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Original Article

EFFECT OF GARLIC (*ALLIUM SATIVUM* L) ON BIOCHEMICAL PARAMETERS AND HISTOPATHOLOGY OF PANCREAS OF ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

Objective: Garlic (*Allium sativum*. L) plays an important dietary role, as well as medicinal, for centuries. Even today the use of garlic is widespread and growing. The present study investigated the effect of garlic extract and glibenclamide on biochemical parameters, enzyme activities, and reduced glutathione (GSH) content in the liver as well as on pancreas tissue in alloxan-induced diabetic rats.

Methods: Diabetes mellitus was induced in 28 out of 35 adult male albino rats, using an intraperitoneal injection of 150 mg/kg body weight of alloxan. The diabetic rats were divided into four groups, two of which were administered orally by garlic extract (250 and 500 mg/kg) and a group composed of diabetic rats was given the standard drug, glibenclamide, orally at a dose of 2.5 mg/kg. The control rats (normal and diabetic) were fed normal saline, once daily for 21 d.

Results: Oral administration of the garlic extract significantly decreased blood glycosylated hemoglobin, serum glucose, total cholesterol, triglycerides, total lipids, glutamic oxalic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) levels, with significant increase in plasma insulin, and GSH content in liver of alloxan-diabetic rats in dose–dependent fashion which was comparable to an antidiabetic standard drug, glibenclamide, given at a dose of 2.5 mg/kg. Concurrent histological studies of the pancreas of these animals have confirmed the changes observed in biochemical parameters and proved the comparable preventive effect of garlic extract.

Conclusion: These results suggest the potential of garlic extract as a histo protective against free-radical-associated diabetes damage, preserving the ability of insulin secretion, and show a concentration-dependent antidiabetic effect.

Keywords: Garlic, Diabetes, Biochemical parameters, Glibenclamide, Pancreas, Rat

INTRODUCTION

Diabetes mellitus is an endocrine disorder that is characterized by hyperglycemia [1]. A number of investigations, of oral anti-hyperglycemic agents from plants used in traditional medicine, have been conducted and many of the plants were found with good activity [2]. The World Health Organization (WHO) has also recommended the evaluation of the plants' effectiveness in conditions where we lack safe, modern drugs [3]. This has leaded an increasing demand of research on natural antidiabetic products which produces minimal or no side effects. Garlic (*Allium sativum* L., Liliaceae) is a common spicy flavoring agent used since ancient times. Garlic has been cultivated for its characteristic flavor and medicinal properties. Although garlic has been used for centuries, and even nowadays is part of popular in many cultures, but until recently there has been little scientific support of its therapeutics and pharmacological properties. In the past decade, some protective effects of garlic have been well established by epidemiological studies and animal experiments. Elkayam, Mirelman and Peleg investigate the commercially available garlic preparations in the form of garlic oil, garlic powder, and pills are widely used for certain therapeutic purposes, including lowering blood pressure and improving lipid profile [4]. Garlic has been largely attributed to the reduction of risk factors for cardiovascular diseases and cancer [5], stimulation of immune function [6], hepatoprotection [7] and antioxidant effect [8]. In addition, garlic contains at least 33 sulfur compounds, several enzymes, 17 amino acids, and minerals such as selenium [9]. It contains a higher concentration of sulfur compounds than any other *Allium* species. The sulfur compounds are responsible both for garlic's pungent odor and many of its medicinal effects.

Therefore, the purpose of the present study is first to examine the influence of oral administration of garlic extract and glibenclamide on biochemical parameters, the activities of some enzymes in plasma, glutathione level in liver and histopathology of the pancreas in the alloxan-induced diabetic rat.

MATERIALS AND METHODS

Preparation of garlic extract

Fresh garlic (*Allium Sativum L.*) was collected from North-East of Algeria (El Tarf Province), in July 2012, and identified by botanists in the herbarium of Badji Mokhtar University, Algeria. Then, the cloves were peeled, sliced, ground into a paste and a homogenate was made in distilled water. Two concentrations of the extract were prepared, 0.1 and 0.2 mg/ml, corresponding to 250 mg and 500 mg/kg body weight of the animal. Oral feeding was done within 30 min of preparation of homogenate.

Animals

Male albino Wistar rats used were 8 w old; they were procured from Pasture Institute, Algiers, Algeria, and were maintained at animal house of animal biochemistry department, Badji Mokhtar university, Annaba. The animals were divided into five groups (n=7 each) and housed in clean cages with temperature (22–24 °C), 12-h light/12-h dark cycle and relative air humidity 40–60%. Rats had free access to food and water.

Induction of diabetes

After one week of adaptation period with a nutritionally complete, rats were fasted overnight and injected intraperitoneally with a freshly prepared alloxan monohydrate solution at a dose of 150 mg/kg body weight [10]. The diagnosis of diabetes was based on hyperglycemia (blood glucose levels above 200 mg/dl) on the 3rd day after alloxan injection.

Experimental protocol

In the present experiment, 35 rats (28 diabetic and 7 normal rats) were used. The rats were randomly divided into five groups of seven males each. Garlic extract and standard drug, glibenclamide, were

fed by gavages every day at fixed time (10.00 a. m) for 3 consecutive weeks as follows:

Group N-C: Normal Control rats were administrated 1 ml of normal saline.

Group DT-250: Diabetic Treated rats were administrated garlic extract (250 mg/kg body weight).

Group DT-500: Diabetic Treated rats were administrated garlic extract (500 mg/kg body weight).

Group D-C: Diabetic Control rats were administrated 1 ml of normal saline.

Group DT-Glb Diabetic Treated rats were administrated standard drug, glibenclamide (2.5 mg/kg body weight).

After three weeks of treatment, total body weights were recorded and animals were sacrificed,

Blood collection and biochemical analyze

Blood was collected with the ethylenediamine tetraacetic acid (EDTA) container and used for the preparation of plasma. Blood collected without anticoagulant was used for serum separation.

-Glucose was measured in 10 µl samples of whole blood by the glucose oxidase method, using an YSI model 27glucose analyzer and the kit constitute of phosphate buffer containing the enzymes (GOD, POD) and D-glucose (Sigma).

-Plasma insulin level was estimated with an enzyme linked immunosorbent assay (ELISA) kit using human insulin as standard [11].

-Lipids, cholesterol, and triglycerides concentrations were determined using commercial test kits for lipids [12], cholesterol [13] and triglycerides [14].

-Plasma was separated and used for glycosylated hemoglobin (HbA1c) assay according to a method of Bisse and Abraham [15].

-GOT, GPT, LDH and ALP activities were also determined using commercial test kits for GOT, GPT [16], LDH [17] and alkaline phosphatase [18].

Protein estimation

The protein contents were determined according to the method of Bradford [19] by using bovine serum albumin as a standard.

Determination of reduced glutathione

The measurement of liver reduced glutathione (GSH) concentration was performed by Weckbercker and Cory method [20] using a colorimetric technique, based on the development of a yellow colour when DTNB [(5,5 dithiobis-(2-nitrobenzoic acid)] is added to compounds containing sulfhydryl groups. In brief, 0.8 ml of liver supernatant was added to 0.2 ml of 0.25% sulfosalicylic acid, and then tubes were centrifuged at 1000 × g for 10 min. Supernatant (0.5 ml) was mixed with 0.025 ml of 0.01M DTNB and 1 ml phosphate buffer (0.1 M, pH 7.4). The absorbance at 412 nm was recorded. Finally, total GSH content was expressed as n mol GSH/mg protein.

Histopathological studies

For histopathological examination, pancreas obtained by dissection was washed with isotonic saline (9g sodium chloride/1 distilled water). It was immediately fixed in Bouin solution for 24 h, processed by using a graded ethanol series, and then embedded in paraffin. The paraffin sections were cut into 5 µm thick slices and stained with hematoxylin and eosin (H&E) [21]. All sections were examined for histological changes as shown in fig. 1. All pictures (microphotography's) have a magnification of 400· and were performed with optic microscopy.

Statistical analysis

Data were expressed as means±SE. Data comparisons were carried out by using one-way ANOVA followed by Student's t-test to compare means between the different treated groups. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Effect of administration of garlic extract on body weight gain, serum glucose, blood glycosylated hemoglobin and plasma insulin levels:

In the present study, we observed after three weeks of treatment a significant decrease in body weight in diabetic control rats (D-C) compared to normal controls (N-C) (table1), whereas diabetic rats orally treated with 250 and 500 mg/kg of garlic extract were maintained a good phenotype in a dose-dependent fashion. Besides, the experimentally induced diabetes increased the level of serum glucose and the percentage of HbA1c compared to the (N-C) group.

However, a decrease in serum glucose and blood glycosylated hemoglobin levels coupled with significant increase in plasma insulin was maximum in the group receiving 500 mg/kg BW of garlic extract which was comparable to an standard antidiabetic drug, glibenclamide, given at a dose of 2.5 mg/kg. (table1). The antidiabetic effect induced by garlic extract, as observed, was dose-related.

Effect of administration of treatment on serum biochemical parameters and liver reduced glutathione

The biochemical parameters (table 2) such as serum lipids, triglycerides, and cholesterol in experimental groups of rats shows a significant increase in the levels of lipids, triglycerides, cholesterol in alloxan-induced diabetic rats (D-C), when compared with normal control rats (N-C). Administration of glibenclamide at dose of 2.5 mg/kg and *Allium sativum* extract to diabetic rats for 21 d resulted in the restoration of biochemical parameters levels towards near normalcy in a dose-dependent fashion.

Liver reduced glutathione concentration of diabetic control animals was lower than this of normal control rats. This alteration was restored back to near normal in diabetic rats orally treated with 250 and 500 mg/kg of raw garlic homogenate and standard drug, glibenclamide (table 02).

Effect of administration of garlic extract and glibenclamide on the activities of pathophysiological enzymes

Table 3 depicts the activities of GOT, GPT, LDH and ALP in the serum of experimental groups of rats. There was a significant increase in the activities of GOT, GPT, LDH and ALP in the serum of diabetic rats when compared with normal control rats. Daily oral administration of glibenclamide at dose of 2.5 mg/kg and *Allium sativum* extract to diabetic rats for 21 at doses of 250 and 500 mg/kg brought down these enzyme activities to near normal.

Table 1: Effect of garlic extract on body weight gain, glucose, blood glycosylated hemoglobin and plasma insulin levels after three weeks of treatment in experimental groups

Groups	Body weight gain (g)	Glucose (mg/dl) (mean±SE)	HbA1c (٪)	Plasma insulin (µU/ml)
N-C	+31.3±11.2	116.10±11.1	2.23±0.5	13.65±4.32
D-C	-06.14$^{(a)}$***±9.92	408.48$^{(a)}$***±10.2	3.72$^{(a)}$***±0.47	5.69$^{(a)}$***±2.75
DT-250	+14.9±19.37	284.8$^{(b)}$**±85.63	2.53$^{(b)}$**±0.53	9.16$^{(b)}$**±2.56
DT-500	+37.3$^{(b)}$***±14.5	166.4$^{(b)}$***±63.8	2.11$^{(b)}$***±0.89	12.06$^{(b)}$***±5.05
DT-Glb	+37.8$^{(b)}$***±20.1	116.4$^{(b)}$***±63.8	2.13$^{(b)}$***±0.65	13.96$^{(b)}$***±4.06

N-C: Normal Control *D-C*: Diabetic Control *DT-250:* Diabetic Treated rats with garlic extract at 250 mg/kg., *DT-500:* Diabetic Treated rats with garlic extract at 500 mg/kg. *DT-Glb* Diabetic Treated rats with glibenclamide at 2.5 mg/kg., Results are expressed as mean±SE (n=7). One-way ANOVA followed by Student's t-test.** *P≤0, 01, *** P≤ 0,001* (a): compared to (N-C) (b): compared to (D-C).

Table 2: Effect of garlic extract and glibenclamide on serum biochemical parameters and liver reduced glutathione after three weeks of treatment in experimental groups

Groups	Lipids (mg/100 ml)	Cholesterol (mg/100 ml)(mean±SE)	Triglycerides (mg/100 ml)	Liver GSH(nM/mg prot)
N-C	334.9±75.1	77.61±4.39	75.2±18.25	122.4±17.8
D-C	753$^{(a)}$***±130	105.69$^{(a)}$***±11.63	114.61$^{(a)}$**±16.13	94.2$^{(a)}$**±11.4
DT-250	588$^{(b)}$**±80,1	88.61$^{(b)}$*±12.72	65.75$^{(b)}$***±18.85	149.3$^{(b)}$***±13.7
DT-500	480.6$^{(b)}$***±102	79.89$^{(b)}$***±6.29	40.96$^{(b)}$***±14.22	186.6$^{(b)}$***±21.1
DT-Glb	380.3$^{(b)}$***±92	76.59$^{(b)}$***±7.19	70.91$^{(b)}$***±14.82	145.6$^{(b)}$***±11.1

N-C: Normal Control *D-C*: Diabetic Control *DT-250:* Diabetic Treated rats with garlic extract at 250 mg/kg., *DT-500:* Diabetic Treated rats with garlic extract at 500 mg/kg. *DT-Glb* Diabetic Treated rats with glibenclamide at 2.5 mg/kg., Results are expressed as mean±SE (n=7). One-way ANOVA followed by Student's t-test. ** P≤0, 05, ** P≤0, 01, *** P≤ 0,001* (a): compared to (N-C) (b): compared to (D-C)

Table 3: Effect of garlic treatment on pathophysiological enzymes activities after three weeks of treatment in experimental groups

Groups	GOT (U/l)	GPT (U/l)(mean±SE)	LDH (U/l)	ALP (U/l)
N-C	31.48±5.2	25.99±5.32	1419±110	283.1±32.9
D-C	45.4$^{(a)**}$±10.8	35.73$^{(a)}$**±5.6	1970$^{(a)}$***±273	610$^{(a)}$***±46.7
DT-250	37.73±6.58	32.39±3.92	1252$^{(b)}$ ***±352	373.3$^{(b)}$ ***±119
DT-500	29.76$^{(b)}$**±3.93	26.38$^{(b)}$ **±4.29	1295$^{(b)}$***±235	243$^{(b)}$ ***±95.1
DT-Glb	29.56$^{(b)}$**±3.13	24.33$^{(b)}$ **±3.19	1315$^{(b)}$***±135	199.2$^{(b)}$ ***±75

N-C: Normal Control *D-C*: Diabetic Control *DT-250:* Diabetic Treated rats with garlic extract at 250 mg/kg., *DT-500:* Diabetic Treated rats with garlic extract at 500 mg/kg. *DT-Glb* Diabetic Treated rats with glibenclamide at 2.5 mg/kg., Results are expressed as mean±SE (n=7). One-way ANOVA followed by Student's t-test., ** P≤0, 05, ** P≤0, 01, *** P≤ 0,001* (a): compared to (N-C) (b): compared to (D-C)

Effect of garlic extracts on pancreas histopathology

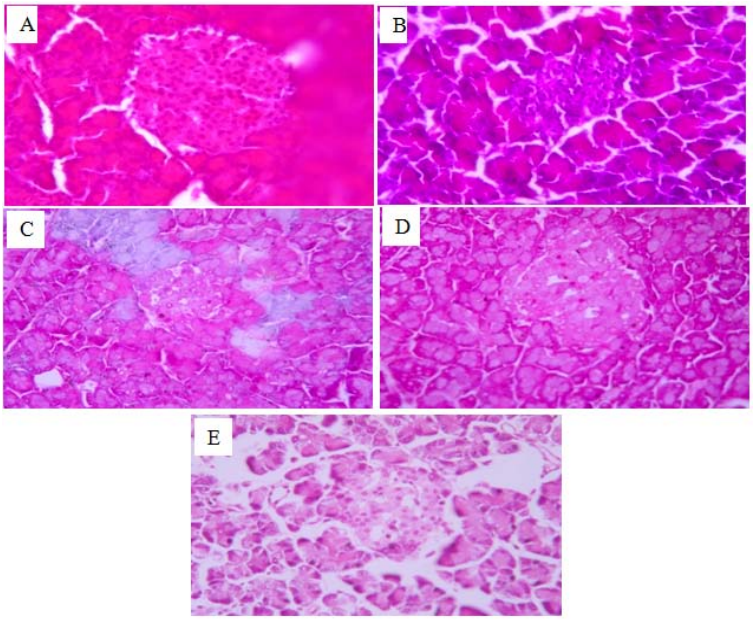

Fig. 1: Effect of garlic extract on histopathological damages in the pancreas after three weeks of treatment in experimental groups: A: Section of pancreas tissue from normal control rats (N-C) showing normal architecture. B: Section of pancreas tissue from diabetic control rats (D-C) showing degenerative vascular changes in the islets. C: Section of pancreas tissue from DT-250 group showing initial stages of regenerating islets. D: Section of pancreas tissue from DT-500 group showing apparently normal architecture. E: Section of pancreas tissue from DT-Glb group showing the apparently normal population of pancreatic islets. Optic microscopy: sections were stained using the hematoxylin-eosin method (400x)

DISCUSSION

Alloxan has been widely used for inducing type I diabetes in a variety of animals by affecting degeneration and necrosis of pancreatic β-cells [22]. The present results showed that alloxan-induction results in a decrease in body weight of diabetic rats which is possible due to catabolism of fats and protein, even though the food intake is more in diabetic rats than normal control. Due to insulin deficiency protein content is decreased in muscular tissue by proteolysis [23]. Daily oral administration of *Allium sativum* extract to diabetic rats for 21 d at doses of 250 and 500 mg/kg significantly improves body weight in diabetic rats. The present data indicated that the garlic extract significantly decreased serum glucose in treated diabetic rats in a dose-dependent fashion as compared with diabetic control rats.

Furthermore, the observed effects of the extract on weight loss compared favorably with glibenclamide. The hypoglycemic potency of garlic has been attributed to the sulphur compounds [di (2-propenyl) disulphide and 2-propenyl propyl disulphide,

respectively] [24]. The mechanism of hypoglycemic action probably involves direct or indirect stimulation of insulin secretion [25]. Further, Augusti suggested that these disulphide compounds have the effect of sparing insulin from–SH inactivation by reacting with endogenous thiol-containing molecules such as cysteine, glutathione, and serum albumins [26]. The garlic extract might enhance glucose utilization because it significantly decreased the blood glucose level in glucose-loaded rats.

During diabetes, the excess glucose present in the blood reacts non-enzymatically with hemoglobin to form glycosylated hemoglobin (HbA1C). As a result, the rate of glycosylation is proportional to the concentration of blood glucose [27]. Hence, estimation of glycosylated hemoglobin is a well-accepted biochemical parameter useful for the diagnosis and management of the disease. The increased glycated hemoglobin is associated with loss of β-cell function and has been implicated in the complications of diabetes mellitus [28]. Oral administrations of *Allium sativum* tend to decrease the level of glycosylated hemoglobin by improving the blood glucose homeostasis.

Lipids play a vital role in the pathogenesis of diabetes mellitus. The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia. In our study, we have noticed elevated levels of serum lipids such as cholesterol and triglycerides in diabetic rats. The levels of increased serum lipids in diabetes represent a risk factor for coronary heart disease. Under normal circumstances, insulin activates lipoprotein lipase and hydrolyzes triglycerides [29]. Insulin increases uptake of fatty acids into adipose tissue and increases triglyceride synthesis. Moreover, insulin inhibits lipolysis. In the case of insulin deficiency, lipolysis is not inhibited, and we have increased lipolysis which finally leads to hyperlipidemia. In insulin-deficient diabetes, the concentration of serum free fatty acids is elevated as a result of free fatty acid outflow from fat depots, where the balance of the free fatty acid esterification–triglyceride lipolysis cycle is displaced in favor of lipolysis.

The administration of garlic extract significantly decreased serum triglycerides and cholesterol in diabetic rats which were comparable to an standard antidiabetic drug, glibenclamide, given at a dose of 2.5 mg/kg. Incontinence with the present data, other workers have reported that administration of fresh garlic or etheric garlic extracts was shown to improved lipid profile including reduction of serum cholesterol levels [30]. Short-term experiments using primary hepatocyte cultures, which have proved useful as tools for screening the anticholesterolemic properties of garlic. With respect to the cholesterol-lowering property of garlic, it has been suggested that some constituents of garlic may act as inhibitors for some enzymes such as hydroxy methyl glutaryl-CoA reductase, which participates in cholesterol synthesis [31]. Consistent with this idea, it has been shown that *in vivo* treatment of garlic extract reduces the lipid peroxidation products [32].

Serum enzymes including GOT, GPT, LDH and ALP are used in the evaluation of hepatic disorders. An increase in these enzymes activities reflects active liver damage/inflammatory hepatocellular disorders [33]. In accordance with these findings, increase in the activities of GOT, GPT, LDH and ALP in serum may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream [34], which gives an indication on the hepatotoxic effect of alloxan. On the other hand, Daily oral administration of glibenclamide at dose of 2.5 mg/kg and *Allium sativum* extract to diabetic rats for 21days at doses of 250 and 500 mg/kg caused reduction in the activity of these enzymes in serum compared to the mean values of the diabetic group and consequently may alleviate liver damage caused by alloxan-induced diabetes; these results were in agreement with other findings [35].

Reduced glutathione is a potent free radical scavenger. GSH within the islet of β-cell is an important factor against the progressive destruction of the β-cells following partial pancreatectomy [36]. Depletion of GSH results in enhanced lipid peroxidation. This can cause increased GSH consumption and can be correlated to increase in the level of oxidized glutathione (GSSG). Administration of garlic extract resulted in the elevation of the GSH level, which protects the cell membrane against oxidative damage by regulating the redox status of protein in the cell membrane similar results [37]. The hepatoprotective activity of garlic extract was higher at 500 mg/kg than glibenclamide. The increase in the GSH content may protect the tissues against diabetes associated tissue injury by reducing the susceptibility to toxic radicals.

The pathological changes observed in pancreas (fig. 1) of alloxan diabetic rats may be due to the hyperglycemia and its mediated oxidative stress. *Allium sativum* extract resulted in glucose homeostasis and attenuation of oxidative stress by optimization of antioxidant status [38], which could have protected tissue damage. The histological evidence authenticated the extent of tissue injury by alloxan and the protection offered to pancreatic β-cells by garlic extract in dose-dependent fashion preserving the ability of insulin secretion [39]. These results are in agreement with those obtained by Banerjee and Dinda [40] wherever they visualized the efficiency of this garlic extract on the protection of the heart against oxidative stress induced by ischemic reperfusion injury.

The present results showed that local spices of *Allium sativum* exerted antioxidant and antihyperglycemic effects and consequently may alleviate and protect pancreas damage caused by alloxan-induced diabetes which was comparable to an standard antidiabetic drug, glibenclamide, given at a dose of 2.5 mg/kg. The effects induced by the extract, as observed were dose-related. Further, it is concluded that the plant must be considered as an excellent candidate for future studies on diabetes mellitus. In addition, comprehensive pharmacological investigations, including chronic experimental studies, should be carried out.

CONFLICT OF INTERESTS

Declared none

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REGULAR ARTICLE

In vitro assessment of the bioactive potential of *Artemisia campestris* L. fractions growing in Khenchela (Algeria)

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ABSTRACT

In order to valorize Algerian medicinal plants, this work aims to characterize quantitatively and qualitatively *Artemisia campestris* L. fractions, a medicinal plant from the Aures-Algeria area, and investigate its biological activities *in vitro*. During preliminary phytochemical screening, flavonoids, saponins, tannins, alkaloids, reducing compounds, and coumarins were discovered. Total polyphenols and flavonoids are greater in the n-butanolic fraction than in ethyl acetate and petroleum ether. The resulting antioxidant capability was assessed *in vitro* using three methods: DPPH free radical scavenging, hydrogen peroxide trapping, and iron reduction. The n-butanolic extract suppresses DPPH oxidation with an IC50 of 2.239±0.32mg/ml, which is comparable to that of standard (1.824±0.97mg/ml). Despite the fact that similar findings were seen in the neutralization of hydrogen peroxide and the iron chelating activity, The anti-inflammatory action was proven i*n vitro* by inhibiting protein denaturation and increasing HRBC membrane stability (Human Red Blood Cells). The n-butanolic fraction was more effective than diclofenac in preventing BSA degradation. It also inhibited membrane hemolysis in human erythrocytes by up to 83%. Activated partial thromboplastin and prothrombin times were used to analyze extrinsic and intrinsic coagulation pathways in *A. campestris* in order to determine its anticoagulant activity. The n-butanol fraction had the greatest impact on PT and aPTT lengthening, with 75.2s and 351s at 3 mg/ml concentration, respectively. The n-butanolic fraction of the *A. campestris* aerial part exhibited antioxidant, anti-inflammatory, and anticoagulant activities. As a result, it may be a viable natural resource for mitigating the impact of stress, which causes inflammatory and cardiovascular disorders.

1. Introduction

Artemisia L. is a genus of tiny shrubs and herbs native to northern temperate climates. It belongs to the large *Compositae* (*Asteraceae*) family, which has about 20,000 species and 1,000 genera. More than 500 species of *Artemisia* are part of the Anthemideae tribe, including *Artemisia campestris*

L (Bora and Sharma, 2011), known locally as "Tagouft," is a polymorphic plant with a large number of subspecies and variants used in North African recipes to treat various diseases. It has medicinal, pharmacological, and culinary qualities (Sijelmassi, 1993). Antivenom, antirheumatoid and antidiabetic characteristics are widely documented in traditional medicine for this species (Bnouham et al. 2002, Dib et al., 2016a), likely because of the presence and extravagance of bioactive metabolites such as flavonoids, chromones, acetophenones, coumarins, and essential oils (Vasconcelos et al. 1998, Pereira et al. 2018). Oxidative damage to bioactive systems is a common cause of many chronic illnesses, including cancer and heart disease. Hydrogen peroxide, superoxide anion, and hydroxyl radical are examples of reactive oxygen species (ROS). They have been associated with age-related health problems such as carcinogenesis and coronary heart disease (Marnett, 2000).

In living organisms, there are a lot of ways to keep the level of ROS and the problems it causes from getting too high. Antioxidants seem to be vital in the protection of a large variety of ailments and health problems since they defend against both direct ROS impacts and reactionary oxidative processes (Lekouaghet et al., 2020). Both reactions are inhibited by the synthetic antioxidants butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT). Since their introduction to the food industry, questions have been raised about their safety and effectiveness because of their unstable and extremely volatile nature (Nabavi et al., 2008). Toxic chemicals and physical trauma may cause inflammation, which is a natural response to tissue damage. It is also associated with pain, as well as an increase in protein denaturation, an increase in vascular permeability, and membrane disruption. One of the most common treatments for inflammation is the non-steroidal anti-inflammatory drug (NSAIDs), but it has a number of side effects, the most serious of which is stomach irritation and the subsequent development of gastric ulcers (Ferrero-Millani et al., 2007). The biggest cause of death worldwide is cardiovascular disease. Coagulation problems may be caused by viral infections. Recently, a number of studies have indicated that patients with severe new coronavirus pneumonia exhibit coagulation malfunction (Hireche et al., 2021). Anticoagulants such as heparin and anti-vitamin K compounds have life-threatening adverse effects (Kishore, 2013). Thrombolytic therapy and mechanical treatments are typically used to treat thrombotic disorders. Slow and inadequate thrombolysis, such as the risk of hemorrhage, is a disadvantage of current thrombolytic therapy (Kumar et al., 2011 and Dib et al., 2017b). As a result, there is a growing need for novel remedies derived from plants, which constitute the major source of drugs for the treatment of many diseases. In this regard, this research has focused on the anti-oxidant, anti-inflammatory and, for the first time, anticoagulant properties of *Artemisia campestris* fractions.

2. Materials and Methods

2.1. Chemicals and Reagents

Several chemical reagents and solvents were used in our experiments, including methanol, n-butanol, petroleum ether, ethyl acetate, chloroform, toluene, acetone, acetic acid, glacial acetic acid, FeCl3, HCl, NaOH, NH4OH, I2, NaCl, AlCl3, Wagner's reagent, formic acid, dichloromethane, NaCl, dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), Tp (thromboplastin), TCK (cephalin Kaolin + $CaCl_2$), quercetin, gallic acid, and DPPH (2,2 diphenyl-1-picrylhydrazyl). The remainder of the chemicals and reagents were all of analytical quality.

2.2. Plant material and preparation of extracts

The aerial part (leaves and twigs) of the plant *Artemisia campestris L. was* collected in 2019 from M'toussa-Ain Touila (Khenchela region) (Lat: 35° 35'58 "S e Long. 7 ° 14'42" W) and identified by Dr. Zeraib Azzeddine, from the Faculty of Nature and Life Sciences of Khenchela University. The sample was dried, ground, and macerated (30g/100ml) in a hydromethanolic (30/70 methanol-water) solution for 24 hours at room temperature. With the regeneration of the solvent, this procedure was repeated three times. A rotary evaporator was used to concentrate the crude extract after that. Sequential extraction with increasing polarity solvents: petroleum ether (Petet), ethyl acetate (EtOAc), and n-butanol (BuOH) was used to partition the crude extract (Markham, 1982). Each fraction was weighed and represented as a percentage of the overall weight of air dried plant material. It was stored at 4°C until examined chemically and assessed for bioactivity.

2.3. Phytochemical Investigation

2.3.1. Phytochemical screening

We used typical phytochemical tests to search for flavonoids, saponins, tannins, alkaloids, and reducing chemical compounds in the plant's crude extract (Edeoga, 2005).

2.3.2. Assessment of phenolic content

The phenolic content in extracts is determined utilizing Folin-Ciocalteau technique (Li et al. 2007). The liquids were combined for 4 minutes before incubation using 200 µl of each extract (dissolved in methanol) and 1 ml of Phenolphthalein (diluted at 10%). After the incubation period was completed, we added a solution of sodium carbonate (75 mg/ ml) to the mixture. The incubation period was 2 hours in the dark condition. A spectrophotometer calibrated to 765 nm was used to measure the absorbance. Gallic acid equivalent per mg of extract was used as the unit of measurement.

2.3.3. Assessment of flavonoids content

The aluminum trichloride method is used to measure the flavonoids in our extracts (Djeridane et al. 2007b). At the appropriate dilutions, 1 ml of each extract and the standard (dissolved in methanol) were added in equal amounts to a solution of $AlCl_3$ (2% in methanol). After 10 minutes of incubation, the absorbance of the samples was determined at 430 nm. Calculation of the flavonoid content was done using a calibration curve as the quercetin equivalent in µg per mg of extract.

2.4. In vitro evaluation of antioxidant activity

A single antioxidant test model should not be used to establish that a substance has antioxidant properties. FRAP (Ferric reducing antioxidant power), $DPPH^+$ (2,2-diphenyl-1-picrylhydrazyl) and other in vitro tests, depending on the substance used as a source of free radicals, are often used in practice to assess the antioxidant capacity of medicinal plants.

2.4.1. Radical-scavenging activity of DPPH

Stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used with minor modifications to measure the extract's free radical scavenging activity (Brand-Williams et al. 1995). The test sample (15 µl) was incubated for 30 minutes in the dark with 1.5 ml of DPPH reagent (100 µM) prepared in absolute methanol. A measurement of absorbance was taken at 517 nm. Ascorbic acid was the standard antioxidant. The percentage of free radical inhibition may be represented using the following formula:

I% = [(Abs Control Negative-Abs Sample) / Abs Control Negative] X 100

The IC50 value was established to be the sample concentration necessary to scavenge 50% of DPPH free radicals.

2.4.2. Hydrogen peroxide scavenging activity

The capacity to reduce hydrogen peroxide from the extract was tested using the Dehpour method (Dehpour et al., 2009). A 10mM hydrogen peroxide solution was prepared in phosphate buffer (pH 7.4). A 0.6ml hydrogen peroxide solution was treated with 1.4 ml of a diluted extract in distilled water. After 10 minutes, the absorbance of hydrogen peroxide at 230nm was measured in comparison to a buffer solution without hydrogen peroxide. The percentage of H_2O_2 trapped (I%) is calculated by the following formula:

$$\mathbf{I\% = [(A_o - A_1) / A_o] \times 100}$$

The absorbance of the sample or standard solution was A1, whereas that of the control solution was A_o.

2.4.3. Ferric Reducing Antioxidant Power (FRAP)

Benzie's et al. (1996) approach was used to calculate the reducing power. For each extract concentration, 1 ml was mixed with 2.5 ml of buffer solution (pH 6.6; 0.2M) and 2.5ml of 1% potassium ferricyanide solution. The mixes were heated to 50° C for 30 minutes. The mixture was then treated with 2.5 ml of 10% trichloroacetic acid. For 10 minutes, the mixture was centrifuged at 3000 rpm. To 2.5 ml of distilled water and 2.5 ml of the supernatant from each concentration, we added 0.5 ml of FeCl3 (0.1%). The combination's absorbance was measured using a spectrophotometer at 700 nm. Vitamin C was used as a positive control, whereas extraction solvents were used as negative controls.

2.5. In vitro evaluation of anti-inflammatory activity

The anti-inflammatory effect of *A. campestris* extracts was demonstrated *in vitro* using the protein denaturation inhibition method and anti-hemolytic activity, which tests the membrane stability of HRBCs (Human Red Blood Cells).

2.5.1. Method of Bovine Serum Albumin (BSA) Denaturation

The *in vitro* anti-arthritic activity was studied using

the albumin stability test (Rahman et al. 2012) at different concentrations. To make the standard solution, 0.45ml of BSA (0.5%) and 0.05ml of diclofenac sodium (0.05%) at varied concentrations are used.

Procedure

A 0.45 ml (0.5% w/v BSA) was combined with 0.05 ml of each extract and diclofenac sodium at various concentrations (50, 100, 250 µg/ml). After 20 minutes at 37°C, they were heated to 57°C for 3 min. When the aforementioned solutions have cooled, add 2.5 ml of phosphate buffer. A UV-Visible spectrophotometer was used to measure the absorbance at 660 nm. The control represents a 100% percentage of protein denaturation. The percentage inhibition of protein denaturation may be calculated as follows:

Inhibition percentage = 100-(Test solution ABS-Product control solution ABS)/Control test solution ABS

2.5.2. Anti-hemolytic activity (HRBC Membrane Stabilization Method)

Different extracts of *A. campestris* were tested for their anti-inflammatory effects on HRBC membranes *in vitro* using a spectrophotometric method (Yang, Z.G., *et al*. 2005). A healthy person's blood was drawn into EDTA vials and centrifuged at 1000 rpm for 5 minutes. Afterwards, the pellet was resuspended in saline solution after being rinsed three times in PBS (0.2 M; pH 7.4; 0.5%). Extracts (0.25–1 mg/ml in PBS) were added to erythrocyte solution and incubated for 20 minutes at room temperature. The resulting mixture should be supplemented with a 2% NaCl buffered saline solution. After 10 minutes of centrifugation at 1000rpm, the samples' absorbance at 540 nm was measured with a spectrophotometer. As a positive control, phosphate buffer saline was used. To figure out the percentage of hemolysis inhibition in each extract, we used this formula:

Inhibition percentage = (Ac-Aext/Ac) x100

A_{ext} was the absorbance of the extract or standard (Aspirin at 100µg), whereas A_c was the absorbance of the negative control.

2.6. In vitro evaluation of anti-coagulant activity

The extracts' anticoagulant activity was determined using the (Brummel-Ziedins et al. 2005) and (Pawlaczyk et al, 2009) techniques. External and internal clotting systems were tested using the activated partial thromboplastin time (aPPT) and prothrombin time (PT). The tests were performed on a fully automated coagulometer. It is made up of plasma that has been taken from healthy, untreated adults who have normal or similar TQ and TCK. Each volunteer's blood was injected into a solution of sodium citrate at a concentration of 3.2% and at a rate of one volume for every three volumes of blood that was drawn. After centrifuging the blood for 10 minutes at 3000 rpm, plasma devoid of platelets is obtained.

2.6.1. Assessment of the anticoagulant extrinsic pathway

The extrinsic pathway of coagulation's factors may be examined globally utilizing a fast coagulation test (TK) or a prothrombin time (PT). For this, 90 µl of standard plasma was mixed with 10 µl of each extract at various doses of 0.5–12 mg/ml produced in DMSO. Coagulation was started by adding 200 µl of thromboplastin that had been preincubated at 37°C for 15 minutes. The clotting time was tested once again using a coagulometer (Brummel-Ziedins et al. 2005).

2.6.2. Assessment of the anti-coagulant intrinsic pathway

The Kaolin Cephalin Time (TCK) is a test that allows the activity of endogenous plasma factors to be explored. It was evaluated according to the following steps: The platelet-poor plasma was incubated at 37°C for 15 minutes with 10µl of each extract at different doses of 0.5–12 mg/ml produced in DMSO. After 3 minutes at 37°C, the mixture was treated with a Kaolin cephalin solution. Then, 100 µl of an aqueous solution with 0.25 M $CaCl_2$ was added. The mixture was incubated for another 3 minutes before coagulation started. The time until the clot formed was then measured visually using the stopwatch (Pawlaczyk et al. 2009).

2.7. Statistical evaluation

All measurements were replicated three times, experimental results are expressed as the mean±SD. The IC50 values were determined through linear regression analysis.

3. Results

3.1. Phytochemical Analysis

The presence of major chemical groups such as

flavonoids, saponins, tannins, alkaloids, reducing compounds, and coumarins was discovered in *A. campestris* L. species by approaches based on solubility tests, staining, and precipitation reactions. The presence of these active phytoconstituents may be responsible for biological activities and therefore be used as a source for future pharmacological research.

3.2. Extraction yield, total polyphenols and flavonoids contents

The percentage yields of dry plant material extraction, and even the total phenolic and flavonoid amounts, are described in Table 1. The gallic acid and quercetin calibration curves were used to figure out how many phenolic and flavonoid compound were in *Artemisia campestris* extracts.

	Petet	*EtocH*	*BuOH*
Extraction yield (w/w %of dry weight)	8.33± 0.22	3.33± 0.09	2± 0.12
Total phenolic content (µg EAG/mg)	47.25± 3.12	312.32± 10.22	468.74± 11.2
Flavonoid content (µg EQ/mg)	3.7± 0.8	11.3± 1.4	74.91± 5.1

Table 1: Yield of extraction and total polyphenols and flavonoids contents of extracts.

3.3. Antioxidant Activity

As indicated in figures 3, 4, 5, and 6, antioxidant activity of *A. campestris* extracts was demonstrated when tested for free DPPH, H_2O_2 scavenging activities, and the FRAP assay.

3.3.1. DPPH radical-scavenging activity

According to the data presented in figure 1, the percentage of reactive oxygen species inhibition increases with increasing concentration, either for ascorbic acid or for the various plant extracts. From the results presented in figure 2, the IC50 obtained for the n-butanolic fraction (9,1±0,09µg /ml) is much lower than those of ethyl acetate (18,7±0,13µg /ml) and petroleum ether extracts (42,5±0,21µg /ml) and even for ascorbic acid (11,2±0,23µg /ml) used as a reference molecule, and thus, a very high antioxidant activity.

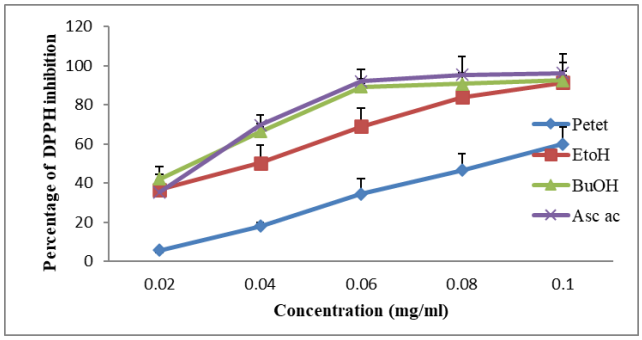

Figure 1: Percentage of DPPH radical inhibition as a function of extract concentration.

3.3.2. Hydrogen peroxide scavenging

The IC50 values for H_2O_2-scavenging activity ranged from 43.9±0.16 to 47.4±0.3µg /ml in the extracts, while the value for ascorbic acid was 31.7±0.147µg /ml (figure 2).

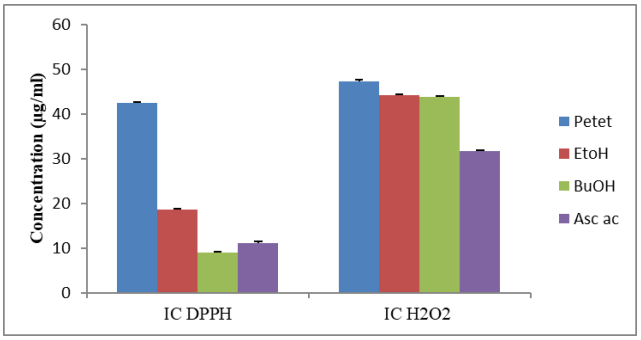

Figure 2: IC50 values of DPPH and H_2O_2 scavenging activity.

In a concentration-dependent way, all *Artemisia campestris* extracts scavenged hydrogen peroxide (figure 3).

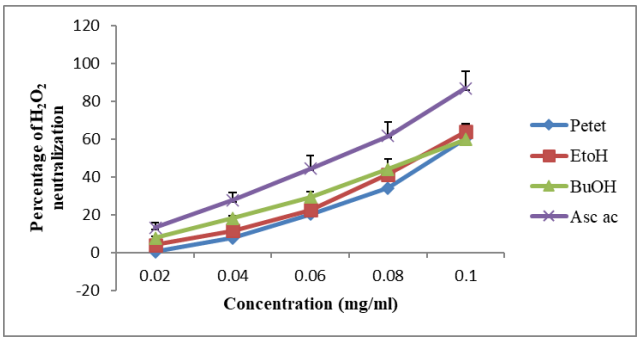

Figure 3: Percentage of H_2O_2 neutralization as a function of extract concentration.

3.3.3. Ferric Reducing Antioxidant Power (FRAP)

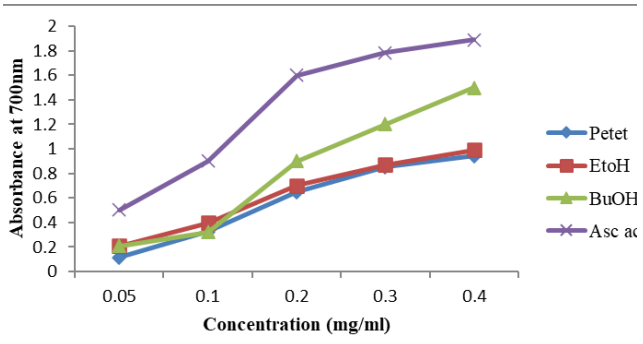

Figure 4: Extracts' reducing efficiency according to the FRAP test.

According to the results given in Figure 4 of the extracts' reduction potency, the n-Butanolic

fraction has a moderate reducing power equivalent to that of ascorbic acid.

3.4. Anti-infammatory activity

3.4.1. Bovine Serum Denaturation Method

Figure 5 shows that the n-butaolic fraction of *A. campestris* at varied doses (50,100, and 250 µg/ml) gave excellent protection against protein denaturation, equivalent to diclofenac sodium at the same concentration.

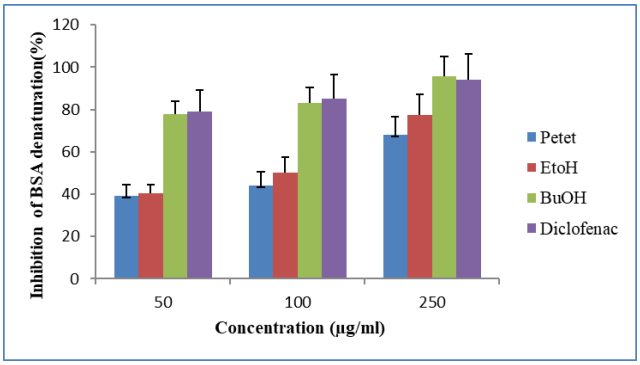

Figure 5: Effect of *A.campestris* extracts and diclofenac on BSA denaturation.

3.4.2. Anti-hemolytic activity

At doses ranging from 100µg/mL to 400µg/mL, all *A. campestris* extracts protect human erythrocyte membranes from hypotonic solution-induced lysis. At a concentration of 100 µg/ml, Petet extract had the lowest percentage inhibition of red blood cell hemolysis (8.07%), compared to 38.84% produced by aspirin at the same concentration (Figure 6). This is what they found: The BuOH fraction can stop the hemolysis of HRBCs in a dose-dependent way, with a very high significance of up to 83.97%.

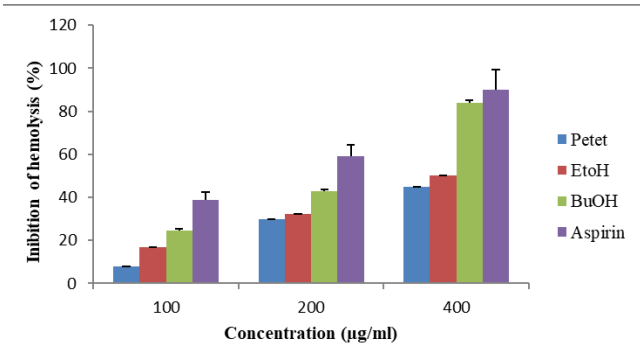

Figure 6: Antihemolytic effect of *A. campestris* fractions in comparison with a positive control (aspirin).

3.5. In vitro evaluation of anti-coagulant activity

PT and aPTT were used to assess the anticoagulant activity of *Artemisia campestris* L. extracts *in vitro* for both exogenous and endogenous pathways (Figure 7,8). The anticoagulant activity of the tested material is confirmed by a longer clotting time when compared to the control.

3.5.1. Evaluation of the anti-coagulant extrinsic pathway

The anticoagulant activity of the samples against exogenous coagulation was assessed using a coagulation assay called prothrombin level or prothrombin time (PT). Figure 7 shows the results, which reveal that PT was significantly prolonged at most of the concentrations tested (1.5, 3, 6, and 12 mg/ml) when compared to the control. If you take butanol at all concentrations, it has a much longer time for your blood to clot than the other extracts.

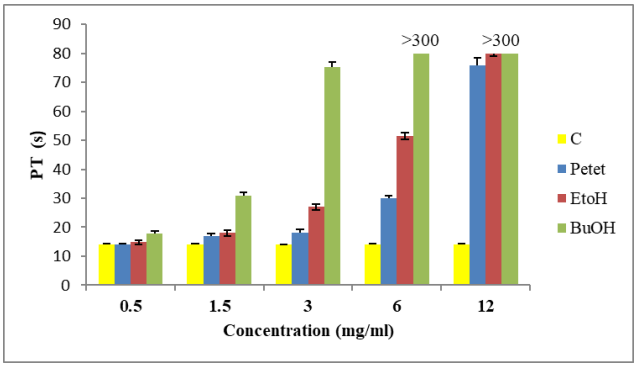

Figure 7: Prothrombin time of normal human plasma treated with *A.campestris* fractions at different concentration.

3.5.2. Evaluation of the anti-coagulant intrinsic pathway

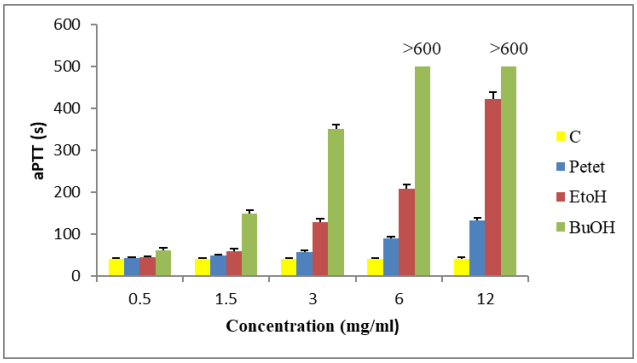

Figure 8: Activated partial thromboplastin time of normal human plasma treated with *A. campestris* fractions at different concentration.

4. Discussion

The Algerian steppe is depicted as a huge regional strip ranging from the Tunisian border to the Moroccan border, stretching over 1000 km long and 300 km wide and covering a total area of 20 million hectares. The aromatic plants of the family *Asteraceae* (*Compositae*) constitute by far the most

important the most important family in our territory (Djellouli, 1990). The present study focuses on valuing *Artemisia campestris*, a species of this native family. The phytochemical investigation revealed the existence of therapeutically effective phytoconstituents such as flavonoids, saponins, tannins, alkaloids, reducing compounds and coumarins, However, saponins and coumarins were absent in the identical plant gathered from the Djelfa region (Saihi, 2011). The method of extraction, the nature of the solvent, the particle diameter of the sample, the storage time and circumstances, the existence of storage conditions, the presence of interfering compounds and/or a delipidation phase, all have an impact on the extraction of polyphenols from plants (Chaudhary et al., 2015). The use of water followed by fractionation against polar solvents may favor the extraction of polar chemicals such as polyphenols and, therefore, affect the biological activity of the sample. More polar chemicals were obtained using aqueous extractions under reflux followed by liquid-liquid fractionation. Extraction yields ranged from 2 to 8.33% on average. Ethyl acetate is used to get mono O-glycosides and some di O-glycosides, but n-butanol is used to get the most polar flavonoids (di-o-glycosides, triglycosides, and tetra-glycosides). The colorimetric Folin-Ciocalteu technique, one of the ancient methods for determining the amount of phenolic compounds in medicinal herbs and foods (Blasa et al. 2007). In the Folin-Ciocalteu technique, gallic acid is the most frequently used reference (Maisuthisakul et al. 2008). However, the n-butanolic fraction presents the highest content of 468.737±83.049 µg EAG /mg compared to the other fractions, ethyl acetate and petroleum ether, with 319±50.119 and 47.25±3.12 µg EAG /mg respectively. In a study on eleven medicinal herbs, including *Artemisia campestris*, Djeridane et al. (2006a) calculated flavonoids concentrations in 70% and 80% (v/v) ethanolic extracts to be 7.46 and 5 mg ER/g, respectively. When the extraction is performed with a 50% alcoholic solution, this concentration can exceed 450 mg GAE/g of extract (Akrout et al., 2011). This difference in content can be explained by the geographical location of the plant species, its maturity, genetics, climate, harvesting period, drying conditions, metabolite content of each species (metabolism), and the nature and polarity of the solvent used for extraction or fractionation (Iloki-Assanga et al., 2015). Bennour et al.(2020) found that evaporating the solvent in an oven at 40 °C was the optimal evaporation method for extracting phytochemicals from *M. oleifera* leaves, utilizing a cold maceration method with 70% aqueous methanol as the solvent. The phenolic compound group is one of the most extensively dispersed and ubiquitous groups in plants, flavonoids, tannins, and phenolic acids are the most common phenolic elements found in plants as antioxidants (Pobłocka-Olech et al., 2016). The aluminum trichloride method is used to quantify flavonoids, with quercetin serving as the standard. Flavonoids are the most important polyphenolic class, with over 5000 chemicals already reported (Gomez-Caravaca et al., 2006). The n-butanolic extract has the highest flavonoid content (74.91±5.1µg EQ/mg of extract), followed by the ethyl acetate extract (11.3±1.4µg EQ/mg), and finally the petroleum ether extract (3.7±0.8µg EQ/mg). Djeridane et al. (2006a, 2007b) determined the concentration of flavonoids in ethanolic extracts at 70% and 80% (v/v), estimated at 7.46 and 5 mg ER/g respectively. However, Akrout et al. (2011) estimated this value to be 56.31 mg ER/g extract when extraction is performed with a 50% alcoholic solution. While Saoudi et al. (2010) found a content of 131.89 mg RE/g aqueous extract of the leaves, Djeridane et al. (2006a) discovered that flavonoids account for 98 percent of total polyphenols in the aerial part of plants, which is not surprising given that the aerial part is important for solar radiation protection (Ryan et al., 2002) and that this group of phenolic compounds is involved in the coloration of leaves and flower petals (Havsteen, 2002 and Gervaise, 2004). Today, there is a great deal of interest in examining the antioxidant activity of plant extracts or foods to discover whether they have therapeutic characteristics, which is one of the most important criteria for drug development (Ghani et al., 2019). By reducing excess free radicals, antioxidants are needed as an additional defense mechanism for our bodies to heal conditions such as aging, cancer, inflammation, cardiovascular and neurological diseases (Moskovitz et al., 2002). Antioxidant testing may be divided into methods based on electron transfer processes and those based on hydrogen atom transfer reactions due to the diversity of antioxidants and their reactivity The scavenging ability of physiologically relevant oxidants has been tested in several ways (Huang et al., 2005 and Hayouni et al., 2007). DPPH (2,2-diphenyl-1-picrylhydraza) radical scavenging is one of the in vitro techniques used, and it is effective,

simple, and predictable, and it may sequester free radicals (Fabri et al., 2011). A lower IC50 value indicates that an antioxidant's ability to scavenge free radicals is more active (Chen et al., 2013). As a control, ascorbic acid was utilized, the n-butanolic fraction of *A. campestris* showed the greatest free radical scavenging activity in the present study. Numerous investigations have revealed that *A. campestris* essential oils have good antioxidant activity by scavenging DPPH radicals (Gherib, 2009 ; Saihi, 2011 ; Dib et al., 2017b). According to Boudjouref (2011), the ethanolic extract of *Artemisia campestris* has the most active antiradical activity of the three. The search for new anticancer agents by the SRB method revealed the activity of methanolic extract of *A. campestris* stems against some lineages responsible for epithelial ovarian cancer (EOC), which are (OVCAR-4, FOUV-1, and COV-362), where normal epithelial cells (HOE) and carboplatin (CBPT) were used as negative and positive controls, respectively (Maafia, 2019). According to our findings, the antiradical activity of the extracts is depending on their total polyphenol and flavonoid concentration. Indeed, phenolic elements, particularly flavonoids, are known as potential antioxidants that may accumulate radical oxygen atoms and reactive oxygen forms (Javanovic et al., 1994). Flavonoids have a low redox potential, which allows them to be thermodynamically able to reduce free radicals by transferring hydrogen atoms from hydroxyl groups to free radicals, this makes them scavengers (Douaouya and Bouzerna, 2016).

In the body, hydrogen peroxide may be transformed into oxygen and water and reactive species (-OH) can be produced that may lead to ADN damage even if hydrogen peroxide itself is not a highly reactive compound. The elimination of H_2O_2 from food systems is thus of paramount importance (Rj et al., 1989). As a result, plant extracts' ability to scavenge hydrogen peroxide was investigated to see if they showed the same pattern of activity as the ability to reduce OH radicals. In a concentration-dependent way, *Artemisia campestris* fractions scavenged hydrogen peroxide. This H_2O_2 piégeage can be attributed to their ability to give H_2O_2 electrons and therefore neutralize it in water (Ebrahimzadeh et al., 2009). Furthermore, another technique for testing antioxidant activity was the Ferric Reducing Antioxidant Power (FRAP) method, which is based on the presence of reductant compounds in the extracts and causes the complex Fe3+/ ferricyanure to be reduced to ferrous form, allowing the amount of polyphénols involved in the redox reaction to be determined (Amarowicz et al., 2004, Bougandoura and Bendimerad, 2012). In fact, n-butanolic extract outperformed the other extracts. This might be explained by its high content of phenols and flavonoides, which play a key role in the Fenton reaction's chélation of transition metals (Valko et al., 2007).

Inflammation is caused by the denaturation of proteins, which leads to their loss of function (Nargund et al., 1993). As a result, Alzheimer's disease as well as rheumatoid arthritis may be treated with medications that limit denatured protein aggregation production and protein condensation (Saso et al., 2001). The capacity of *A. campestris* fractions to suppress protein denaturation has been examined in this context. In comparison to the other fractions, the n-butanolic fraction was shown to be the most efficient, and the inhibitory effect of BSA on thermal denaturation was dose-dependent. The outcomes of this extract are comparable to those of sodium diclofenac, a popular anti-inflammatory prescription. Inflammatory disorders may be caused by denaturation of proteins *in vivo*, which might lead to the production of autoantigens. A possible process of denaturation is the modification of electrostatic, hydrogen, hydrophobic, and disulfide linkages, which retain the three-dimensional structure of proteins (Chatterjee et al., 2012). Polyphenols in *Artemisia campestris*, like flavonoids and tannins, help make it an anti-inflammatory food (Sangeetha, 2011). The most abundant cells in the human body are erythrocytes, which possess several biological and physical properties and thus have been frequently used for drug delivery (Kumar, 2011). Toxic substances such as hypotonic liquid may injure red blood cells (RBCs), heat, methyl salicylate, or phenylhydrazine, the membranes lyse, causing hemolysis and hemoglobin oxidation (Feirrali et al., 1992). In the HRBCs membrane stabilization experiment, the anti -inflammatory effect was dose related. The BuOH fraction elicited a stronger response, with a percentage reduction in red cell hemolysis equivalent to that of aspirin. The erythrocyte membrane was stabilized by all *A. campestris* extracts examined, particularly n-butanolic, which inhibited hypotonia-induced lysis. To minimize the inflammatory response, stabilizing the lysosomal membrane, which is comparable to the erythrocyte membrane, is essential. This is because it stops

activated neutrophils from releasing lysosomal components such as lytic enzymes and active mediators of inflammation, which cause tissue damage upon their extracellular release (Yurugasan et al., 1981; Vadivu and Lakshmi, 2008). On the other hand, hypotonicity-induced hemolysis may result from cellular shrinkage caused by osmotic loss of electrolytes and intracellular fluid components. The extract has the ability to inhibit or accelerate the flow of these intracellular components (Yang et al., 2010). Flavonoids and tannins, alone or in combination, may be responsible for the impact of the anti-inflammatory extract/fraction (Sudharshan et al., 2010). Coagulation was proven to be a crucial lethal component throughout the COVID-19 pandemic's progression (Ackermann et al., 2020 and Schulman, 2020). Determining the active ingredient in plants might also help researchers find safer alternatives to anticoagulants. In this context, utilizing prothrombin and activated partial thromboplastin times, the effects of *A. campestris* fractions on human blood coagulation have been examined *in vitro*. In this study, a prolonged aPTT was seen at all concentrations of extracts. This means that these extracts may have an inhibitory or deficient effect on intrinsic pathway coagulation factors (Hireche et al., 2021). The n-butanolic fraction was shown to be the most potent extract. It is possible, however, that this extract might have an effect on factors Xa and Va, or on the interaction between thrombin and the fibrinogen substrate, which would prevent the formation of thrombosis. An inhibitor of proteases has been found in *A. campestris*, limiting the production of active factors such as Xa, Va, and andthrombin from zymogens in the organism (Félix -Silva et al., 2014). The anticoagulant pathway may be stimulated by the binding of the active agent to antithrombin III, producing a conformational shift in protein C (PC), resulting in the activation of protein C (APC), which inhibits factors Va and VIIIa (factor Va and VIIIa cofactors) with its cofactor (protein S). Anticoagulants may be found in secondary metabolites generated by plants (Luo et al., 2017).

5. Conclusion

To conclude, the antioxidant, anti-inflammatory, and anticoagulant properties of the n-butanolic fraction of the *A. campestris* aerial part were found. As a result, it might be a valuable natural resource for lowering the effects of oxidative stress, which can lead to inflammatory and cardiovascular disorders. Furthermore, the results of this study are preliminary, and they are just the beginning of the search for physiologically active natural compound. As a consequence, this research generates a wide range of experimental perspectives. In reality, more research is needed in a number of areas, including more efficient separation techniques (HPLC, LC/MS, GC/MS...) for qualitative characterization of flavonoids and evaluating and testing these active compounds *in vivo* for additional biological features in the context of pharmacological and industrial applications.

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REGULAR ARTICLE

Phytochemical characterization and biological activities of butanolic extract of aerial parts of *Tetraclinis articulata* from Algeria

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ABSTRACT

Tetraclinis articulata is a forest plant species that is widely used in North African folk medicine due to its immense bioactive potential. The objective of the present study was to characterize flavonoids present in butanolic extract of aerial part of *T. articulata* (TABE) and to test its antibacterial synergistic effect, anticoagulant and antioxidant properties. The analysis of the TABE by colorimetric tests and TLC revealed the presence of flavonoids. The dosage showed that the proportions of total flavonoids and flavonols were 19.25 ± 0.07 and 13.6 ± 1.6 mg QE/g DW, respectively. Besides, the greatest antibacterial activity of the extract was shown against *S. aureus.* In contrast no effect was observed against *E. coli*. Furthermore, the most *synergistic potential* inhibitory effect was noted in the case of *E. coli*, when the extract was combined with penicillin. In addition, the MICs was determined used microdilution method, the values obtained were ranged from 2.5 to 20 mg/ml. Moreover, the *in vitro* anticoagulant activity was assessed by PT and KCT tests. The results showed interesting anticoagulant activity, with KCT value of 55.4 s which is superior to that obtained by the anticoagulant drug –Lovenox- (39.37s). The antioxidant activity was evaluated *in vitro* using DPPH• method. The result expressed in terms of IC50 was found to be 0.04 mg/ml. To conclude, the TABE constitutes a valuable source of anticoagulant, antioxidant and antibacterial synergism metabolites, which makes it a potential candidate to isolate responsible flavonoids and to develop novel pharmaceutical formulations.

1. Introduction

Tetraclinis articulata (Vahl.) is an evergreen small

Figure 1: Pictures of *T. articulata* species with their different organs: (A) Whole tree, (B) Tree bark and (C) Cones.

coniferous tree (height: 6-8 m) (Figure 1), that belongs to the *Cupressaceous* family. It is endemic species of Northen Africa, predominantly in the three Maghreb countries (Algeria, Morocco and Tunisia), as it covers approximately 1 million hectares. Yet, it is also found in other parts of Europe such as Malta and Spain (Montanari, 2014; Bourkhiss et al., 2007).

Due to the existence of numerous valuable bioactive phytochemicals, the various parts of this tree are widely used in folk medicine. The leaves and twigs are used mainly to treat the, respiratory and intestinal infections, stomach pain, rheumatism, diabetes and arterial hypertension (Jouad et al., 2001). Besides, the seeds are also used for diabetes, hypertension, childhood fevers and anti-diarrheal (Ziyyat et al., 1997). However, the whole plant, including branches with leaves, cones and resins, has been used for skin illness (Bourkhiss et al., 2007).

Prior studies proved some biological properties of its essential oils such as antibacterial and antifungal (Bourkhiss et al., 2007), cytotoxic (Buhagiar et al., 1999) and anti-inflammatory (Djouahri et al., 2014). Another study showed that the crude aqueous extract of *T. articulata* twigs induces the endothelium dependent relaxation of the isolated rat aorta (Zidane et al., 2014).

Thus, this plant may be used as an alternative natural solution to treat various diseases and avoid the harmful side effects of synthetic antibiotic, anticoagulant, and antioxidants drugs.

Since their discovery, antibiotics have been indispensable in the treatment of bacterial infections, but over many years of use, the efficacy of these antibacterial agents has been reduced due to their side effects and the emergence of antibiotic- resistant bacteria (Lee and Warren, 2014; Levy and Marshall, 2004). On the other hand, sometimes, the use of single antibiotic does not produce the desired inhibitory effects. Therefore, to overcome this, a combination of two or more antibacterial agents often lead to an efficient strategy in treating infectious diseases caused by resistant microorganisms.

Moreover, anticoagulant drugs are used for the short term treatment of arterial and venous thrombotic disorders and for the long-term prevention of recurrences. The existing anticoagulants agents consist of heparins (like lovenox), vitamin K antagonists (such as Warfarin), and their derivatives, which correspond to principle drugs used in clinical practices. Most of these synthetic anticoagulants have harmful life-threatening side effects, like thrombocytopenia and immune suppression, after a long-term heparin application (Juliana et al*.,* 2014).

The antioxidants can scavenge free radicals, which are recognized as principle causes of various chronic and degenerative diseases such as cancer and cardiovascular problems. Currently, the use of synthetic antioxidant molecules is being questioned due to potential toxicological risks (Shakeriet al., 2012).

In Algeria, *T. articulata,* commonly known as sandarac Gum tree and Thuya of Barbary, is the most popular herb used traditionally to treat various diseases. It is located essentially in the western region of the country where it covers around 160 000 hectares (Benabid, 1977).

As most studies on *T. articulata* in Algeria have focused on the essential oils, the present work, to the best of our knowledge, is the first to test the biological activities of its butanolic extract that is obtained from the aerial parts.

The main objective of the present work is to determine the flavonoids (one of the largest and most widespread group of plants secondary

metabolite) composition of butanolic extract of aerial parts of *T. articulata* originated from Algeria, and to evaluate its antibacterial synergistic effect, anticoagulant and antioxidant activities.

2. Materials and methods

2.1. Drugs and chemicals

In the present work, three drugs were used; penicillin (6µg) and ofloxacin (5µg) for the antibacterial activity, and Lovenox (20 mg) for the anticoagulant activity. All other chemicals used in the current work were purchased from (Sigma-Aldrich) (USA) and Fluka Chemie (Buchs, Switzerland).

2.2. Tetraclinis articulata fractionation

T. articulata was collected from Zakor, 800 meters above sea level and located at 35°23'59" North and 000°08'24" East in the wilaya of Mascara in North west Algeria. The taxonomic identification was performed by Pr. Belgharbi Benamer (Mustapha Stambouli University, Mascara- Algeria) and Pr. Righi Kada (Ibn Khaldoune University, Tiaret - Algeria).

Fresh aerial plant parts (stems, leaves and flowers) were first air-dried in shade at room temperature of 25°C; then mechanically powdered and sieved. 1000 g of the obtained powder were macerated during 24 h at room temperature in a mixture of distilled water–methanol (3:7, v/v). The resulting crude preparation was filtered three times and concentrated, under reduced pressure, in a rotary evaporator, at 55 °C to obtain the crude methanolic extract. This crude extract was dissolved in water and the resulting aqueous phase was extracted with butanol. All bioactivity tests, in the present study, were carried out using *T. articulata* butanolic extract (Markham, 1982).

2.3. Qalitative analysis of flavonoids

2.3.1. Phytochemical tests

Phytochemical analyses were performed to test the presence of flavonoids, anthocyanins, flavonols, flavons and flavonons (Paris and Moyse, 1969; El Haci et al ., 2012).

For ***flavonoids*****,** a few drops of AlCl3 (1%) were added to 5 ml of TABE. The apparition of a yellow color indicated the presence of a targeted molecule. On the other hand, the formation of a pink-red color, after mixing 2 ml of TABE, 2 ml of HCl (2N) and few drops of NH4OH, showed the presence of ***anthocyanins***. Concerning, ***flavonols*****,** 0.5 ml of the concentrated HCl were added to 5ml of TABE. This mixture was then incubated between 80-90 C° for 30 min, so the appearance of a red-purple color confirmed the presence of flavonols. For ***flavons*** test, a few drops of KOH were added to 1ml of TABE. The appearance of an orange color indicated their presence. Finally, for the presence of ***flavonons***, a few drops of FeCl3 were added to 1ml of the extract. The positive result was indicated by the formation of a red purple color.

2.3.2. Thin Layer Chromatography (TLC) analysis

TLC principle depends on molecules solubility and consists in a mixture separation of polar, non polar, and mid polar compounds from the extracts on a stationary phase (silica gel) and in a mobile phase which is generally a mixture of solvents, adapted to the type of targeted substances. The movement of compounds from the mixture relies on their physical properties, molecular structure, and functional groups (Bele and Khale, 2011). Briefly, 2µl-5µl of TABE were deposited at 1.5 cm from the bottom of TLC plate (20 x 20 cm) using a capillary spotter. Five different solvent systems specific to flavonoids were used in this investigation:

System 01: Ethyl acetate -Formic acid - Glacial acetic acid - Distilled water (100:11:11:26,v/v/v/v);

System 02: Butanol - Acetic acid -Distilled water (04:01:05, v/v/v);

System 03: Butanol - Acetic acid -Distilled water (02:03:05, v/v/v);

System 04 : Acetone - Distilled water (1:1, v/v).

At the end of the solvent migration, spots were visualized separately under UV at 365 nm. The frontal ration was calculated using the following formula:

Frontal ration **= distance travelled by a compound / distance travelled by a solvent**

The corresponding frontal ration values with the color of each spot may give preliminary information on a detected flavonoid structure.

2.4. Quantitative analysis of flavonoids

2.4.1. Dosage of total flavonoids

The total flavonoids concentration in TABE was determined by a spectrophotometer, using a

method based on the formation of a complex "flavonoid–aluminium" whose absorbance is maximum at 430 nm. 1ml of diluted TABE was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm, in triplicate. The calibration curve was prepared by preparing quercetin solutions at different concentrations from 0 to 40 µg /ml in methanol. The total flavonoids content was expressed in mg per g of quercetin equivalents per g of dry weight (mg QE/ g DW) (Bahorun et al., 1996).

2.4.2. Dosage of flavonols

The content of flavonols was determined by the method described by Kumaran et al. (2007). 0.25 ml of the extract (prepared in methanol) were mixed with 0.25 ml of AlCl3 (2 mg/ ml) and 1.5 ml of sodium acetate (50 mg/ ml). The absorbance was measured at 440 nm after 2.5 h, in triplicate. The content of flavonols was expressed as mg of quercetin equivalents per g of dry weight (mg QE/ g DW).

2.5. Biological activities of TABE

2.5.1. Antibacterial activity

2.5.1.1. Disc diffusion on Muller-Hinton agar

To assess the antibacterial activity of TABE and its synergistic effect with antibiotic drugs, five bacterial strains were used: two Gram positive; *Staphylococcus aureus* (ATCC 25923) and *Listeria monocytogenes* (ATCC 7644), and three Gram-negative; *Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC 700603)* and *Pseudomonas aeruginosa (ATCC 27853)*. All the tested bacteria were obtained from the Institute of Nutrition Food and Agro-Food Technology of Constantine (INATAA), Algeria. The antibacterial activity of the TABE was carried out by the disc diffusion method on Muller-Hinton agar as recommended by Bauer et al. (1966).

First, the TABE was dissolved in dimethyl sulfoxyde (DMSO) at a concentration of 100 mg/ml. Then, sterile Wattman paper n°3 discs (6 mm in diameter) were impregnated with the TABE (10 µl per disc). On the other hand, 100 µl of bacterial inoculums prepared in sterile saline water (equivalent to a 0.5 Mc Farland standard) were spread over Petri dishes containing Mueller Hinton agar.

Finally, the discs prepared previously were transferred aseptically on the media surfaces and discs impregnated with DMSO used as negative controls. The Petri dishes were incubated for 24 h at 37 °C, and the experiments were performed in triplicate. Bacterial strains presenting inhibition zones diameters (D) less than 8mm, 9 ≤D ≤ 14mm; 15≤D ≤ 19mm; and D>20mm , were considered respectively; resistant (-), sensitive (+), highly sensitive (++), and extremely sensitive (+++).

2.5.1.2. Determination of TABE minimum inhibitory concentrations

The determination of TABE minimum inhibitory concentrations (MICs) was carried by employing a micro-dilution method described by Oussou et al. (2004). 90 µl of broth Mueller-Hinton medium was distributed in a sterile 96-well plate. Binary serial dilutions of TABE dissolved in DMSO were performed to get concentrations ranging from 40 mg/ml to 0.3125 mg/ml. Then, 100 µL of each dilution were distributed in the three first wells of plate, which were seeded after wards with 10 µl of microbial suspension to reach, consequently, a final volume of 200 µl of preparation. Control tests correspond to wells containing exclusively a mixture of a culture medium and a microbial suspension, whereas sterile control tests wells contained exclusively culture medium. On the other side, a positive control wells contained the antibiotics -Ofloxacin (5µg) or Penicillin (6µg) (100 µl each), 90µl of sterile Mueller-Hinton, and 10 µl of microbial suspensions. Plates were incubated for 24 hours at 37°C.

Bacterial growth (expressed as turbidity) was examined in each well. The MICs values were defined as the lowest concentration of TABE, which completely inhibited microbial growth. The results were expressed in milligrams per milliliter.

2.5.1.3. Synergistic effect of TABE with antibiotics against bacterial strains

The cumulative antibacterial effect of TABE with Penicillin and Ofloxacin was tested by the disk diffusion method on Muller-Hinton agar as described above (Bauer et al., 1966). Standard discs of antibiotics were impregnated with 10 µl of TABE, and transferred aseptically on the surface of Muller -Hinton agar medium inoculated previously by bacterial inoculums. The inhibition zones diameters were recorded after incubation at 37ºC/24h.

The synergistic effect was evaluated by comparing the inhibition zones diameters in Petri dishes

containing discs impregnated with the mixture of antibiotics and TABE to control Petri dishes containing discs exclusively impregnated with tested antibiotics**.** The test was performed in triplicate and the results were expressed as mean ± standard deviation.

2.5.2. In vitro anticoagulant activity of TABE

In the present study, the anticoagulant effect of TABE were tested for both intrinsic and extrinsic anticoagulation pathways using plasma, collected from blood samples of normal individuals by using the test of Prothrombin time (PT) or Quick time (QT) and the test of Kaolin-Cephalin time (KCT), as described by Rizzo et al. (2008). The principle of the two tests was based on prolongation of clotting time of the plasma, when it was incubated with a reagent necessary to allow clotting to proceed, and the compound which inhibits blood coagulation, as compared to the control (no inhibitor added) as stated by Visioli et al. (1999) and the anticoagulant activity was expressed in term of clotting time in seconds.

2.5.2.1. Preparation of plasma pool

Six blood samples were collected from healthy volunteers, using sterile syringes. Then, these samples were anti-coagulated using 3.2% tri-sodium citrate in a polypropylene container (3volumes of blood to 1 volume of tri-sodium citrate solution). Preparations were immediately centrifuged at 3000 rpm for 10 min and plasma samples were pooled and stored at 4°C until their use.

2.5.2.2. Intrinsic pathway (Kaolin -Cephalin Assay)

The inhibitory effect of the TABE extract on the intrinsic pathway of a coagulation cascade was determined using KCT. Briefly, 90 µl of plasma were mixed with 10 µl of TABE prepared in DMSO (0.5 mg/ml), and incubated for 15 min at 37°C. Then, 100 µl of kaolin -cephalin reagent were added and the mixture was re-incubated for 3 min at 37°C. The clotting time was immediately recorded with a chronometer just after the addition of 100 µl of calcium chloride CaCl2 (0.025M) used to initiate the coagulation. Plasma alone without the TABE was tested to determine the normal KCT. The solvent DMSO and the anticoagulant drug Lovenox (20mg) dissolved in DMSO were used as a negative control and a positive control; consecutively. Each experience was carried out in triplicate and data were expressed as means +/- standard deviations.

2.5.2.3. Extrinsic pathway (Prothrombin time assay)

TABE extract inhibitory effect of the coagulation extrinsic pathway cascade was determined using PT test or QT test. 10 µl of TABE dissolved in DMSO (0.5mg /ml) were added to 90 µl of plasma. The mixture was incubated at 37°C for 15 min. Next, 200µl of thromboplastin reagent (pre-incubated at 37°C for 15 min before use) were added to the mixture in order to neutralize the sodium citrate and initiate the coagulation.

The PT was recorded with a timer which was stopped as soon as the clot formation began. Plasma alone without the extract was tested to determine the normal PT; the solvent DMSO alone as a negative control, and the anticoagulant drug Lovenox (20mg) dissolved in DMSO as a positive control. Each experience was carried out in triplicate and data were expressed as means +/- standard deviations.

2.5.3. Antioxidant activity

The DPPH• (1-diphenyl-2-picryl hydrazyl radical) was used for the determination of free radical scavenging activity (RSA) of the TABE extract. Dilutions of TABE extract in methanol were prepared to obtain solutions with concentrations ranging from 0.005 to 0.1 mg /ml. Then, 15 µl of these TABE dilutions were added to 1.5 ml of a 100 µM DPPH• (dissolved in methanol). The mixtures were shaken vigorously and incubated in the dark for 15 min. After that, the reduction of DPPH• absorption was measured at 517 nm.

At the same time, a blank solution of DPPH• with methanol were screened to estimate the DPPH• decomposition during the time of measurement. The experiment was repeated 3 times. Quercetin was used as standard control. The percentage of free radical scavenging activity was calculated as below:

(Blank OD – Sample OD / Blank OD) x 100

The activity of TABE was determined in terms of IC50 value which denotes the concentration of a sample required to scavenge 50% of DPPH• free radicals (Koleva et al., 2002).

2.6. Statistical analysis

All assays here were performed in triplicates and results were expressed as means ± standard deviations. *In vitro* antioxidant assay data were

analyzed with computerized Graph pad prism 5 software to determine the IC50 value.

3. Results and discussion

The qualitative phytochemical analysis is an essential step for the discovery of new drugs as it provides the information regarding the presence of a particular secondary metabolite in a Phytotherapeutic plant extract (Samah et al., 2016). The results of phytochemical analyses carried in this work, on TABE revealed the presence of flavonoids including different classes like, flavonols, flavones and anthocyanins. However, no trace of flavonons has been detected, which explain the use of *T.articulata* in traditional medicine (Fatima Zahra et al., 2019).

The results of phytochemical analyses found in the present study accord with the finding of Wahiba et al. (2018) which confirmed that *T. articulata* is a good source of flavonol such as quercetin. In another study, Herzi et al. (2013) reported the presence of anthocyanins on the leaves extract from *T. articulata.*

In fact, the different classes of flavonoids identified in TABE are known to be biologically active compounds and they possess different pharmacological properties. It has been used by humans for many therapeutic purposes; they act as strong antioxidants by their ability for protecting the human body against the ill-effects of oxidative stress, anti-inflammatory, antibacterial, anti-cancer and anti-allergic activities (Kim et al., 2003; Vermaa and Pratap, 2010 ; Konga et al., 2003).

The different classes of flavonoids identified in TABE indicate the necessity of their separation through suitable chromatographic techniques. In the present study, the results of thin layer chromatography using different solvent systems revealed the presence of promising spots as shown in (Table 1).

Solvent system	Spot no	Colors under UV light	Rf values
1	01	Yellow Orange	0.81
	02	Orange	0.93
2	01	Yellow orange	0.20
	02	Yellow	0.54
3	01	Yellow- green (brown)	0.67
	02	Blue	0.77
4	01	Yellow	0.41

Table 1: RF values and colors of TABE spots detected by TLC under 365nm UV light, using four different solvent systems.

TLC flavonoids analysis giving different Rf values in different solvent systems may help in understanding their polarity in addition to the selection of appropriate solvent efficient in pure compounds separation (Gujjeti et al., 2013). On the other hand, the presence of numerous fluorescent colors such as orange, yellow, blue and green on the chromatograms under UV at 365 nm (Table 1) are characteristics of flavonoids (Mamyrbékova-Békro et al., 2008).

Indeed, the yellow spots confirm the presence of flavonols. While the yellow-green (brown) color is due to the presence of flavone glycoside, the appearance of blue spots indicate the existence of anthocyanidins-3-glycosides, methylated isoflavones, flavanones, chalcones and methylated flavones (Gwatidzo et al., 2018).

This richness in flavonoids of the TABE forced their quantification. The total flavonoids content in TABE was estimated in terms of quercetin equivalents (QE) per gram of dried extract at 19.25 mg (Figure 2). This value is more important than that reported by Meryem et al. (2016), which estimated about 11.78 mg QE/ g DW in water extract of *T. articulata* leaves.

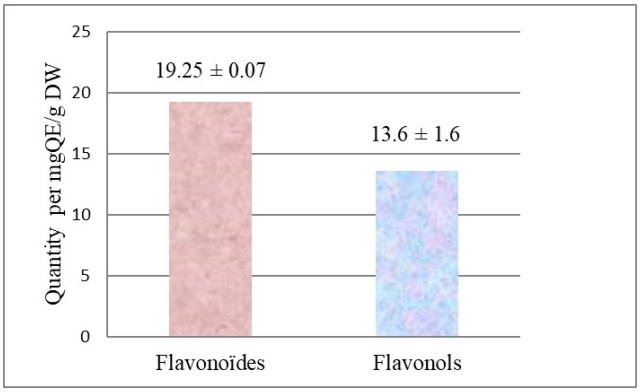

Figure 2: Total flavonoid and flavonols contents of TABE.

The difference in flavonoid content in the same plant species may be due to plant growth conditions such as soil, geographic location, organ developmental conditions, degree of maturity, and genetic differences, in addition to the extraction method and the nature of solvents used (Agata et al., 2009). It is important to signal that the contents of flavonols in terms of quercetin equivalents (13.6 QE/g DW) in TABE were quantified, in this study, for the first time for *T. articiculata* plant species.

Three main biological activities of TABE were studied in the present work: anticoagulant, antioxidant, and antibacterial synergistic effects.

Results of the antimicrobial activity of TABE, *in vitro*, on Muller-Hinton agar, showed no activity against *E. coli* while positive effect with inhibition zones diameters (mm) of 13.5, 10, 12.5, and 11.5, against *S. aureus L. monocytogenes, P. aeruginosa and K. pneumonia*, respectively, was found (Figure 3 and 4). Fouad et al. (2015) showed that the essential oils of *T. articulata* were active in all the pathogenic strains tested; *S. aureus*, *E. coli*, *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa* and *C. albicans*. In another study, the crude aqueous extract, ethyl acetate and butanol fractions of *T. articulata* leaves exhibited a good activity against all the tested bacterial strains especially *E. coli, M. morganii,* and *P.aeruginosa* (Wahiba et al., 2018).

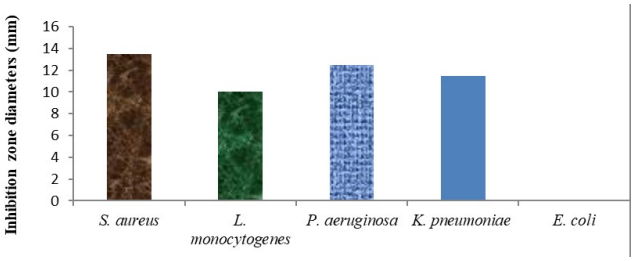

Figure 3: Inhibition zones diameters of TABE against some pathogenic bacteria.

The observed differences concerning *E. coli* may be explained by the dissimilarity in the chemical composition of various plant parts.

The antimicrobial mechanisms of plant extracts are versatile. In fact, flavonols explained specific interactions such as establishing hydrogen bridges with proteins from cell walls or enzymes, metal ion chelation, inhibition of bacterial metabolism, and the sequestration of substances necessary for the bacteria growth (Nuria et al., 2003).

On the other side, results of TABE MICs determination showed that *L. monocytogenes* was the most sensitive bacterium, with the MIC of 2.5 mg/ml, followed by *S. aureus* and *K. pneumonia* (5 mg/ml). While the lowest effect was noted against *P. aeruginosa* with 20 mg/ml MIC value, in a study of Fouad et al. (2015), the MIC of essential oil from fresh and dried leaves of *T. articulata* against *E. coli, K. pneumonia, P. mirabilis, P. aeruginosa, S. aureus and C. albicans,* ranged from 64.5 and 516 µg/ ml.

Demeterio et al. (2015) demonstrated that the MICs of crude ethanol extracts from 12 Philippine medicinal plants against methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus,* extended spectrum b-lactamase-producing, carbapenem-resistant *Enterobacteriaceae* and metallo b- lactamase-producing, *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* varied between 19-625µg/ml, exception with ethanol extract from the *P. guajava* leaf which showed no any activity against the Gram-negative tested bacteria.

Furthermore, Oussou et al. (2004) found that MICs of *Psidium guajava Linn*. stem extracts against

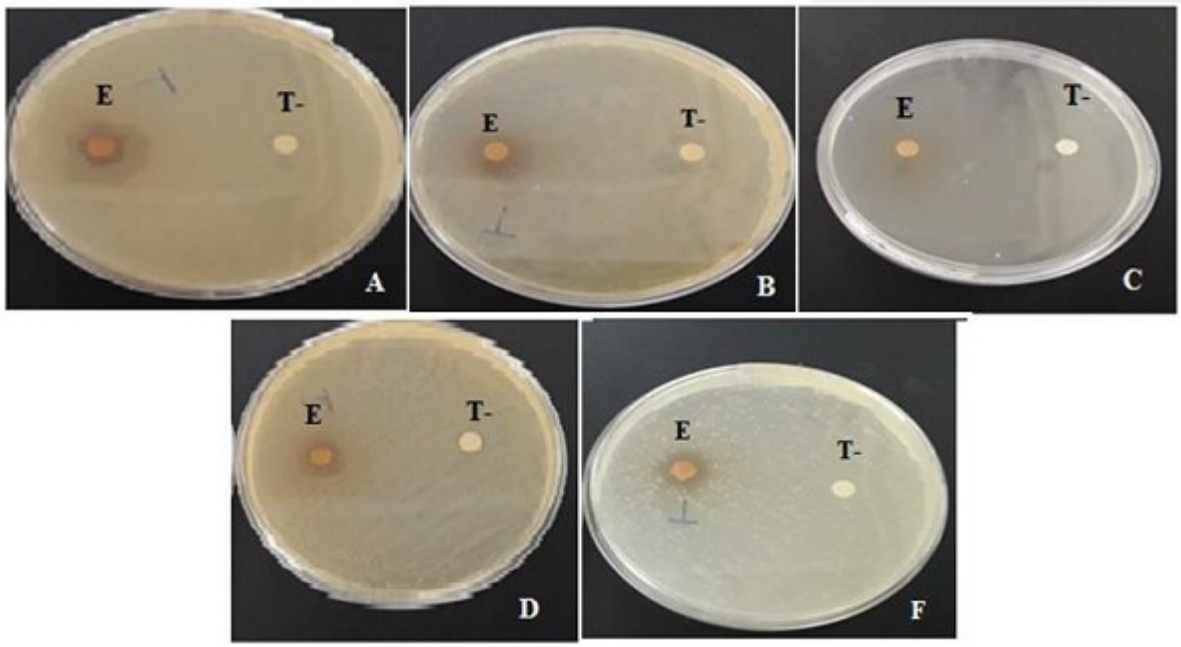

Figure 4: Antibacterial effect of TABE (E) against bacterial strains: *P. aeruginosa* (A), *S. aureus* (B), *E. coli* (C), *L. monocytogene* (D), *K. pneumonie* (F).
Note: (e): Extract, (t-): Negative controls

Bacterial strains	TABE concentration (mg/ml)							
	40	20	10	5	2.5	1.25	0.625	
S. aureus	-	-	-	-	+	+	+	Bacterial turbidity
L. monocytogenes	-	-	-	-	-	+	+	
P. aeruginosa	-	-	+	+	+	+	+	
K. pneumoniae	-	-	-	-	+	+	+	

Table 2: Minimum Inhibitory Concentration (MIC).

methicillin-resistant *S. aureus* ranged between 1250–5000 µg/ml and 625–2500 µg/ml for aqueous and methanol extracts, respectively. These results were almost conformed to those obtained in the current study. The slight difference in MICs values is due to the use of several compounds found in various extracts.

The inhibition effect produced by the combinations between TABE and antibiotics against bacterial strains tested in the present work, to study their synergistic effect. Results from this part has shown that this combination was antagonistic in the case of *L. monocytogenes* (21*vs*. 23mm for ofloxacin and 16 *vs.*17 mm, for penicillin), and *E. coli* (14 *vs.*25 mm for ofloxacin) (Figure 5 and Table 3). In contrast, all combinations were partially synergistic, notably, *S. aureus,* (20 vs. 14 mm for penecilin), *P. aeruginosa* (23 vs 11.5; and 22 vs 20 mm, for penicillin and ofloxacin, respectively) and *K. pneumonia* (18vs 10; and 31vs 25 mm, for penicillin and for ofloxacin, respectively). On the other hand, a potential synergistic inhibitory effect was noted in the case of *E. coli* (13 vs. 0 mm, for Penicillin).

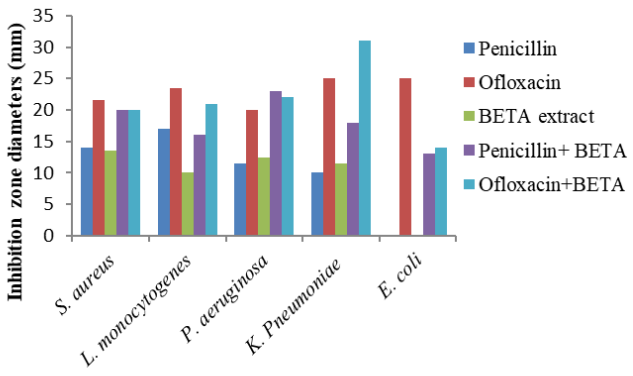

Figure 5: The comparison between inhibition zones diameters produced by the antibiotics, TABE and their combination, against bacterial strains.

Bacterial strains	Zone inhibitions diameters (mm)					Effect
	TABE (100 mg/ml)	Antibiotics (5µg for Ofloxacin and 6µg for Penicillin)		Combination between TABE and antibiotics		
S. aureus	13.5	Penicillin	14	20	*Partial synergistic**
		Ofloxacin	21.5	20	*Partial synergistic*
L. monocytogenes	10	Penicillin	17	16	Antagonistic**
		Ofloxacin	23.5	21	Antagonistic
P. aeruginosa	12.5	Penicillin	11.5	23	*Partial synergistic*
		Ofloxacin	20	22	*Partial synergistic*
K. pneumoniae	11.5	Penicillin	10	18	*Partial synergistic*
		Ofloxacin	25	31	*Partial synergistic*
E. coli	0	Penicillin	0	13	*Potentialsynergistic ****
		Ofloxacin	25	14	Antagonistic

Table 3: Inhibition Zones diameters of TABE, antibiotics and their combinations against *P. aeruginosa*, *S. aureus*, *E. coli*, *L. monocytogene*, *K. pneumonie.*
Note: *Partial Synergistic: the effect of the two agents is less than the sum of the effects of agents simultaneously (the overall effect < the effect of agent A + the effect of agent B); ** Antagonistic: the simultaneous effect of two agents leads to the partial (reduction) or complete (cancellation) inhibition of the action of one of them; * Potential Synergistic: the effect of the two agents simultaneously is greater than the sum of the effects of agents separately. The overall effect > the effect of agent A + the effect of agent B.**

The study of Dawoud et al. (2013) suggested that the synergistic effect occurred between *Rehum* methanol extract and different antibiotics (Gentamycin, Ceftasidine, Tobramycin, Cefoperazone and Spictinomycin) have shown a potential antibacterial effect against *S. aureus* and *A. xylosoxidans*. In addition, Liu et al. (2011) concluded that flavonoids such as biochanin A (BCA) displayed synergistic activity in combination

with the antibacterial agent CPFX against *S. aureus*.

Furthermore, several studies in literature demonstrated that when β-lactam antibiotics were synergized with plant extract, it displayed diverse antimicrobial mechanisms such as the inhibition of bacterial enzyme located on the cell membrane, inhibition of cell growth, inhibition of ribosomal protein synthesis, and inhibition of bacterial cell wall synthesis (Dawoud et al., 2013; Aronson and Meyler's, 2010).

Importantly, it has been noted that the synergistic effect of plant extracts is exploited to reduce antibiotics doses, and, consequently, reducing undesirable side effects, mainly antibiotic resistance (Olgica, 2018).

The *in vitro* anticoagulant activity of TABE was evaluated by KCT and PT or QT chronometric tests to detect endogenous and exogenous pathways, respectively. A prolonged clotting time compared to the negative control reflected the anticoagulant activity of the tested extract (Visioli et al., 1999).

The coagulation processes via intrinsic pathway are activated by the contact between factor XII and the Kaolin activator (a 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 (Renné et al., 2006).The results of TABE anticoagulant activity by this pathway are shown in figure 6. Control (14.3 s) and negative control (13.96 s) serum samples showed KCT values within the normal range (12-14 s). However, TABE has shown a clotting time prolongation (55.4 seconds), which is superior to that obtained by the positive control (Lovenox -anticoagulant drug-: 39.37 s). These results indicate that butanolic extract of *T. articulata* may carry an anticoagulant property correlating with the intrinsic coagulation processes. (Awad and Binder, 2005) reported that the prolongation of KCT can be due to the presence of coagulation factors inhibitors.

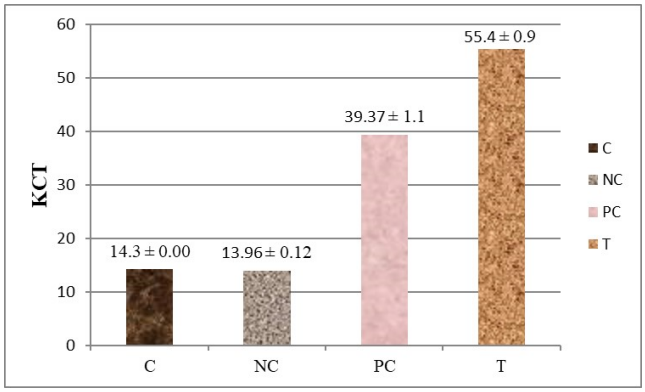

Figure 6: Results of anticoagulant activity via the intrinsic pathway Kaolin Cephalin Time.
Note: (C): control, (NC): Negative Control, (PC): positive control, (T): Test

Natural anticoagulant agents are of potential interest for primary prevention of cardio vascular diseases. Furthermore, Bijak et al. (2011) demonstrated that extracts from the dried fruits of *A.melanocarpa* and *V.vinifera* seeds rich in polyphenols has a dose-dependent anticoagulant effect with KTCs of 42.6 and 41.0 s respectively for a concentration of 50 µg/ml of extract, which is probably due to the richness of these extracts in flavonoids, in particular flavanols and flavonols.

In the current work, the prolongation of KTC was 4 times superior to that of the control, which may be due to the presence of numerous flavonoids especially flavonols whose presence was investigated by phytochemical screening tests. These metabolites are strong anticoagulant molecules (Amira et al., 2017) and they are able to inhibit the endogenous pathway factors of coagulation (Athukorala et al., 2006; Zhang et al., 2008).

Consequently, *T. articulata* flavonoids extract can be used as a supplementary anticoagulant agent to improve and/ or prevent cardiovascular diseases. Further, findings from the present research will be useful to start new studies whose main objectives are to identify anticoagulant potent molecules, to determine their mode of action, and finally to produce new anticoagulant drug.

On the other side, the coagulation process through the extrinsic pathway explored the VII factor and the common factors (X,V, II and fibrinogen) of blood coagulation, where the tissular factor thromboplastin is the initiator (Pawlaczyk et al., 2011). Results of the anticoagulant extrinsic pathway test carried here are shown in figure 7. The TABE anticoagulant activity via the extrinsic pathway give a PT equal to 14.67s, which is near to that of the negative control and the control (15.33s and 13.3s respectively). Therefore, the extract tested does not have a remarkable anticoagulant activity via this pathway.

Guglielmone reported that the anticoagulant activity of flavonoids may be due to their inhibitory action on the intrinsic pathway of coagulation and not on the extrinsic pathway, which corresponds to

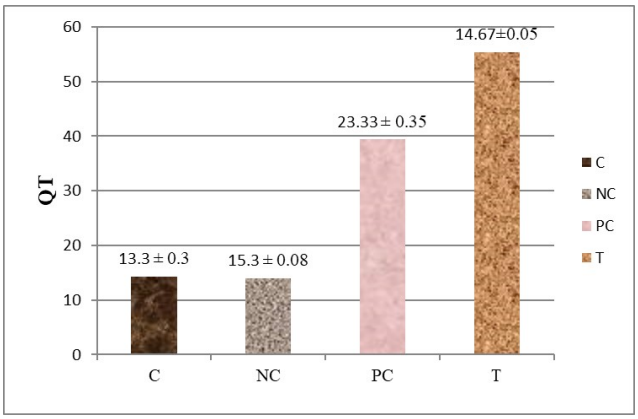

Figure 7: Results of anticoagulant activity via the extrinsic pathway.
Note: (C): control, (NC): Negative Control, (PC): positive control, (T): Test

our findings (Guglielmonea et al., 2002).

Finally, the TABE antioxidant effect at different concentrations (0.1 – 0.05- 0.02- 0.01 and 0.005 mg/ml) was studied spectrophotometrically (517nm), based on the reduction of the DPPH• radical in to DPPH-H (a-a-diphenyl-b-picrylhydrazine), which is accompanied with the change of the color from the purpule (the radical form) to the yellow (reduced form) in the presence of the antioxidant agent (Cai et al., 2003). The obtained results are given in table 4 and figure 8. These results revealed an increase in the percentage of inhibition as a function of the TABE concentrations. The IC50 of TABE is 0.04 mg/ml,

Concentrations of TABE (mg/ml)	% of DPPH• reduction by TABE
0.1	83.58±0.72
0.05	80.68±1.18
0.02	46.35±1.03
0.01	26.23±2.09
0.005	5.71±0.08

Table 4: DPPH• radical reduction percentage by TABE.
Note: Data were expressed as means ± SD.

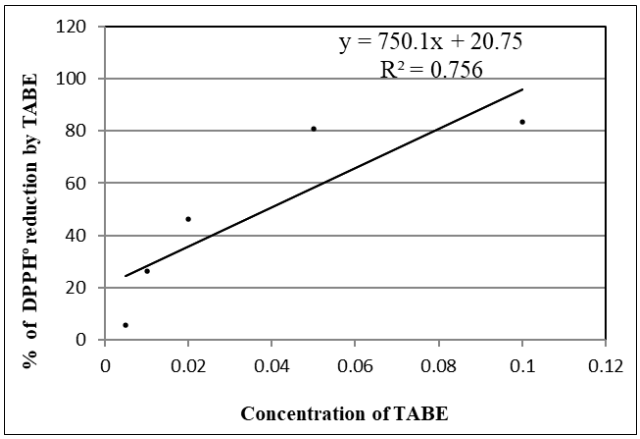

Figure 8: DPPH• radical reduction percentage as function of TABE concentration.

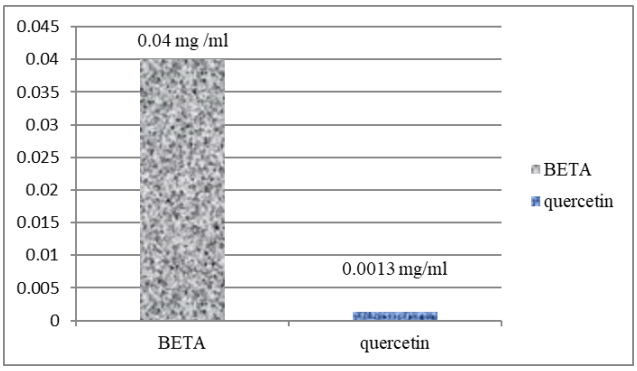

Figure 9: IC50 (mg/ml) values of BETA and quercetin for RSA by DPPH•.

according to the graph mentioned on (Figure 9). However, quercetin IC50 calculated in our previous study was around 0.0013 mg/ml.

The antioxidant activity of plant extracts is associated with its medicinal values (Iqbal et al., 2015). The lower IC50 value corresponds to a higher antioxidant activity, as mentioned in the study of Mariem et al. (2013), which revealed an efficacy in DPPH• radical scavenging by *thuja* from Tunisia with an IC50 of 0.005, and the study of Samira et al. (2016) in which it is observed a strong antioxidant capacity of hydro-methanolic extract of *T. articulata* with IC50 of 0.023 mg/ml.

Comparing IC50 values from literature to that obtained in the present work confirmed that butanolic extract from *T. articulata* displayed a greater antioxidant potential activity with other medicinal plants. For example, our result is more effective compared to the study by Hutadilok-Towatana et al. (2006) that recorded an IC50 value in the order of 0.164 mg/ml from the crude extract of lime peels. Also Bakasataea et al. (2018) found an IC50 value in the order of 0.126 mg/ml of butanolic extract for the wood of *Albizia myriophylla.*

The ability of flavonoids to scavenge free radicals is explained by their chemical structures that comprise a large number of hydrogen atoms, hydroxyl groups and phenyl rings, which would be able to capture free radicals by demobilizing their single electrons (Calliste et al., 2001;Wojdyło et al., 2007). In fact, significant correlations were also found between the antiradical DPPH• activity and levels of proanthocyanidins, flavonols and orthodiphenols (Montoro etal., 2006; Ayoola et al., 2006). The TABE contain the majority of these flavonoids like, flavonols, flavones and anthocyanins, which all of them, were able to scavenge DPPH• radical by their hydrogen donating

ability.

4. Conclusion

Teteraclinis articulata aerial parts butanolic extract (TABE), tested in the current study, revealed the presence of flavonoids incluing flavonols and flavones, known to be responsible for many biological activities. TABE showed a remarkable antibacterial activity against *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa and Klebsiella pneumonia*, in addition to its synergistic inhibitory effect when it was combined with Penecillin and Ofloxacin. Importantly, the most interesting inhibitory synergistic potential was found in *E. coli*, when TABE was combined with penicillin. On the other hand, TABE proved also considerable antioxidant and intrinsic anticoagulant activities. Thus, results from this study could give scientific evidence for the *T. articulata* popular use in ~~a~~ traditional medicine. Therefore, this plant might be used in the future as a promising source or producing therapeutic bioactive molecules.

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