounds of olive oil mill wastewater from Abani, endemic Algerian variety. *Scientific Reports*, 1-16. DOI: 10.1038/s41598-022-10052-y
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2022)**. Anti-inflammatory Activity of Polyphenols from Olive Oil Mill Wastewaters, *Jordanian Journal of Engineering and Chemical Industries*, 5(1), 1-6. DOI: 10.48103/jjeci542022
- **Gueboudji Zakia,** Bagues Mohamed, Kadi Kenza, Nagaz Kamel, Addad Dalila. **(2021)**. Effect of storage time on the biodegradability of olive oil mill wastewater from the cold extraction of olive oil system. *The EuroBiotech Journal*, 5(3), 142-154. DOI: 10.2478/ebtj-2021-0023
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Evaluation of the Anticoagulant effect of phenolic extracts of two olive mill by-products: olive mill wastewater and olive mill pomace. *European Journal Of Science And Technology*, (28), 826-830. DOI: 10.31590/ejosat.1005114
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Extraction and quantification of polyphenols of olive oil mill wastewater from the cold extraction of olive oil in the region of Khenchela-Algeria. *Genetics and Biodiversity Journal*, 116-122. DOI: 10.46325/gabj.v5i2.79
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Étude quantitative et activité antioxydante des molécules bioactives des effluents issues de l'extraction de l'huile d'olive. *International Journal of Natural Resources and Environment*, 3(2), 16-21.

National Communications

- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Physicochemical characterization and assessement of the pollution degree of liquid effluents of oil mills from the cold extraction of olive oil in Khenchela- Algeria. 2ème Séminaire National des Sciences d'Interfaces Chimie – Biologie (2éme SNSI C-B) 20 Février. Université Mohammed Chérif Messaadia (Souk Ahras), Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Physicochemical study and antioxidant potential of olive oil mill wastewater from Khenchela-Algeria. Séminaire National : Ressources végétales, Produits Naturels et Santé (RVPNS) 9,10,11 Juin. Université Saad Dahlab (Blida), Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Effet de stockage sur les caractéristiques physico-chimiques des margines d'huiles d'olive. Séminaire National « La recherche agronomique au cœur de la sécurité alimentaire et au service de l'environnement » 1-2 Décembre. Université Mohamed El Bachir El Ibrahimi (Bordj Bou Arreridj), Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Assessment of the antioxidant power of by-products from the cold extraction of olive oil. 1er Atelier sur l'Evaluation et la Gestion des Ressources Environnementales 14 Juillet 2021, Université des Sciences et de la Technologie Houari Boumediene (USTHB) Alger, Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Valorization of the liquid effluents from olive oil mills into biogas. Journées Nationales de l'Agro-Eco-Biotechnologie (JNAEB), 15-16 Octobre, Faculté des Sciences de la Nature et de la Vie, Blida, Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Étude quantitative et activité antioxydante des margines issues de l'extraction à froid de l'huile d'olive dans la régions de Khenchela-Algérie. Séminaire National sur la Valorisation des Bio-ressources et Environnement SNVBE, 7-8 Avril. Université Ahmed Draia, Adrar, Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2022)**. Study of the Anti-Hemolytic Effect of Polyphenols Extracted From Oil Mill Effluents. The Valorization of Natural Resources and the Environment (VRNE 2022), 30 Mars, Setif, Algeria.

International Communications

- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2020)**. Extraction and quantification of bioactive molecules of olive oil mill wastewater from the cold extraction of olive oil in the region of Khenchela-Algeria. Sixth MGIBR International Workshop (webinar)of Aromatic, Medicinal and condiment plants Virtues and development prospects. 21-22 December, Tlemcen University, Algeria.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Phytochemical screening and assessment of the antioxidant activity of biophenols of olive oil mill wastewater from the cold extraction of olive oil in Khenchela region eastern of Algeria. First International Conference on Renewable Energy Advanced Technologies and Applications (ICREATA), October 25-27. Research Unit of Renewable Energies in Saharan Region, Adrar, Algeria.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Physicochemical and microbiological study of olive oil mill wastewater from the cold extraction of olive oil of Khenchela-Algeria. Séminaire International sur les Sciences Naturelles et de la Vie, 19-20 Février, International Journal of Human Settlements, Oran, Algérie.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Evaluation de l'effet anticoagulant de l'extrait phénolique des margines. lst International Seminar New visions in the sustainable amortization of the biodiversity of uncertain areas, 07-08 April, University Center Salhi Ahmed, Naâma, Algeria.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Evaluation of the Anti-inflammatory Activity of Polyphenols from Algerian Monovarietal Olive Oil Mill Wastewaters. 9th Jordan International Chemical Engineering Conference (JIChEC09), 12-14 October, Amman, Jordan.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Quantitative and qualitative study of olive oil mill wastewater from the cold extraction of olive oil in the region of Khechela-Algeria. 1ère Edition des Journées Internationales en Géoscience en Environnement (JIGE), 26-27 Mars, Agadir, Morocco.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. In vitro evaluation of the antioxidant activity of polyphenols of olive oil mill wastewater in the region of Khenchela-Algeria. International Seminar on Biodiversity, Valorization and Conservation of Urban and Forest Ecosystems: (In support of sustainable development), 28-29 April, M'sila, Algeria.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Evaluation of the anticoagulant effect of the phenolic extracts of olive mill wastewater and olive mill pomace. 1st International Conference on Applied Engineering and Natural Sciences (ICAENS), 1-3 November, Konya, Turkey.
- **Gueboudji Zakia,** Kadi Kenza, Nagaz Kamel. **(2021)**. Evaluation de niveau de pollution des eaux de végétation issues de l'extraction à froid de l'huile d'olive dans la région de Khechela-Algérie. 1ère Edition des Journées Internationales en Géoscience en Environnement (JIGE), 26-27 Mars, Agadir, Morocco.

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Biological activities and phenolic compounds of olive oil mill wastewater from Abani, endemic Algerian variety

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The current study aimed to determination of cytotoxicity, antioxidant, anti-inflammatory, anti-hemolytic, and anticoagulant activities of phenolic compounds extracted from olive oil mill wastewater (OMW) issue from the cold extraction of olive oil from Khenchela eastern in Algeria. The LC–MS (liquid chromatography–mass spectrometry) results were revealed the presence of 20 phenolic compounds in the extract of OMW and mostly consisted of Kaempferol, 4,5-di-O-caffeoyquinic acid, quinic acid, and caffeic acid. The extracts possessed effective reducing power (FRAP) and high radical scavenging activity against DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS + (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radicals, and it inhibited cytochrome c reduction in a dose-dependent manner. They exert a protective effect on red blood cells, and they were found to exhibit the highest inhibitory effect anti-inflammatory activity using inhibition of protein denaturation (IPD) and membrane stabilizing potential (MSP) tests (80.46 ± 3.81 µg/mL and 87.43 ± 0.66 µg/mL) more than the standard used. The extract also showed the greatest anticoagulant activity in both the endogenous and exogenous routes (44.77 ± 0.25 s and 15.84 ± 0.12 s, respectively). Based on these findings, it is reasonable to infer that OMW is a good source of natural phenolic compounds with potential antioxidant, anti-inflammatory, and anticoagulant properties.

Abbreviations

OMW	Olive oil mill wastewater
LC–MS	Liquid chromatography–mass spectrometry
DPPH	2,2-Diphenyl-1-picrylhydrazyl
$ABTS^+$	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric reducing ability of plasma
IC_{50}	Half-maximal inhibitory concentration
IDP	Inhibition of protein denaturation
MSP	Membrane-stabilizing potential
APTT	Endogenous coagulation pathway
PT	Exogenous coagulation pathway
EC	Electrical conductivity
DM	Dry matter
OM	Organic matter
MM	Mineral matter
BOD_5	Biological oxygen demand
COD	Chemical oxygen demand
TPC	Total phenolic content
TFC	Total flavonoids content
TCC	Tannin condensed content
DC_{50}	Death concentration

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pH	EC (mS/cm)	TSS%	DM (g/L)	OM (g/L)	BOD_5 (g/L)	COD (g/L)
4.9 ± 0.01	12.89 ± 0.09	0.9 ± 0.03	110.8 ± 3.17	53.7 ± 1.16	68 ± 2.28	170 ± 8.5

Table 1. Physicochemical properties of OMW studied.

LC_{50}	Median lethal concentration
PBS	Phosphate buffered saline
RBC	Red blood cells
PMA	Phorbol myristate acetate
PUFA	Polyunsaturated fatty acids
DMSO	Dimethyl sulfoxide
ROS	Reactive oxygen species
FRO	Free radicals of oxygen
NADPH	Nicotinamide adenine dinucleotide phosphate

The olive mills activity creates huge quantities of waste in a short period, generally 4 months from October to January. Based on the extraction methods used, an estimated average volume of olive mill wastewater (OMW) ranging from 0.3 to 1.2 m^3/tons of processing olives. Also, an average quantity of solid residue ranging from 500 to 735 kg/tons of processing olives has been observed[1]. The OMW is a turbid, watery, black, and foul-smelling liquid that contains easily fermentable emulsified grease[2,3]. It has a toxic effect on soil, microorganisms, plants, and marine organisms[4,5]. The OMW comprised about 98% of the total phenolic content of olive fruits ranging from 0.5 to 24 g/L[6], including hydroxytyrosol, tyrosol, and flavonoids[7]. Pollution is mainly due to the high concentrations of phenolic compounds[8]. Because of their acidity, high levels of biological oxygen demand, and chemical oxygen need, OMWs are highly contaminating and phytotoxic. On the other hand, OMWs, are a valuable source of molecules such as plant nutrients, anthocyanins, flavonoids, polysaccharides, and several phenolic compounds with industrial applications such as fertilizers, antioxidants, antifungal and antibacterial drugs, cytoprotective agents, gelling and stabilizing agents in food preservation. As a result, significant efforts have been made to shift from OMW detoxification to its commercialization by maximizing the recovery of high added-value bioactive chemicals[9]. Phenolic compounds have many biological properties, particularly antioxidant, anti-inflammatory, and anticoagulant effects, which are used in the pharmaceutical industries[10].

Cell damage caused by free radicals has been linked to several diseases including cancer, arthritis, and diabetes. Some free radicals and reactive nitrogen species can activate and upregulate cell death pathways such as apoptosis. Antioxidants have been linked to a range of therapeutic properties. Several researchers have hypothesized that antioxidants might reduce the harmful free radical effects improving consequently the therapeutic effectiveness[11]. However, oxidative stress is unsteady in the balance between the defense system of antioxidants and the production of reactive oxygen species (ERO)[12]. This causes biochemical damage in the organism's cells due to molecular repercussions, such as changes in proteins, the appearance of DNA breaks, or damage to the cell membrane integrity owing to the induction of lipid peroxidation. Oxidative stress has been described as a crucial etiological factor involved in various chronic human diseases such as cancer, cardiovascular and neurodegenerative diseases, inflammation, diabetes mellitus, and aging[13].

Inflammation and coagulation are two major host defense mechanisms that work in tandem[14]. They are implicated in many cardiovascular diseases such as thrombosis and atherosclerosis[15]. Thromboembolic diseases continue to be the leading cause of death throughout the world[16]. As is well known, thrombosis is closely related to activating platelet adhesion, aggregation, secretion functions, and activation of intrinsic and extrinsic coagulation systems, which cause blood coagulation and fibrin formation[17]. Therefore, anticoagulants play a pivotal role in the prevention and treatment of thrombotic disorders[18]. Diseases arising from blood clotting, including pulmonary emboli, deep vein thrombosis, and cardiovascular diseases are the main causes of death and disability worldwide[19].

Thrombosis is a process in which a blood clot develops within a blood artery and can obstruct blood flow in the afflicted area, eventually creating disorders such as pulmonary emboli, deep vein thrombosis, strokes, and heart attacks. On the other hand, Thrombolytic agents are those that inhibit the development of a blood clot in blood arteries, such as tissue plasminogen activator (tPA), streptokinase (SK) urokinase, and others. Natural thrombolytic medicines derived from traditionally significant medicinal plants may be a viable source in this case, as they have few negative effects than manufactured pharmaceuticals. Nowadays, more than 60% of cytotoxic agents are derived from natural sources such as plants, marine creatures, and microbes, either directly or by chemical modification based on natural lead compounds. Furthermore, natural compounds have a wide range of applications in cancer treatment[20]. Hence, the main objective of the current report is to evaluate the cytotoxicity, antioxidant, anti-inflammatory, and anticoagulant activities of phenolic compounds of OMW obtained from cold extraction of olive oil from Khenchela eastern Algeria.

Results

Physicochemical properties. The physicochemical characteristics of OMW that were studied are summarized in Table 1. The OMW is an acidic liquid effluent (pH = 4.9 ± 0.01) loaded with organic and mineral materials as indicated by a high electrical conductivity value (EC = 12.89 ± 0.09 mS/cm). The total suspended solids, biological (BOD_5) and chemical (COD) oxygen demands were respectively 0.9%, 68 and 170 g/L. The dry (DM), organic (OM) and mineral (MM) matters were 110.8 and 53.7 g/L respectively.

Secondary metabolites	Total polyphenols (mg GAE/mL)	Total flavonoids (mg RE/mL)	Condensed tannins (mg TAE/mL)
Concentration	10.82 ± 0.11	3.11 ± 0.16	2.43 ± 0.15

Table 2. Total polyphenols, flavonoids and condensed tannins of phenolic extract of OMW.

No	Phenolic compounds	Chemical class	Rt (min)	MW	Ionization forms	Concentration (µg/L)
1	Quinic acid	Phenolic acid	2.03	191	$[M-H]^-$	194.53 ± 29.092
2	Gallic acid	Phenolic acid	4.10	169	$[M-H]^-$	65.925 ± 91.78
3	Protocatechuic acid	Phenolic acid	7.40	153	$[M-H]^-$	195.35 ± 58.578
4	Caffeic acid	Phenolic acid	15.98	179	$[M-H]^-$	29.26 ± 9.519
5	*p*-coumaric acid	Phenolic acid	22.11	163	$[M-H]^-$	21.93 ± 5.604
6	Rutin	Flavonol	24.93	609	$[M-H]^-$, $[2M-H]^-$	2.106 ± 0.445
7	Trans-frolic acid	Phenolic acid	24.28	193	[M–H]	2.708 ± 4.69
8	Hyperoside (quercetin-3-*O*-galactoside	Flavonol	25.07	463	$[M-H]^-$, $[2M-H]^-$	4.801 ± 0.655
9	Luteolin-7-*O*-glucoside	Flavone	25.67	447	$[M-H]^-$	15.445 ± 1.365
10	Naringin	Flavanone	27.04	579	$[M-H]^-$, $[2M-H]^-$	16.721 ± 0.532
11	4,5-Di-*O*-caffeoyquinic acid	Phenolic acid	27.64	515	$[M-H]^-$	676.57 ± 83.712
12	Quercetrin (quercetin-3-*O*-rhamonosid	Flavonol	27.92	447	$[M-H]^-$, $[2M-H]^-$	12.310 ± 1.248
13	Apegenin-7-*O*-glucoside	Flavonol	27.90	431	$[M-H]^-$, $[2M-H]^-$	0.732 ± 1.269
14	Salviolinic acid	phenolic acid	28.918	717	$[M-H]^-$	33.82 ± 2.943
15	Kaempferol	Flavonol	32.87	285	$[M-H]^-$, $[2M-H]^-$	906.831 ± 306.164
16	Quercetin	Flavonol	32.87	301	$[M-H]^-$, $[2M-H]^-$	2.457 ± 0.291
17	Naringenin	Flavanone	34.78	271	$[M-H]^-$, $[2M-H]^-$	1.367 ± 2.368
18	Apigenin	Flavone	35.42	269	$[M-H]^-$, $[2M-H]^-$	96.2 ± 10.05
19	Cirsiliol	Flavone	36.46	329	$[M-H]^-$	51.258 ± 2.664
20	Cirsilineol	Flavone	39.01	343	$[M-H]^-$	2.924 ± 5.065

Table 3. LC–MS analysis of phenolic extract of OMW.

	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	FRAP (IC_{50} µg/mL)
Extract	9.62 ± 0.28^{d}	7.10 ± 0.11^{b}	3.59 ± 0.24^{d}
BHT	20.03 ± 0.25^{b}	4.27 ± 0.38^{c}	18.81 ± 0.09^{a}
Ascorbic acid	20.84 ± 0.65^{a}	2.03 ± 0.14^{d}	11.08 ± 0.18^{b}
Rutin	10.5 ± 0.36^{c}	8.48 ± 0.33^{a}	4.72 ± 0.21^{c}

Table 4. Antioxidants activity of phenolic extract of OMW by DPPH, ABTS, and FRAP.

Total phenolic, flavonoids and tannins content. The results of total phenolic, flavonoids and condensed tannin contents were summarized in Table 2. The Student's t-test revealed a significant difference between the means of the phytochemical contents. According to the findings, OMW is distinguished by a high polyphenol (10.82 ± 0.11 mg GAE/mL), flavonoid (3.11 ± 0.16 mg RE/mL), and condensed tannin (2.43 ± 0.15 mg TAE/mL) contents.

LC–MS separation and identification of phenolic compounds. The quantitative analysis results of major phenolic compounds identified in the of OMW extract were summarized in Table 3. Liquid chromatography–mass spectrometry (LC–MS) was used to screen thirty-one (31) compounds. In the extract, only twenty (20) compounds were identified including 8 phenolic acids and 12 flavonoid compounds. The 4,5-di-*O*-caffeoyquinic acid (676.57 µg/mL), protocatechuic acid (195.35 µg/mL), quinic acid (194.53 µg/mL), gallic acid (65.92 µg/mL), salviolinic acid (33.82 µg/mL), caffeic acid (29.26 µg/mL), and p-coumaric acid (21.93 µg/mL) were classified as the most abundant phenolic acids. Among the individual flavonoid compounds in OMW extract, five were detected as predominant: kaempferol (906.83 µg/mL), apigenin (96.2 µg/mL), cirsiliol (51.5 µg/mL), naringin (16.72 µg/mL), and luteolin-7-*O*-glucoside (15.44 µg/mL).

$DPPH^{\bullet}$, $ABTS^{\bullet}$ and FRAP assays. The antioxidant potential of the studied extract are summarized Table 4. The DPPH., ABTS.+, and FRAP free radical scavenging activity is frequently utilized to evaluate the antiradical/antioxidant capability of OMW phenolic compounds and compared the results using a variety of

Concentration (µg/mL)	% of Death of OMW	% of Death of $K_2Cr_2O_7$
0	0	0
1	5.0 ± 0.1^{e}	10 ± 0.6^{f}
5	10 ± 0.1^{d}	20 ± 0.5^{e}
10	42 ± 0.31^{c}	55 ± 0.3^{d}
20	80 ± 0.2^{b}	60 ± 0.2^{c}
40	100 ± 0.3^{a}	80 ± 0.5^{b}
80	100 ± 0.1^{a}	100 ± 0.1^{a}
LC_{50}	23.72 ± 0.1	33.74 ± 0.1

Table 5. Brine shrimp lethality bioassay of phenolic extract of OMW and $K_2Cr_2O_7$ used as standard. *LC_{50}* lethal concentration of 50% mortality.

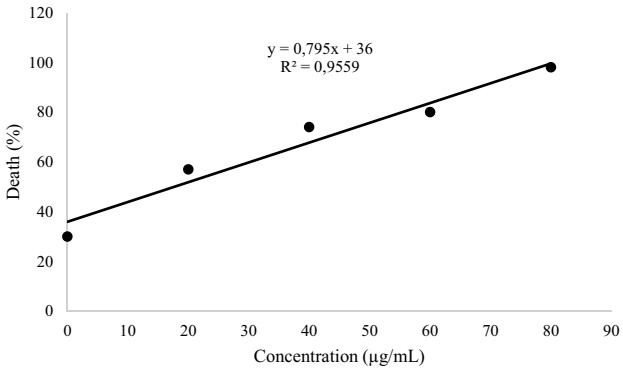

Figure 1. Lineal curve representing the percentage of mortality of *Artemia salina* according to the concentrations of OMW.

reference standards in order to obtain more useful and perhaps required findings. The findings of DPPH scavenging revealed that the phenolic extract of OMW had the highest antioxidant activity ($IC_{50} = 9.62 \pm 0.28$ g/mL), which was comparable to rutin ($IC_{50} = 10.5 \pm 0.36$ µg/mL) and greater than BHT and ascorbic acid (20.03 and 20.84 µg/mL). Additionally, the OMW possessed high scavenging activities against the ABTS free radical which evidenced by low IC_{50} value (7.10 µg/mL). Moreover, the extract exhibited the highest ferric reducing power ($IC_{50} = 3.59 \pm 0.24$ µg/mL) than rutin ($IC_{50} = 4.72$ µg/mL), ascorbic acid (11.08 µg/mL) and BHT ($IC_{50} = 18.81$ µg/mL).

Cytotoxicity effect. *Brine shrimp cytotoxicity test.* The statistical analysis results revealed a very highly significant difference between the percentages of larval mortality concerning the phenolic extract and the $K_2Cr_2O_7$ standard with $p < 0.001$. The results of larval toxicity by "brine shrimp" test of phenolic extracts of OMW studied are presented in Table 5.

The results reveal that the phenolic extracts of OMW have positive lethal effects on the larvae (nauplius) of brine shrimp after 24 h of exposure to the extracts; indicating that they are biologically active with mortality percentages gradually increasing with increasing frequency increase in the concentration of the extracts studied.

Figure 1 showed that the larvae were sensitive to OMW phenolic extracts according to a dose–response relationship. The DC_{50} values were obtained from the regression equation of the linear curve of the percentage mortality of shrimp nauplii as a function of the concentrations phenolic extract of OMW tested, including the DC_{50} equal to (23.72 ± 0.1 µg/mL) that showed the lowest concentration compared to the standard used which gave a DC_{50} (33.74 ± 0.1 µg/mL).

Cytotoxicity test of the phenolic extract of OMW on human neutrophils. The viability of neutrophils is an essential test if it has been plan to use molecules in humans. To test the purified extracts, the purity of the freshly isolated neutrophils is checked with crystal violet (dye-based on gentian violet and acetic acid) and they are counted. Cells are suspended for the second time in phosphate buffer and stored at 4 °C before using them. Before testing the extracts of the polyphenols on the neutrophils, the cytotoxicity of the product is checked by incubating the neutrophils in the presence of high concentrations of polyphenols ranging from 0 to 300 µg/mL for 30 min, the dead cells allow the blue of trypan, which stains them blue, unlike living cells which remain transparent.

The results (Table 6) showed that the phenolic extract had no toxic effect on neutrophils, even at higher concentrations; the neutrophil viability rate exceeds 95%, so the rate of dead cells is not significant. The obtained

Phenolic extract (µg/mL)	% viability of PMNs
0	100
50	98
100	96
200	95
300	98

Table 6. Cytotoxicity test of phenolic extract of OMW on human neutrophils.

	IPD IC_{50} µg/mL	MSP IC_{50} µg/mL
Extract	80.46 ± 3.81^a	87.43 ± 0.66^a
Sodium diclofenac	83.83 ± 0.21^b	95.31 ± 0.69^b

Table 7. Anti-inflammatory activity of phenolic extract of OMW.

	APTT (second)	PT (second)
Negative control	28.17 ± 0.06^c	13.4 ± 0.1^c
Extract	44.77 ± 0.25^a	15.84 ± 0.12^a
Positive control	33.1 ± 0.1^b	14.1 ± 0.13^b

Table 8. Anticoagulant activity of phenolic extract of OMW.

solvent-free extract shows that no toxicity to cells; the trypan blue exclusion test showed a viability average greater than 96% even at high concentrations.

Anti-inflammatory activity. *Inhibition of protein denaturation (IPD).* Table 7 present the findings of the denaturing effect of proteins. The studied phenolic extract of OMW has an inhibitory efficiency of thermal denaturation ($IC_{50} = 80.46 \pm 3.81$ µg/mL), superior to that of the reference anti-inflammatory drug diclofenac sodium ($IC_{50} = 83.83 \pm 0.21$ µg/mL).

Membrane stabilizing potential (MSP). Table 7 showed the findings of the OMW phenolic extract's membrane-stabilizing action. The extract had a higher inhibitory concentration, according to the data ($IC_{50} = 87.43 \pm 0.66$ µg/mL) than diclofenac sodium ($IC_{50} = 95.31 \pm 0.69$ µg/mL).

Anticoagulant activity. The anticoagulant activity of OMW phenolic extract was evaluated in vitro by two different methods: activated partial thromboplastin time (APTT) and prothrombin time (PT)[21].

Endogenous coagulation pathway (APTT). The anticoagulant activity data (Table 8) show that the extracts have a dose-dependent anticoagulant effect. The clotting time (APTT) in the presence of polyphenolic extracts of OMW and their compounds and heparin had been determined. The findings indicated that the polyphenolic extract might considerably increase the APTT ($p \leq 0.01$). The phenolic extract of Wastewater obtained (44.77 ± 0.25 s) had a longer clotting time than the negative (28.17 ± 0.06 s) and positive (33.1 ± 0.1 s) control.

Exogenous coagulation pathway (PT). The results showed that the time spent incubating polyphenolic extracts with plasma has a substantial ($p \leq 0.05$) effect on their anticoagulant activity. From the results obtained (prothrombin time) (Table 8), it appears that the extract is capable of significantly increasing PT. The coagulation times of the phenolic extract of OMW (15.84 ± 0.12 s) was higher than that of the negative (13.4 ± 0.1 s) and positive (14.1 ± 0.13 s) controls.

Hemolysis test. *Anti-hemolysis activity induced by salicylic acid.* From the results obtained (Fig. 2), very significant hemolysis ($p < 0.05$) of ($81 \pm 0.71\%$) is obtained at a concentration of (0.3–0.5 mg/mL) of salicylic acid. Salicylic acid induced hemolysis is concentration dependent, 50% hemolysis is obtained for a concentration of ≈ 0.15 mg/mL of salicylic acid.

The statistical study also showed a highly significant erythrocyte lysis rate for the positive control and a very low significant for the negative control.

The study of the effect of phenolic extracts from OMW on hemolysis induced by salicylic acid showed that the extracts exhibit effective anti-hemolytic effects of the order of IC_{50} of (100–600 µg/mL) of extracts depending on the concentration (Fig. 3) with more or less significant differences ($p < 0.05$). The phenolic extract gave the

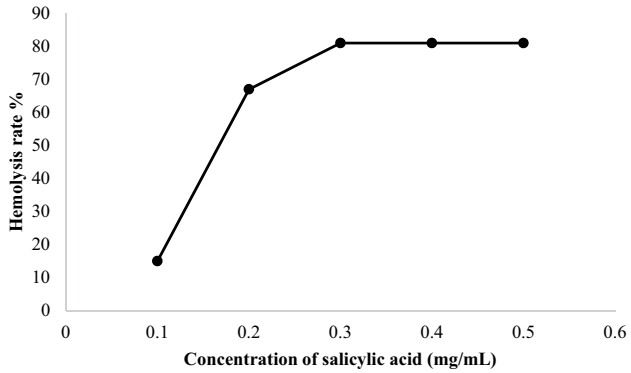

Figure 2. Rate of hemolysis induced by different concentrations of salicylic acid.

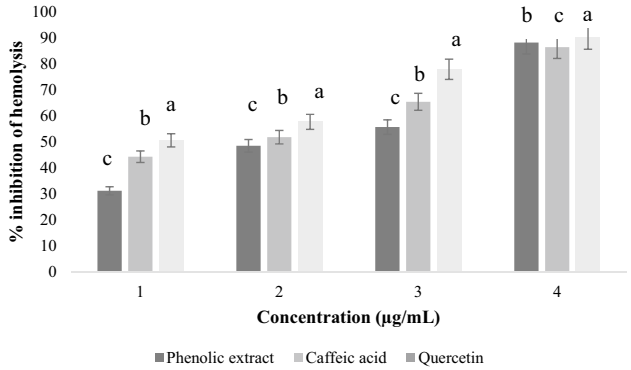

Figure 3. Effect of phenolic extracts of OMW on salicylic acid-induced hemolysis.

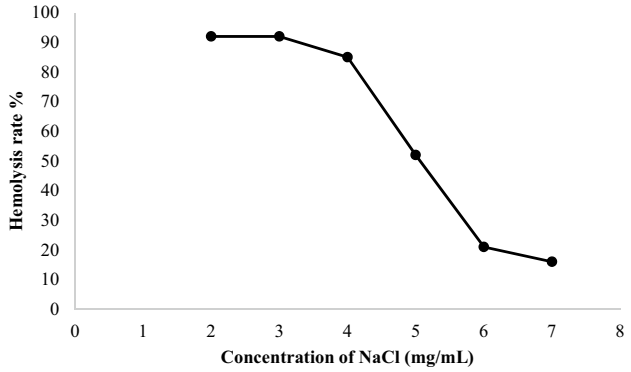

Figure 4. Rate of hemolysis induced by different concentrations of NaCl.

greatest percentage inhibition of hemolysis, which is of the order of (88.2 ± 0.07%) for a concentration of 600 µg/mL. At the same maximum concentration, caffeic acid gave a percent inhibition of (90.1 ± 0.11%). Finally, an inhibition was recorded for quercetin with a percentage of (86.4 ± 0.17%).

Hypotonic anti-hemolysis activity. The in vitro hemolysis results carried out on human red blood cells by the hypotonic solution of NaCl (Fig. 4) showed that the maximum hemolysis was found at the concentration between 2 and 3 mg/mL with a percentage of (92 ± 0.08%).

The inhibitory effect of phenolic extracts from OMW studied against the lysis of red blood cells showed significant differences ($p < 0.05$) between the different concentrations (Fig. 5). According to the results, the phenolic extracts of OMW showed significantly large differences depending on the concentration ($p < 0.05$). These extracts protect human red blood cells against sodium chloride-induced hemolysis. The percentage inhibition of each extract increases with the concentrations used. For example, the phenolic extract with a concentration

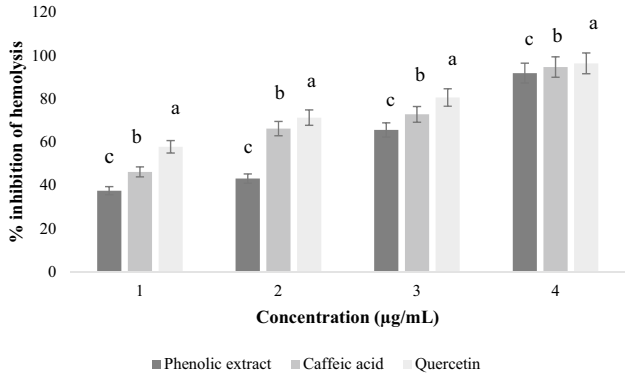

Figure 5. Effect of phenolic extracts of OMW on NaCl-induced hemolysis.

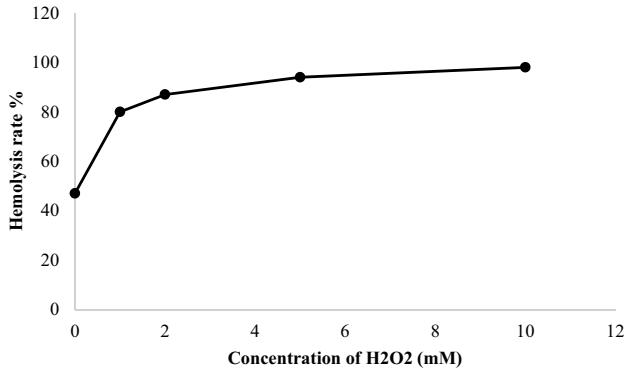

Figure 6. Rate of hemolysis induced by different concentrations of H_2O_2.

of 800 µg/mL exhibits a maximum inhibition rate of 91.9 ± 0.17%, while at a concentration of 200 µg/mL, it exhibits a minimum rate of 37.5 ± 0.12%. The inhibition percentage the standards (quercetin and caffeic acid) present the maximum inhibition rates with a rate of 96.4 ± 0.22% for quercetin and 94.7 ± 0.08% for caffeic acid, at a concentration of 800 µg/mL.

Anti-hemolysis activity induced by the H_2O_2 radical. The study of the hemolytic power of hydrogen peroxide was carried out at different concentrations of H_2O_2, an essential step to set the concentration of H_2O_2, which causes optimal hemolysis. The results in Fig. 6 showed that the rate of hemolysis increases with increasing hydrogen peroxide concentration. Statistical analysis of the results of the hemolytic activity induced by the H_2O_2 radical showed a significant difference between the different dilutions of H_2O_2 and the two controls negative and positive for ($p < 0.05$) H_2O_2 concentrated 10 mM causes almost complete hemolysis (98 ± 2.1%). Even at a concentration of 0.1 mM of H_2O_2, the hemolysis rate is (74 ± 0.2%). By comparing the lysis rates at the different concentrations of H_2O_2 with that of the positive control, the differences are more or less significant, so the differences are highly significant with the negative control. It is observed that the osmotic fragility increases with the increase in the concentration of H_2O_2. A hemolysis percentage of (47.00 ± 0.06%) was obtained in the absence of H_2O_2 and the presence of a physiological NaCl concentration of 0.9% (w/v).

The results obtained on the anti-hemolytic effect of the compounds tested are presented in Fig. 7. The percent inhibition of hemolysis increases with the concentration of the compounds tested. The phenolic extract of OMW showed an inhibition of hemolysis of (41.1 ± 6.89%), (57.5 ± 4.22%), (74.7 ± 4.3%) and (82.8 ± 1.1%) at concentrations of (100, 200, 300 and 400 µg/mL), respectively. Caffeic acid shows the greatest anti-hemolytic activity with a rate of (89.1 ± 3.55%) followed by quercetin with a rate of (86.4 ± 1.24%). As for the phenolic extracts of OMW also exhibit significant anti-hemolytic activity at concentrations of (300 and 400 µg/mL) compared with the two standards caffeic acid and quercetin.

Effect of phenolic extract of OMW on the production of superoxide anion by neutrophils. To specify the effect of the phenolic extract of OMW on the FRO, the production of $O_2^{\cdot-}$ under the same conditions with an appropriate cytochrome c probe was measured, which exclusively detects the superoxide anion. The superoxide anion production is measured; the reduction of cytochrome c is monitored at 37 °C in kinetics at 550 nm with a UVIKON 860 brand spectrophotometer.

The results shown in Fig. 8 showed that the phenolic extract of OMW inhibits cytochrome c reduction in a dose-dependent manner. This result suggests that either the polyphenol extract inhibits the activity of the

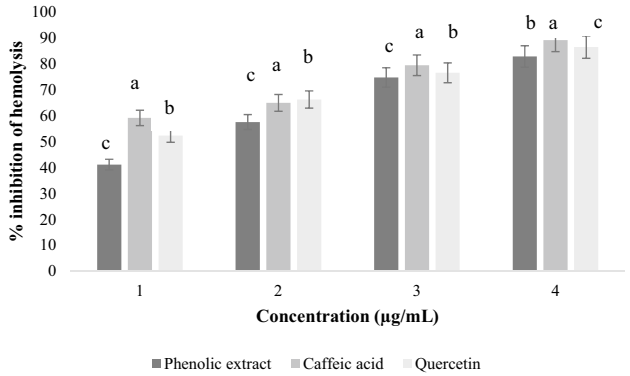

Figure 7. Effect of phenolic extracts of OMW on H_2O_2-induced hemolysis.

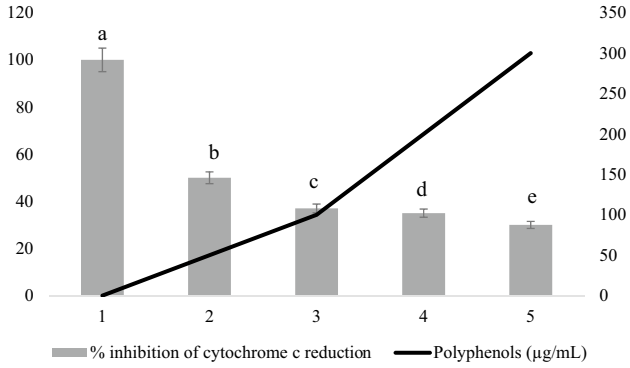

Figure 8. Effect of phenolic extract on the production of superoxide anion by neutrophils.

neutrophil NADPH oxidase, which produces this FRO precursor (superoxide anion), or it scavenges the superoxide anion. This effect can be specified by further tests using an enzyme system in which only the superoxide anion is produced, such as the xanthine and xanthine oxidase system.

Discussion

The present study demonstrates cytotoxicity, antioxidant, anti-inflammatory, anti-hemolytic, and anticoagulant activities of phenolic compounds extracted from olive oil mill wastewater have been tested. This topic is relatively new because the aforementioned activities were studied together, as well as for the olive cultivar "Abani" from which OMW is extracted.

The composition of OMW is frequently studied in the literature, and our findings are consistent with numerous established findings. OMW is an acidic effluent with a pH range of 3–5 and a conductivity of 16.79 mS/cm. Generally, it composed of dry matter (6–17%), organic matter (4–16%), chemical oxygen demand (COD) (40–220 g/L) BOD_5 (35–110 g/L) and the presence of several phenol-type molecules (0.5–24 g/L)[22,23]. The quality and quantity of OMW are very different and are influenced by different factors, such as type of production process, olives, use of pesticides and fertilizers, the area cultivated, weather conditions and stage of ripening olives[24].

According to the findings, OMW is distinguished by a high concentration of phenolic components. Furthermore, the results that we obtained were higher than those obtained by[22,23].

Several researchers were identified phenolic compounds by HPLC as[22] that were identified ten peaks by LC–MS. They were as follows: quinic acid (23.940 ppm), Kaempferol (3.635 ppm), cirsiliol (2.352 ppm), *p*-coumaric acid (1.427 ppm), quercetin (0.470 ppm), apegenin (0.843 ppm), rutin (0.255 ppm), luteolin-7-*O*-glucoside (0.197 ppm), naringin (0.154 ppm) and quercetrin (quercetin-3-*O*-rhamnosid) (0.084 ppm).

The DPPH., ABTS.+, and FRAP free radical scavenging activity is frequently utilized to evaluate OMW phenolic compounds' antiradical/antioxidant capability, and compared the results with several reference standards that were developed to produce more informed and perhaps required results.

Stoclet et al.[25] showed that a strong antioxidant activity characterizes thz phenolic compounds of OMW. The in vitro antioxidant activity of bioactive compounds has received a lot of attention in the literature. The existence of oxidizing species such as free radicals or metal complexes oxidized in the presence of an extract containing antioxidants capable of preventing the formation of radicals is used in these approaches. Several studies have found that antioxidant activity is influenced by overall polyphenol content, and the type and structure of the antioxidants in the extract. Furthermore, the antioxidant capacity measured by the DPPH and ABTS assays was strongly associated with total polyphenol content.

According to[26], at the cellular level, certain flavonoids can act on transmitting signals by protein kinases, including the expression of antioxidant and anti-inflammatory genes and vice-versa, the inhibition of oxidative and inflammatory genes.

The high antioxidant activity of the extract can be explained by the overall phenolic content and the kind of phenolic compounds present.

In addition, this strong antioxidant activity is due to the flavonoid contents, especially Kaempferol, which is the most abundant flavonoid in our extract (906.831 ± 306.164 ppm).

That means that phenolic extracts of OMW were very toxic according to the criteria of Clarkson[27]. These extracts are considered very toxic indicating the presence of powerful cytotoxic components such as heavy metals, polyphenols, etc. However, previous studies have shown that plant extract lethality against brine shrimp nauplii with a value of DC_{50} below 100 g/mL is reasonably correlated with cytotoxic and antitumor properties and may constitute potential antitumor and anticancer agents[28].

Therefore, this "brine shrimp" larval toxicity test then constitutes a preliminary screening to determine the degree of product toxicity. Indeed, during a toxicological evaluation of plant extracts by this toxicity test, an LC50 value of < 1000 g/mL is considered bioactive[29].

The extract showed anti-inflammatory potential due to its protein stabilizing action, but further in vivo testing is needed to validate it. Indeed, the conformation of a protein is linked to the secondary and tertiary structure; it is carried out using lower energy bonds (hydrogen bonds, electrostatic, hydrophobic and disulfide bridges), therefore fragile. Denaturation results from modifying the quaternary, tertiary and secondary structures without fragmentation of the peptide chain under the effect of various chemical or physical agents[30,31]. The denaturation of a protein causes the induction of the inflammatory reaction by producing auto-antigens, important factors for developing chronic inflammation[30]. Many flavonoids and related polyphenols have been demonstrated in recent research to have substantial antioxidant and anti-inflammatory properties. This anti-inflammatory action may be aided by the presence of these bioactive chemicals in the OMW extract. Therefore, the use of agents that can prevent protein denaturation would be helpful for the development of anti-inflammatory drugs[32].

Stabilization of the red blood cell membrane has been used as a method to study anti-inflammatory activity in vitro because the erythrocyte membrane is analogous to the lysosomal membrane[31]. According to the findings, in comparison to diclofenac sodium, the extract demonstrated a substantial stability of the red blood cell membrane. This means that the OMW phenolic extract can effectively stabilize the lysosomal membrane. The lysosome must be stabilized to minimize the inflammatory response by limiting the release of lysosomal components such as bacterial enzymes and protease from active neutrophils. Nonsteroidal drugs such as diclofenac sodium work either by inhibiting lysosomal enzymes or by stabilizing lysosomal membranes[33]. The strong anti-inflammatory of the phenolic extract obtained in this study is probably due to Kaempferol, 4.5-di-*O*-caffeoyquinic acid, and protocatechuic acid, which are abundant in the extract.

APTT assay was used to assess the inhibition of intrinsic factors of blood coagulation pathways such as F XII, XI, V, III IX, and prekallikrein[34]. The APTT test detects VIII, IX, XI, and excitatory releasing enzymes in the endogenous coagulation pathway using brain lipids and activators rather than platelets to represent the impact of endogenous variables on coagulation time. The results obtained were in the range of those obtained from[35]. As a result, the extract examined exhibits high anticoagulant action in terms of the endogenous route.

PT assay is assessed to examine the inhibition of the extrinsic coagulation pathway, especially factors V, VII, and X[34]. To represent the influence of external variables on coagulation time, PT tests are performed by adding thromboplastin to plasma. In order to research an elongation at the level of the coagulation time, which is defined by an anticoagulant activity of the polyphenolic extracts of OMW with respect to the cascade of this pathway. Depending on the reagents employed, a typical PT lasts between 12 and 14 s. The results obtained were in the range of those obtained from[35]. As a result, the extract examined exhibits anticoagulant solid effect in the exogenous route. Heparin's anticoagulant action is due to the inhibition of endogenous coagulation enzymes when it forms a compound with anti-thrombin III.

Because thromboplastin time is a coagulation test that investigates all coagulation components in the exogenous route, it is quite possible that the anticoagulant effect of the OMW extract is related to the blockage of one of these components, which are stimulated in a cascade[36]. In addition, the phenolic extract of OMW has an anticoagulant impact on the two coagulation pathways in a dependent manner, based on the findings.

Anticoagulant medications are desperately needed since thrombotic disorders have become the leading cause of mortality. Based on the findings, the phenolic extract of OMW exhibits an anticoagulant action in vitro and might be developed as an anticoagulant medication to treat coagulation-related disorders. The extract's high anticoagulant action might be attributed to its high levels of Kaempferol, caffeic acid, and its derivatives.

Erythrocytes constitute an excellent cellular model for evaluating the effects of ROS and antioxidants[37] due to their richness in iron and polyunsaturated fatty acids. This cell type is one of the cells most exposed to O_2 and, therefore, to oxidation risk. Hydrogen peroxide is frequently used to trigger the formation of free radicals in red blood cells due to its ability to easily cross cell membranes[38].

According to[39], the exposure of human erythrocytes to oxidizing agents such as salicylic acid (aspirin precursor) only promotes hemolysis in the event of large intakes, among the side effects of Aspirin, it can cite its oxidizing effect, which can lead to the production of free radicals, the latter attacking the membrane. Aspirin is indicated in most cases in the induction of hemolytic anemia in subjects with an enzymatic deficiency in G6PD, which alters the functioning of the voice of pentose, one of the main pathways of energy metabolism which induces dysfunction sodium pump (ATPase dependent magnesium), causing a massive release of Na^+ ions, which makes the environment hypotonic[39]. Therefore, the deficientrefore, the deficient has erythrocytes unable to resist oxidative stress because they cannot produce reduced glutathione due to lack of NADPH, therefore more protection against oxidative stress[40].

The rate of hemolysis of red cells depended on the concentration of NaCl. In fact, the most pronounced hemolytic activity is observed in the hypotonic medium (3.5 mg/mL). In contrast, in the isotonic medium, from the concentration of 5 mg/mL, the hemolysis rate decreases until it reaches a minimum at the concentration of 7 mg/mL. If an erythrocyte is placed in a hypotonic medium, water enters the cell and gradually creates a deformation of the blood cells; beyond a certain threshold, the membrane bursts and intracellular hemoglobin passes into the medium exterior (hemolysis)[41].

The results showed that the osmotic fragility increases with increased oxidative stress induced by hydrogen peroxide. Low-concentration hydrogen peroxide-induced hemolysis results from destructive oxidative damage to the cytoplasmic membrane following lipid peroxidation of Polyunsaturated Fatty Acids (PUFA) present therein[42]. When H_2O_2 crosses this membrane, it can cause the breakdown of hemoglobin hem and Fe^{2+} ions, which generates the hydroxyl radical, which is very reactive by the Fenton reaction. These two radicals induce a chain of lipid peroxidation leading to the lysis of erythrocytes[43]. On the other hand, at high concentrations, the rate of hemolysis decreases, which may be linked to the insolubility of hemoglobin following its polymerization and the aggregation of erythrocytes[43].

The results of anti-hemolytic tests have shown that the phenolic extract exerts a protective effect on red blood cells and provides protection against hemolysis of red blood cells and denaturation of hemoglobin with a dose dependent.

From work carried out, it is possible to suggest that the anti-hemolytic effect of the phenolic extracts of OMW is due to the prevention of methemoglobin formation, following the trapping of hydrogen peroxide. This phenomenon prevents or decreases the formation of the hydroxyl radical and therefore prevents oxidative damage to membrane constituents, thus preventing the induction of hemolysis. Another possible protection mechanism would be the trapping of the hydroxyl radicals formed and a chelating action of the metals of these molecules.

Methods

Ethics statement. All experiments were conducted in accordance with the guidelines of the Declaration of the World Medical Association of Helsinki. The experiments were approving by the scientific committee of the Faculty of Nature and Life Sciences, Abbes Laghrour University of Khenchela, Algeria. They were performed in accordance with relevant guidelines and regulations. The blood donation for this in vitro study was approved by the Ethics Committee of the Central Laboratory of Ahmed Ben Bella Hospital Khenchela, Algeria. Written consent to participate and approval for publication was obtained from each volunteer. Informed consent was obtained from all subjects.

Physicochemical properties. The olive oil mill wastewater (OMW) from Abani variety was obtained from a modern olive mill situated in Khenchela, eastern Algeria, in November 2019. It was collected directly from the decanter, frozen immediately and kept at − 20 °C until use. Standard Methods[44] were used to measure pH, electrical conductivity (EC), dry matter (DM), total suspended solids (TSS), organic matter (OM), mineral matter (MM), biological oxygen demand (BOD_5), and chemical oxygen demand (COD). The pH level was measured using a pH meter (AdwaAD1000). Electrical Conductivity (EC) was determined by conductivity meter type (inoLab WTW). Dry matter content (DM) was measured by drying at 105 °C for 24 h. Organic matter (OM) was calculated by the difference between the dry weight of the OMW and its weight after the calcination. Mineral matter (MM) was determined by weighing after ignition in a muffle furnace type (Nabertherm) at 550 °C, for 24 h. The chemical oxygen demand (COD) was determined using potassium dichromate, as described by BOD_5 (biological oxygen demand) is determined by the respirometric method. Analyzes were carried out in triplicate.

Polyphenol extraction methods. The phenolic compounds were extracted using the maceration method. 1 g of OMW powder was mixed with 10 mL of pure methanol. Then, vortexed for 15 min and kept to macerate overnight at 4 °C in the dark. After maceration, filtering using filter paper is performed. The macerate was collected and added to 10 mL of methanol (90%) for a second time; the combination was vortexed for 15 min before being left to macerate for 1 h. The two filtrates are mixed and filtered through sodium sulfate-containing cellulose paper. The solution was condensed in a rotary evaporator (HAHNVAPOR) at 40 °C, and the dry material was stored.

Total phenolic content (TPC). The total phenolic content was determined following the Folin–Ciocalteu method[45]. Taking 125 µL of the extract diluted 100 times is put in the presence of 500 µL of distilled water and 125 µL of the FCR After stirring and standing for 3 min, 1250 µL of a 7% CO_3Na_2 solution was added to the mixture. The volume of the mixture was adjusted to 3 mL with ultrapure water and then left in the dark at room temperature for 90 min. The results were expressed as milligrams of gallic acid equivalents per milliliter of extract (mg GAE/mL). Gallic acid calibration curve was used to quantify the total phenolic content (TPC) of extracts ($y = 0.0046\ x + 0.0108$, $R^2 = 0.9967$).

Total flavonoids content (TFC). The quantification of total flavonoids content was performed by the method of[46]. To a dose of 250 µL of the extract diluted 100 times with methanol, is added 75 µL of a 5% $NaNO_2$ solution. After 6 min of incubation at room temperature, 150 µL of an aluminum chloride solution $AlCl_3$ was added to the mixture. After 5 min of incubation at room temperature, 500 µL of sodium hydroxide was added to the mixture and then the volume was adjusted to 2500 µL with distilled water. The results were expressed as milligrams rutin equivalents per milliliter of extract (mg RE/mL). The total flavonoids content (TFC) was calculated following the calibration curve prepared from rutin ($y = 0.0103\ x + 0.0061$, $R^2 = 0.9963$).

Tannin condensed content (TCC). The quantification of condensed content was performed according to the method of[47] by reaction with vanillin in the presence of sulfuric acid. A volume of 0.5 mL intake of the suitably diluted extract is mixed with 2 mL of 1% vanillin and then added with 2 mL of concentrated sulfuric acid. After homogenization, the mixture is incubated at room temperature. The results were expressed as milligrams of tannic acid equivalent per milliliter of extract (mg TAE/mL). The tannins condensed content (TCC) was calculated using tannic acid calibration curve ($y = 0.0066\,x + 0.0113$, $R^2 = 0.9969$).

LC–MS separation and identification of phenolic compounds. The OMW extracts were analysed using a Shimadzu UFLC XR system consisted of an electrospray ionization source (ESI) equipped with two LC-20ADXR solvent delivery units, a SIL-20AXR autosampler, an SCL-10A system controller, a CTO-20 AC column oven, a DGU-20AS degasser (Shimadzu, Kyoto, Japan). A volume of 5 µL each extract was injected at 0.5 mL/min to a Discover BIO Wide Pore C18 column (150 mm × 3 mm, 3 µm) at 40 °C and separated with two mobile phases: A (0.1% formic acid in water v/v) and B (0.1% formic acid in methanol v/v) following the programmed linear gradient elution : 0–14 min, from 10 to 20% B; 14–27 min, from 20 to 55% B; 27–37 min, from 55 to 100% B; 37–45 min, 100% B; and 45–50 min 10% B[48,49]. The ionization mode was negative and The ESI conditions were set as follows: capillary voltage of − 3.5 v, a nebulizing gas flow of 1.5 L/min, a dry gas flow rate of 15 L/min, a DL (dissolving line) temperature of 280 °C, a block source temperature of 400 °C, and a voltage detector of 1.35 V. Compounds were identified by comparing their retention time and mass spectra with those of reference standards. The validation of the method was determined as detailed in[50].

Antioxidant assays. *DPPH free radical-scavenging activity.* The antioxidant activity of different extractions was evaluated following[51] method using the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) with some adjustment. A test sample of 0.5 mL of the extract at different concentrations is mixed with 0.5 mL of a solution of DPPH (0.2 mM in methanol). After vigorous shaking of the mixture, it is left to stand for 30 min in the dark. The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid and rutin).

$ABTS^+$ free radical scavenging activity. The ABTS (2,2-Azino-bis-3-ethyl benzothiazoline-6-sulfonic acid) scavenging activity was determined according to the method of[51] with some adjustment. A volume of 10 µL of the extract is added to a volume of 990 µL of ABTS solution. The discoloration relative to the control, containing $ABTS^+$ and the solvent (ethanol), is measured with a spectrophotometer at 734 nm after 30 min of incubation in the dark. The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid, and rutin).

FRAP ferric reducing antioxidant power. The FRAP activity was evaluated following[51] with some adjustment. It consists of mixing 1 mL of each solution of extracts or of the standard antioxidant at different concentrations with 1 mL of phosphate buffer (0.2 M, pH 6, 6) and 1 mL of a 1% solution of potassium ferricyanide[$K_3Fe(CN)_6$]. The mixture obtained is incubated at 50 °C for 20 min, and then 1 mL of 10% trichloroacetic acid (CCl_3COOH) is added to stop the reaction. The mixture is centrifuged at 2000*g* for 10 min. To 1 mL of the supernatant are added 1 mL of distilled water and 0.5 mL of 0.1% iron chloride ($FeCl_3$). The reaction medium is incubated at room temperature for 10 min. The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid, and rutin).

Brine shrimp cytotoxicity test. The brine shrimp lethality bioassay was performed using the method of[29]. *Artemia nauplii* were obtained by hatching brine shrimp eggs (*Artemia salina*) in artificial seawater (3.8% NaCl solution) for 48 h. The dissolution of 30 mg of OMW was carried out in 3 mL of artificial seawater containing 20% DMSO to give a concentration of 10 µg/mL. From this solution 0.1, 5, 10, 20, 40, and 80 µL were transferred to each 10 mL vial and using artificial seawater the volume was adjusted to 10 mL per artificial seawater. *Artemia nauplii* were cultivated in these solutions and their mortality was observed after 24 h. The number of surviving larvae is counted in each tube and the mortality is calculated at each concentration as follows:

$$\%\ \text{Deaths} = (\%\ \text{test deaths} - \%\ \text{control deaths})/(100 - \%\ \text{control deaths}).$$

The control mortality should not exceed 15%. The artificial seawater medium containing DMSO used for the analysis was used as a negative control. $K_2Cr_2O_7$ was used as a standard in this test.

The 50% Death Concentration (DC50) reflecting the toxicity of the products is estimated as: (−)Toxicity when DC50 ≥ 100 µg/mL, (+) Toxicity when 100 µg/mL > DC50 ≥ 50 µg/mL, (++) Toxicity when 50 µg/mL > DC50 ≥ 10 µg/mL, and (+++) Toxicity when DC50 < 10 µg/mL.

The number of dead and alive nauplii was recorded after 24 h. Nauplii were considered dead if no internal or external movement was observed within 30 s. The percent mortality of salted shrimp and the LC 50 (median lethal concentration) was then calculated. This was done by plotting the percent mortality against the logarithm of the extract concentration. The LC 50 value was derived from the regression equation[29].

Cytotoxicity test of the phenolic extract of OMW on human neutrophils. *Isolation of neutrophils by the Dextran–Ficoll method.* The purpose of this method is to separate blood cells from fresh blood taken on an anticoagulant. The principle is based on the separation of blood components according to their density. The number of neutrophils recovered from a 400 mL blood bag after isolation ranges from 6 to 10×10^8 neutrophils. Isolation of Polynuclear Neutrophils begins with mixing one volume of blood with one volume of 2% Dextran-

T500 (prepared in advance in 0.9% NaCl and filtered). After 30 min incubation at room temperature, the majority of erythrocytes precipitate at the bottom of the tube. The upper phase contains the white blood cells. The latter is collected in clean tubes and the red blood cells of the lower phase are discarded. The phase containing the white blood cells is centrifuged for 8 min at 400 g at 22 °C. The pellet is suspended a second time in phosphate buffer, then deposited on a Ficoll cushion and centrifuged for 30 min at 400*g* and at 22 °C. After this step, three phases are distinguished, the pellet corresponds to the granulocytes (eosinophils, neutrophils and basophils) with the contaminating erythrocytes, the mononuclear cells (monocytes and lymphocytes) form a ring between the pellet and the supernatant is a mixture of plasma, buffer phosphate and Ficoll. Lysis of residual erythrocytes is carried out by adding a cold 0.2% NaCl hypotonic solution to the pellet, mixing for 40 s, isotonicity is restored by adding the same volume of 1.6% NaCl. The solution is buffered by adding cold phosphate buffer. The neutrophils are recovered in a small volume of phosphate buffer, after centrifugation for 8 min at 400*g* and 4 °C. Neutrophils are stored at 4 °C until use.

Neutrophil viability and purity test. The neutrophil viability test is performed by the Trypan blue exclusion test, which stains dead cells blue. While the purity of Polynuclear Neutrophils is checked with another dye, crystal violet stains the nuclei of cells, making it possible to distinguish the polylobed nucleus characteristic of neutrophils from other cell nuclei. The count is done on a Malassez slide by light microscopy at magnification ×40. Their effect has been verified on the viability of neutrophils, this test is very important if one plans to use these molecules in humans. To test the purified extracts, the purity of the freshly isolated neutrophils is checked with crystal violet (dye-based on gentian violet and acetic acid) and they are counted. The cells are resuspended in phosphate buffer and stored at 4 °C before using them. Before testing the extracts of the polyphenols on the neutrophils, it was been checked the cytotoxicity of the product, by incubating the neutrophils in the presence of high concentrations of polyphenols ranging from 0 to 300 µg/mL for 30 min, the dead cells allow the blue of Trypan, which stains them blue, unlike living cells which remain transparent.

Anti-inflammatory activity. *Inhibition of protein denaturation (IPD).* It is determined by the method described by[52] with slight modifications. The concept is that the phenolic extract of OMW inhibits denaturation of BSA induced by heat (72 °C) 0.1 mL of each concentration of extract added to 1 mL of 0.2% BSA solution prepared in Tris–HCl pH 6.6, then incubated at 37 °C for 15 min then in a water bath at 72 °C for 5 min. After cooling, the turbidity is measured at 600 nm in a cell spectrophotometer (SPECORD 210 plus). Diclofenac sodium standard (injectable form) was produced using the same technique in ultra-pure distilled water from a 500-ppm mother solution, with distilled water serving as a negative control.

Membrane stabilizing potential (MSP). It was measured according to[53]. An equivalent volume of blood was collected from healthy human volunteers who had not taken any NSAIDs for two weeks before blood collection and combined with an equal volume of sterile Alsever solution. This blood solution was centrifuged for 10 min at 3000 rpm, the packed cells were separated and washed with iso-saline solution, and a 10% (v/v) suspension was made using an iso-saline solution.

1 mL phosphate-buffered saline, 0.5 mL 10% blood suspension, 0.5 mL phenolic extract of OMW with various concentrations, and 2 mL hypotonic saline make up the dose combination. All test mixtures were incubated at 37 °C for 30 min and then centrifuged at 3000 rpm for 20 min. The hemoglobin concentration was measured using a spectrophotometric measurement at 560 nm after the supernatant was separated.

The negative control was distilled water and the positive control was diclofenac sodium at the final concentration. The IC_{50} was measured once again using a graph that showed inhibition at various doses.

Anticoagulant activity in vitro. *Endogenous coagulation pathway (APTT).* Activating partial thromboplastin time (APTT) was determined according to[29]. A platelet plasma pool comprises a plasma combination from ten healthy, untreated individuals with normal APTT and PT.

The activity of the phenolic extract was established on a volume of 100 µL whose plasma is 90 µL was mixed with 10 µL of extract. After 15 min of incubation at 37 °C 100 µL cephalin kaolin was added to the mixture, which was re-incubated for 3 min with agitation at 37 °C. Using a coagulometer, the coagulation time was measured by adding 100 µL of warmed calcium chloride (0.025 M). In parallel, a positive control of calciparine (unfractionated heparin) and a negative control test (substituting the samples with a 0.9% NaCl solution) were performed under identical circumstances. An increase in APTT in the presence of polyphenols compared to the negative control implies an anticoagulant impact at this route level. Clotting time was determined by an automatic coagulation analysis system (Coa DATA 4004).

Exogenous coagulation pathway (PT). Prothrombin time (PT) was determined according to the protocol described by[54]. The coagulation time of citrated plasma in the presence of an excess of calcium thromboplastin is measured in this activity using platelet-poor plasma in the presence of calcium thromboplastin. The phenol extract (90 and 10 µL, respectively) was combined with 100 µL of platelet-poor plasma that had been warmed for 2 min at 37 °C. After 15 min of incubation at 37 °C, 200 µL of calcium thromboplastin was added to the mixture, which had been warmed for at least 15 min at 37 °C. Coagulation time was determined by an automatic coagulation analysis system (CoaDATA 4004).

Study of antioxidant activity on a cell model. *Hemolysis test.* The anti-hemolytic effect of plant extracts is evaluated in vitro using the Erythrocyte model. The latter is easily isolated from blood and its membrane shows similarities to other cell membranes[55].

Preparation of the erythrocyte suspension. The blood used to prepare the erythrocyte suspensions was taken from healthy people in heparin tubes. Serological analyses were carried out to exclude any risk of contamination of any pathology. After centrifugation of the blood at 3000 rpm/5 min, the recovered pellet is washed 3 times with the Phosphate Buffered Saline (PBS) solution formed from 10 mM potassium phosphate buffer, pH = 7.4 and 154 mM of NaCl. Each wash consists of a suspension of the cells in iso-saline PBS and centrifugation at 3000 rpm/5 min. After the last centrifugation, the pellet is suspended for the second time again in a solution of iso-saline PBS at the rate of 1 volume of the pellet and 9 volumes of PBS, thus obtaining a hematocrit at 10%[56].

Development of in vitro induced hemolysis tests. The exposure of red blood cells (RBC) to certain physicochemical parameters such as the hypotonic medium, the use of a membrane disruptor such as detergents or reactive oxygen species, causes a rupture of its cytoplasmic membrane thus causing the release of the hemoglobin, which will then be determined by visible absorbance spectrophotometry at 540 nm. To test the anti-hemolytic effect of the phenolic extracts of OMW, tests on an erythrocyte model with hemolysis induced by three different agents (hypotonic medium, salicylic acid and H_2O_2) were carried out.

Induction with salicylic acid. In test tubes each containing 4.5 mL of hypotonic NaCl (4.5 mg/mL), 50 µL of salicylic acid in different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) were added. The control tube receives the same volume of PBS buffer, and then a volume of 500 µL of the erythrocyte suspension is added to each tube. After that, the tubes are homogenized, incubated at 37 °C for 30 min in a water bath, and centrifuged at 3000 rpm for 5 min. The absorbance is then measured at 540 nm[39].

Hypotonic induction. To determine the concentration of NaCl, which causes the lysis of red blood cells, 100 µL of the erythrocyte suspension (10%) were added to 5 mL of NaCl at different concentrations (2, 3, 4, 5, 6 and 7 mg/mL), as well as a negative (Isotonic NaCl 9 mg/mL) and positive (distilled water) control. After incubation for 30 min at room temperature, the mixture was centrifuged at 3000 rpm/10 min and the DO was read at 540 nm[57].

Induction by hydrogen peroxide H_2O_2. A volume of 500 µL of H_2O_2 at different dilutions (0, 1, 2, 5 and 10 mM) were mixed with a volume of 250 µL of the suspension of red blood cells. After 3 h of incubation at 37 °C, PBS was added and then the mixture was subjected to centrifugation for 10 min with a speed of 3000 rpm. The absorbance of the supernatant was read at 540 nm. Controls were prepared by replacing H_2O_2 with distilled water for the positive control and with PBS for the negative control[48].

The percent hemolysis for all tests was calculated using the following formula[55]:

$$\%\ \text{hemolysis} = (\text{Absorbance of the test}/\text{Absorbance of the control}) \times 100.$$

Measurement of the oxidative explosion of polynuclear neutrophils by the cytochrome c reduction technique. The production of $O_2^{\cdot-}$ by activated neutrophils is measured by the cytochrome c reduction technique[49]. The principle of this test is based on the use of oxidized cytochrome c "Fe^{3+}" in the presence of a source producing superoxide anions ($O_2^{\cdot-}$) such as neutrophils or by an acellular system composed of xanthine/hypoxanthine and xanthine oxidase. In the presence of this very reactive and unstable $O_2^{\cdot-}$ radical, cytochrome c is reduced to Fe^{2+}. This test exclusively detects the extracellular superoxide anion because cytochrome c does not cross the cell membrane; the reduced cytochrome is measured by spectrophotometry at 550 nm. In practice, neutrophils at 10^6 cells/mL in phosphate buffer are pretreated with increasing concentrations of polyphenols (0, 50, 100, 200 and 300) µg/mL for 10 min at 37 °C. The cells are incubated in the presence of cytochrome c at 1 mg/mL final and then stimulated with Phorbol Myristate Acetate (PMA) (100 ng/mL) and the absorbance is measured at 550 nm using a thermostated spectrophotometer brand UVIKON 860. The production of $O_2^{\cdot-}$ is measured for 10 min and the results are expressed in nanomoles of $O_2^{\cdot-}$ produced per minute and per million neutrophils using Beer Lambert's law: $A = \varepsilon . C . l$, where ε is the molar extinction coefficient ($l . Mol^{-1} . Cm^{-1}$), A being the absorbance (without unit), C is the concentration of the solute (mol/L).

Statistical study. Data obtained were presented as mean ± SD of three dependent determinations. Significant differences between means of total phenolic, total flavonoids, tannins and LC–MS analysis results were determined by Student t-test, and p values (< 0.05) were regarded as significant. Results of antioxidant, anti-inflammatory and anticoagulant activities were subjected to statistical analysis of variance (ANOVA) using ECXEL STAT (version 2014) package at $p < 0.05$ significant levels.

Conclusions

The current study aims to determine cytotoxicity, antioxidant, anti-inflammatory, anti-hemolysis, and anticoagulant activities, of phenolic compounds extracted from OMW from Abani cultivar. Because of few scientific works have been carried out on the enhancement and evaluation of the biological potential of polyphenols of OMW, this topic is considered that be relatively new. It shows for the first time that phenolic extracts of OMW

have anti-hemolytic and anti-inflammatory properties. In addition, it demonstrates that they possess potent dose-dependent toxicity was observed in brine shrimp lethality assay, antioxidant and anticoagulant activity, and can stabilize human erythrocyte membranes in a dose-dependent manner. Chemical examination reveals the presence of polyphenols, flavonoids and tannins that may be responsible for the aforementioned properties. The major phenolic compound revealed by LC–MS is Kaempferol. The overall results of the current study demonstrate that OMW offers very promising prospects for valorizing polyphenols and reducing their polluting impact on the environment and OMW bioactivities increase the valorization of these by-products. Concluding, the obtained results are interesting, however, the mechanisms underlying the observed effects are unknown. It would therefore be wise in the future to deepen the phytochemical study of this effluent by trying to identify and purify the phenolic compounds responsible for these biological activities and to test them in vivo. In addition, more extensive research is needed to identify the secondary metabolites responsible for the reported biological activities and discover the underlying mechanism behind these therapeutic potentials.

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Author contributions

Z.G.: design, performed the experiment, writing of the project. K.K. and K.N.: help in writing and supervising the project. D.A. and K.K.: do the statistical study. M.S. L.B.Y., B.L. and A.A.: contribute to the laboratory experiments. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Abstract

The objective of this work is to evaluate the effect of storage time on the quality of olive oil mill wastewater (OMW) obtained from the cold extraction of olive oil in the region of Khenchela, Eastern Algeria, and their biological properties. The physicochemical quality of OMW from two types of separated and mixed showed that the OMW storing could help decrease the pollution produced by this waste over time. During one year of storage, the reduction rate of chemical oxygen demand, biological oxygen demand, total oxidizable matter, and biodegradability index is correspondingly (29.4%), (54.8%), (39.16%), and (54.2%); however, C/N, BOD_5/COD, and pH continue to decrease. The highest phenolic concentration (961.11 ± 65.95 µg GAE/mL) was recorded in OMW of Abani variety after 12 months of storage. The highest concentration of flavonoids (27.96 ± 3.71 µg QE/mL) was recorded in OMW of Zlitni, just after olive oil extraction. The highest concentration of total tannin (90.47 ± 21.24 µg CAE/mL) was recorded in Zlitni after 1 month of storage. For condensed tannin, the highest concentration (8.66 ± 3.22 µg TAE/mL) was recorded in Abani after 6 months of storage. Qualitative analysis by LC-MS revealed the presence of 28 constituents, of which kampherol was the major constituent and rosmarinic acid was detected for the first time in OMW. The analysis of the antioxidant activity showed a very highly significant difference for DPPH, ABTS, FRAP, and H_2O_2. The best results were obtained in OMW of Abani after 12 months of storage for the four tests; DPPH (IC_{50} = 151.12 ± 0.22 µg/mL), ABTS (IC_{50} = 129.32 ± 26.09 µg/mL), FRAP (IC_{50} = 72.42 ± 8.59 µg/mL) and H_2O_2 (IC_{50} = 75.52 ± 0.85 µg/mL). The anti-inflammatory activity of the phenolic compounds showed a very highly significant difference between the two tests, IPD and MSP. The best value obtained for the IPD test (IC_{50} = 25.47 ± 1.50 µg/mL) was recorded after 1 month of storage of OMW from the Abani variety. The best value obtained for the MSP test (IC_{50} = 16.11 ± 0.10 µg/mL) was recorded in OMW from Abani after 12 months of storage. For the anticoagulant activity, the finding showed, for the time effect, a very highly significant difference, as regards APTT and PT. The best values obtained for APTT (80.07 ± 0.15 s) and PT (37.13 ± 0.31 s) were recorded in Zlitni OMW just after olive oil extraction. It concluded that the storage time affects the physicochemical and biological properties of OMW. In addition, OMWs are a source of molecules with biological activity, and their extraction can constitute one of the strategies for recovering this waste.

Keywords: Storage time, olive oil mill wastewater, cold extraction, Khenchela, biological properties, olive oil.

Résumé

L'objectif de ce travail est d'évaluer l'effet du temps de stockage sur la qualité des margines issues de l'extraction à froid de l'huile d'olive dans la région de Khenchela, à l'Est de l'Algérie, et leurs propriétés biologiques. La qualité physico-chimique des margines issues de deux types séparés et mélangés a montré que le stockage peut contribuer à diminuer la pollution produite avec le temps. Pendant un an de stockage, le taux de réduction de la demande chimique en oxygène, de la demande biologique en oxygène, de la matière oxydable totale et de l'indice de biodégradabilité est de (29.4%), (54.8%) (39.16%) et (54.2%), cependant, C/N, DBO_5/DCO et le pH continuent de diminuer. La concentration phénolique la plus élevée (961.11 ± 65.95 µg EAG/mL) a été enregistrée dans les margines de l'Abani, après 12 mois de stockage. La concentration la plus élevée de flavonoïdes (27.96 ± 3.71 µg EQ/mL) a été enregistrée dans les margines de Zlitni, juste après l'extraction de l'huile d'olive. La concentration la plus élevée de tanin total (90.47 ± 21.24 µg EAC/mL) a été enregistrée à Zlitni après 1 mois de stockage. Pour le tanin condensé, la concentration la plus élevée (8.66 ± 3.22 µg EAT/mL) a été enregistrée pour Abani après 6 mois de stockage. L'analyse qualitative par LC-MS a révélé la présence de 28 constituants, dont le kamphérol était le constituant majeur et l'acide rosmarinique est détecté pour la première fois dans les margines. L'analyse de l'activité antioxydante a montré qu'il y avait une différence très hautement significative pour le DPPH, l'ABTS, le FRAP et le H_2O_2. Les meilleurs résultats ont été obtenus dans les margines d'Abani après 12 mois de stockage pour les quatre tests ; DPPH (IC_{50} = 151.12 ± 0.22 µg/mL), ABTS (IC_{50} = 129.32 ± 26.09 µg/mL), FRAP (IC_{50} = 72.42 ± 8.59 µg/mL) et H_2O_2 (IC_{50} = 75.52 ± 0.85 µg/mL). Les résultats de l'activité anti-inflammatoire montrent qu'il y avait une différence très hautement significative entre les deux tests IPD et MSP. La meilleure valeur obtenue pour le test IPD (IC_{50} = 25.47 ± 1.50 µg/mL) a été enregistrée après 1 mois de stockage des margines de la variété Abani et aussi pour le test MSP (IC_{50} = 16.11 ± 0.10 µg/mL) après 12 mois de stockage. Pour l'activité anticoagulante, la constatation a montré, pour l'effet temps, une différence très hautement significative, en ce qui concerne l'APTT et le PT. Les meilleures valeurs obtenues pour APTT (80.07 ± 0.15 s) et PT (37.13 ± 0.31 s) ont été enregistrées chez la variété Zlitni juste après l'extraction de l'huile d'olive. D'après les résultats trouvés, il a été conclu que les propriétés physico-chimiques et biologiques des margines sont affectées par la durée de stockage. De plus, les margines présentent une source de molécules à activité biologique et leur extraction peut constituer une des stratégies de valorisation de ces déchets.

Mots clés : Durée de stockage, margine, extraction à froid, Khenchela, propriétés biologiques, huile d'olive.

الملخص

الهدف من هذا العمل هو تقييم تأثير وقت التخزين على جودة المرجين المتحصل عليه من الاستخلاص البارد لزيت الزيتون في منطقة خنشلة، شرق الجزائر، وخصائصها البيولوجية. أظهرت الجودة الفيزيائية والكيميائية للمرجين من نوعين منفصلين ومختلطين أن تخزين المرجين يمكن أن يساعد في تقليل التلوث الناتج عن هذه النفايات بمرور الوقت. خلال سنة واحدة من التخزين، كان معدل خفض الطلب على الأكسجين الكيميائي، والطلب على الأكسجين البيولوجي، وإجمالي المواد المؤكسدة، ومؤشر التحلل البيولوجي المقابل (29.4٪)، (54.8٪)، (39.16٪)، و (54.2٪)؛ ومع ذلك، يستمر C / N و BOD_5/COD و pH في الانخفاض. تم تسجيل أعلى تركيز فينولي (961.11 ± 65.95 ميكروغرام مكافئ حمض الغاليك / مل) في المرجين من صنف عباني بعد 12 شهرًا من التخزين. تم تسجيل أعلى تركيز للفلافونويد (27.96 ± 3.71 ميكروغرام مكافئ الكارستين / مل) في المرجين من صنف زليتني بعد استخلاص زيت الزيتون مباشرة. تم تسجيل أعلى تركيز من إجمالي التانين (90.47 ± 21.24 ميكروغرام مكافئ حمض الكاتشيك / مل) في زليتني بعد شهر من التخزين. بالنسبة للتانين المكثف، تم تسجيل أعلى تركيز (8.66 ± 3.22 ميكروغرام مكافئ حمض التانيك /مل) في صنف عباني بعد 6 أشهر من التخزين. كشف التحليل النوعي بواسطة الكروماتوغرافيا (مطياف الكتلة اللوني السائل عالي الأداء-LC MSعن وجود 28 مكونًا ، كان الكومفيرول هو المكون الرئيسي وحمض الروزمارينيك تم اكتشافه لأول مرة في المرجين. أظهر تحليل نشاط مضادات الأكسدة أن هناك فرقًا مهمًا للغاية في DPPH و ABTSو FRAP و H_2O_2 .تم الحصول على أفضل النتائج في صنف عباني بعد 12 شهرًا من التخزين للاختبارات الأربعة؛ 151.12 DPPH (IC_{50} = 0.22 ±ميكروغرام / مل), ABTS (IC_{50} = 26.09 ± 129.32 ميكروغرام / مل), FRAP (IC_{50} = 72.42 ± 8.59 ميكروغرام / مل) و H_2O_2(IC_{50} = 75.52 ± 0.85 ميكروغرام / مل). أظهرت نتائج النشاط المضاد للالتهابات للمركبات الفينولية وجود فرق كبير للغاية بين الاختبارين IPD و MSP للتأثير. تم تسجيل أفضل قيمة تم الحصول عليها لاختبار IPD (IC_{50} = 25.47 ± 1.50 ميكروغرام / مل) بعد شهر واحد من تخزين المرجين من صنف عباني تم تسجيل أفضل قيمة تم الحصول عليها لاختبار MSP (IC_{50} = 16.11 ± 0.10 ميكروغرام / مل) في صنف عباني بعد 12 شهرًا من التخزين. بالنسبة للنشاط المضاد للتخثر، أظهرت النتائج، بالنسبة للتأثير الزمني، فرقًا مهمًا للغاية فيما يتعلق بـ APTT وPT. تم تسجيل أفضل القيم التي تم الحصول عليها لـ APTT (80.07 ± 0.15 ثانية) و PT(37.13 ± 0.31 ثانية) في صنف زليتني بعد استخلاص زيت الزيتون مباشرة. وخلصت هذه الدراسة إلى أن الخصائص الفيزيائية والكيميائية والبيولوجية للمرجين تتأثر بوقت التخزين. بالإضافة إلى ذلك، يعد المرجين مصدرًا للجزيئات ذات النشاط البيولوجي ويمكن أن يشكل استخلاصها إحدى الاستراتيجيات لإعادة تدوير واسترداد هذه النفايات.

الكلمـــات المفتاحيـــة: مدة التخزين، المرجين، الاستخلاص البارد، خنشلة، الخصائص البيولوجية، زيت الزيتون.