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Evaluation of putative cytotoxic activity of crude extracts from *Onopordum acanthium* leaves and *Spartium junceum* flowers against the U-373 glioblastoma cell line

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Abstract: Crude hydromethanolic (80% methanol) extracts produced by maceration of *Onopordum acanthium* leaves and *Spartium junceum* flowers were tested for cytotoxic effects against glioblastoma U-373 tumour cells. *Onopordum acanthium* extract was found to be ~5 times more cytotoxic than *Spartium junceum* (IC_{50} values of 309 and 1602µg/ml, respectively). Similar to most chemotherapeutic agents killing through the intrinsic pathway, *Onopordum* killed the cells via apoptosis, which was confirmed by the activation of caspase-3. *Spartium* exerted its weak cytotoxic effect, presumably by a caspase-independent, non-apoptotic form of necrotic-like programmed cell death. *Onopordum acanthium* is considered a promising plant for the researchers investigating putative biological activities, particularly antitumour and immune-related activity.

Keywords: *Onopordum acanthium*, *Spartium junceum*, glioblastoma U-373, cytotoxicity, apoptosis.

INTRODUCTION

Most chemotherapeutic agents cause tumour cell death by inducing apoptosis and resistance to chemotherapy is believed to be due to a series of events (that may include mutations) that ultimately lead to the suppression of the apoptosis control mechanisms (Cotter, 2009). Apoptosis has typical hallmarks that distinguish it from other forms of programmed cell death, and is characterized by chromatin condensation into complete or apparently compact globular or crescent-like geometric fig. DNA fragmentation, cell membrane blebbing (zeiosis), cytoplasmic shrinkage, phosphatidylserine exposure and most importantly, activation of caspases - particularly caspase-3 (Kerr *et al.*, 1972; Leistand Jaattela, 2001; Saraste and Pulkki, 2000). Apoptosis occurs *via* two pathways: extrinsic (initiator caspases-8 & -10) and intrinsic (initiator caspases-9 & -2), and it is believed that the latter pathway is involved in the cytotoxic effect of the chemotherapeutic agents. Briefly, in the intrinsic pathway, the mitochondria release cytochrome *c,* which has a role in activating initiator caspases by dimerization, the initiator caspases (such as caspase-9) then activate executioner caspases (-3, -6, and -7) by interdomain cleavage (Boatright and Salvesen, 2003).

Onopordum acanthium (Scotch thistle) and *Spartium junceum* (Spanish broom) belong to *Asteraceae* (*Compositae*) and *Fabaceae* (*Legumenosae*), respectively the second and the third largest plant families. *O. acanthium* is a flowering biennial plant native to Europe and Western Asia with coarse spiny leaves 20-50 cm in width with conspicuous and spiny-winged stems (Stace CA 2010; Mucina L, 1989). *S. junceum* is a perennial leguminous shrub native to the Mediterranean region of southern Europe, southwest Asia and northwest Africa. *S.junceum* is cultivated as an ornamental plant, preferring sunny sites with dry and sandy soils and growing to a height of 2-4 m. It has thick succulent grey to green rush-like shoots with sparse small deciduous leaves and yellow flowers~1-2 cm in diameter (Treatment from Jepson Manual, 1993). In this study, crude hydromethanolic extracts of *O. acanthium* (OACE) leaves and of *S. junceum* (SJCE) flowers were tested for their putative cytotoxicity against the glioblastoma U-373 cell line.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin mixture, glutamine were supplied from Euroclone S.p.A. (Pero, Milano, Italy), trypan blue was from Gibco BRL (Carlsbad, CA, USA), phosphate buffered saline solution (PBS), and other general laboratory chemicals were obtained from Sigma-Aldrich S.r.l. (Milan, Italy).

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Identification and collection of plant material

Plants were identified by Professor Benayache Samir (Laboratory of Development of Natural Resources and Synthesis of Bioactive Substances, University Mentouri, Constantine, Algeria). The *O. acanthium* leaves were collected on June 12, 2012 from the wild plants in Khenchela (north east of Algeria) and the flowers of *S. junceum* were collected on June 20, 2012 from wild plants in the City of Constantine. The plant material was dried for several days at room temperature, ground and stored in a dry and dark place at 25ºC pending further processing.

Preparation of the crude extract

O. acanthium leaves (400g) and *S. junceum* flowers (300 g) were extracted by maceration as follows. The plant material was added to 1.5 L of a hydromethanolic solvent (8: 2 methanol: water ratio) and extracted for 24 hours at room temperature with continuous shaking. The solvent was evaporated using a rotavapor (Buchi R-200, Germany) and the recuperated solvent used to re-extract the plant material (twice for *O. acanthium* leaves and 3 times for *S. junceum* flowers). The particulate plant material was removed and discarded leaving the extract behind to be dried and used. The plant material was removed by filtration before the extracted material was dried; *O. acanthium* leaves yielded 3.5% and *S. junceum* flowers yielded 8% by weigh of the crude extract.

Stock solutions

Crude extracts were weighed and dissolved in dimethyl sulfoxide (DMSO, not exceeding 1%) to make a stock solution from which other dilutions were made. The following concentrations were screened for putative cytotoxicity: 100, 200 and 300µg/ml for *O. acanthium*, and 100, 200 and 400µg/ml for *S. junceum*, in addition to the control (0 µg/ml).

Cell culture

The human glioblastoma U-373 cell line was purchased from American Type Culture Collections (ATCC) (Rockville, MD, USA). The cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2mM) and penicillin/ streptomycin (100 U/ml, 100µg/ml) and were incubated at 37°C in humidified air with 5% CO_2.

Cell Treatment

Cells were seeded in six-well culture plates at a density of 4 x 10^4 cells/ml, and after a 12h attachment period (time 0), the culture medium was replaced with 2.0ml of fresh medium containing either *O. acanthium* extract (100, 200, 300µg/ml) or *S. junceum* extract (100, 200, 400µg/ml). Cells underwent biochemical evaluation 24h after treatment.

Cytotoxicity assay

The trypan blue dye exclusion test was used to quantify the effect of the crude plant extracts on the cells. Microscopically, dying cells are seen with blue cytoplasm (these cells cannot exclude the dye since the integrity of the cell wall has been adversely affected), while intact (living) cells have a clear cytoplasm. After 24 hours of treatment, cell viability was determined evaluated using a Photozoom inverted microscope (Optech GmbH, Munchen, Germany) connected to a digital camera (Coolpix 4500, Tokyo, Japan). The numbers of dead and viable cells were calculated from several randomly chosen areas of each well (Krischel *et al*, 1998).

The percent cytotoxicity was calculated as:

$$\text{Cytotoxicity}\,(\%) = \frac{\text{Number of dead cells}}{\text{Number of total cells (dead and viable)}} \times 100$$

The average from 3 trials of the percent cytotoxicity was plotted versus extract concentration. The IC_{50} values (the concentration that is cytotoxic to 50 % of the cells) were calculated from the equation of the graph with the best fit between y and x values.

Caspase-3 assay test

Caspase-3 activity in glioblastoma U-373 cells was evaluated to confirm the occurrence of apoptosis. Analysis was performed according to the manufacturer's protocol included with the Caspase-3 Colorimetric Activity Assay Kit (Catalog No. APT131, Millipore Corp. Billerica, MA, USA). The Colorimetric Caspase 3 Assay Kit is based on the ability of caspase 3to recognize and cleave a substrate containing the DEVD motif. Briefly, activated caspase 3 from lysed cells cleaves the para-nitro aniline (pNA)-DEVD substrate and pNAis quantified by measuring absorbance at 405 nm and comparing the results to a standard curve obtained from serial dilutions of the pNA standard provided in the kit. Values are expressed as relative optical density (OD)/100µg protein and converted into enzyme level-expressing values (U/ml). The latter were plotted versus the concentrations for both plant crude extracts.

Graphs and statistics

Data are expresses as mean ± SD. Graphs and histograms were plotted using Microsoft Office Excel 2007. ANOVA single-factor test (add-in for Microsoft Office Excel 2007) was used for evaluating the significance between the means of the control and the various concentrations of the extracts. Significance levels of 0.001 and 0.05 were selected for the cytotoxicity and caspase-3 assay tests, respectively.

RESULTS

Microscopic evaluation of cytotoxicity

Figs. 1 and 2 show light microscopy images of glioblastoma U-373 cells after 24 hours of incubation with various concentrations of *O. acanthium* crude extract

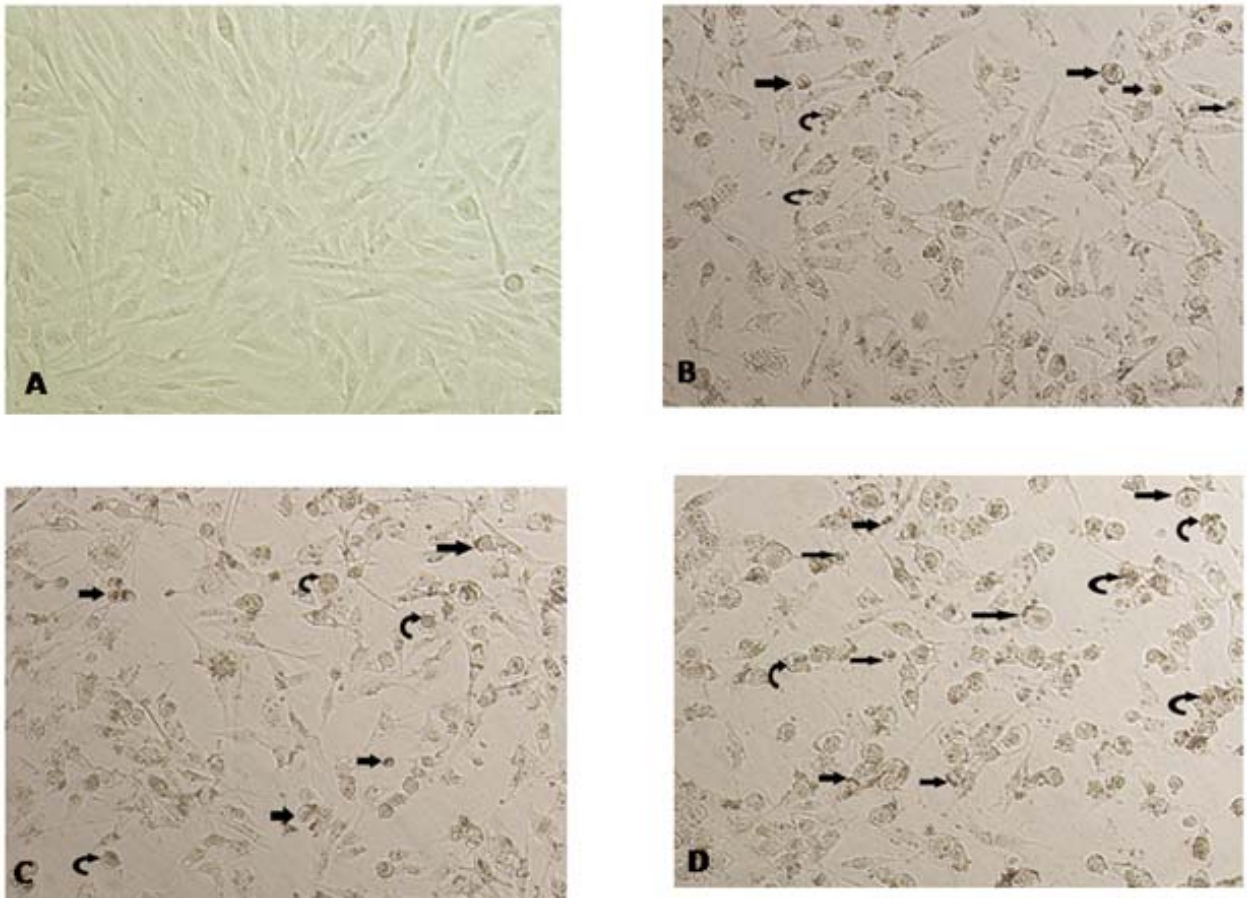

Fig. 1: Light microscopy images (Photozoom inverted microscope, Optech GmbH, Munchen, Germany) showing the cytotoxic (presumably apoptotic effect) of OACE on glioblastoma U-373 cells after a 24-hour incubation. A: Control (no treatment), B: Treatment with 100µg/ml, C: 200µg/ml and D: 300µg/ml. Solid straight arrow (➡) indicated presumed examples of apoptosis, while curved arrows (↶) indicated presumed examples of caspase-independent programmed cell death.

(OACE) and of *S. junceum* crude extract (SJCE), respectively. By examining the pictures, it can be noticed the formation of dark spots (condensed chromatin) in the dead cells as they could not exclude the dye, and consequently appeared dark cytoplasm. For OACE, the occurrence of condensed chromatin was extremely abundant compared to that of SJCE, indicating the several-fold cytotoxicity of OACE when compared to SJCE. Particularly for OACE, by comparing these geometric shapes of the condensed chromatin with those of documented apoptotic cells from the literature, it can be postulated that the cytotoxicity occurred via apoptosis. However, other apoptosis-like cell death forms are not precluded.

Quantification of extract cytotoxicity (trypan blue exclusion test)

The cytotoxic effects of OACE and SJCE were quantified using the trypan blue exclusion test for cell viability. The data for concentration-dependent cytotoxicity after the 24-hour incubation period are shown in fig. 3. The cytotoxicity of both plant extracts showed statistically significant differences from control (P<0.001 in one-way ANOVA test). OACE was ~5 fold more cytotoxic to the glioblastoma U-373 cells than the SJCE, with the IC50 for OACE and SJCE estimated at 309 and 1602 µg/ml, respectively.

Caspase-3 assay

In an attempt to elucidate a possible mechanism by which both plant extracts exerted their cytotoxic effects, caspase-3 activity (executioner caspase in the apoptotic process) was evaluated. OACE was found to exert its cytotoxic effect via activation of Caspase 3 (P<0.05), while the variation between control and treatments were not significant for SJCE, suggesting a possible caspase-independent programmed cell death. See fig. 4

DISCUSSION

Cancer remains a major worldwide health concern as the leading cause of death in economically developed

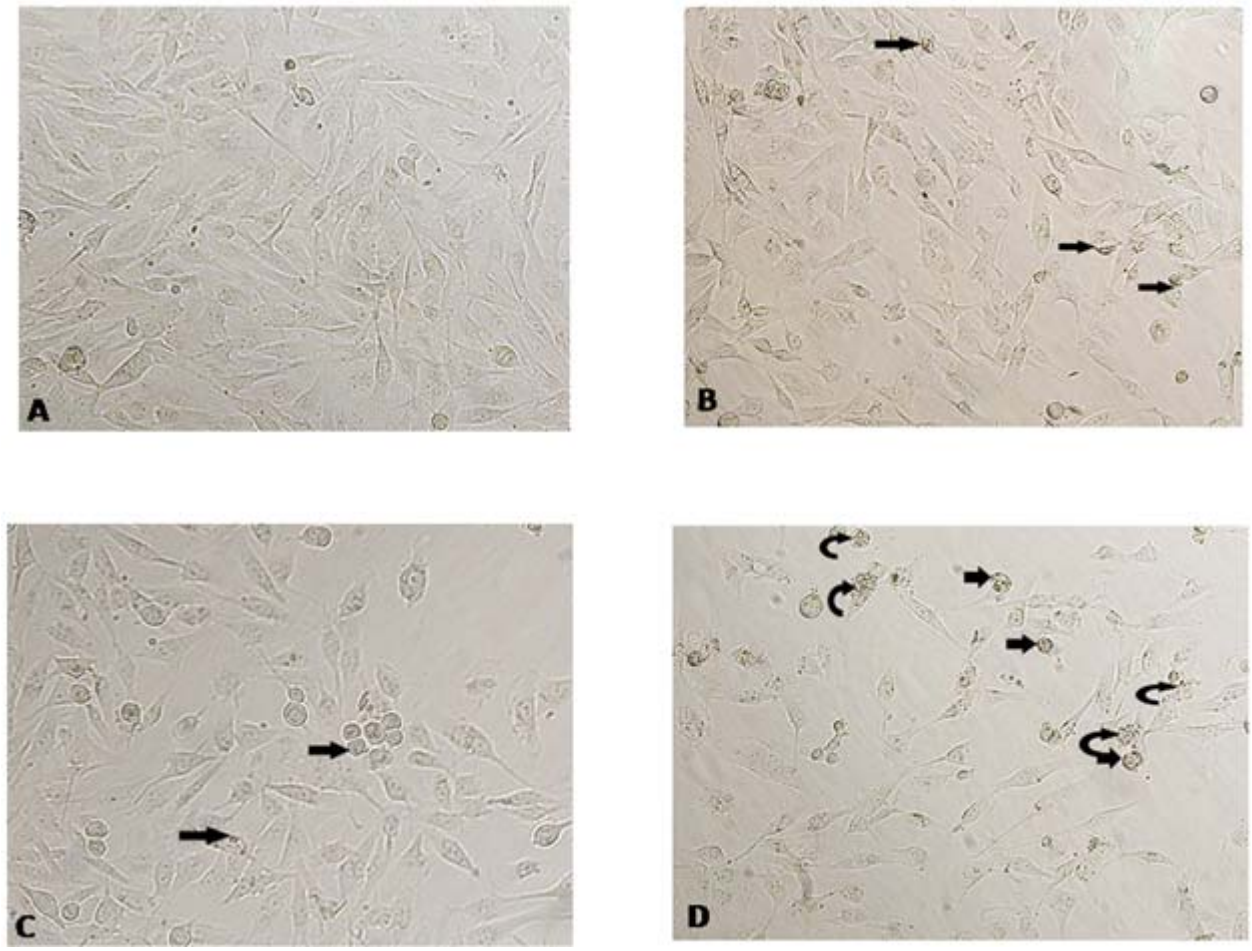

Fig. 2: Light microscopy images (photozoominvertite microscope, Optech GmbH, Munchen, Germany) showing the cytotoxic effect of the SJCE on glioblastoma U-373 cells after a 24-hour incubation. A: Control (no treatment), B: Treatment with 100µg/ml, C: 200µg/ml and D: 400µg/ml. The solid arrow (➡) points to slight chromatin condensation, whereas curved arrows (↶) denoted presumed nuclear fragmentation, which was more obvious at the highest used concentration (400µg/ml).

countries and the second leading cause of death in the developing countries (World Health Organization, 2008). Although several reports describe a general decrease in incidence rates and mortality which are attributed to increased knowledge of cancer prevention (the adoption of tobacco control programs, vaccinations for liver and cervical cancers, recognition of the effects a healthy diet and exercise), it is obvious that there are increasing trends for numerous types of the other diseases linked with gender, age group, ethnicity or geographical area. As an example, brain tumours demonstrate differing incidence and mortality patterns, though the rate has stabilized and mortality decreased owing to the methods of diagnosis and treatment (Legler *et al.*, 1999). In Canada, the incidence and mortality rates increase considerably for the elderly (above 65 years), though the sudden increase in the incidence rate could be ascribed to progress in diagnostic techniques and ease of diagnosis for glioblastomas (Mao *et al*., 1991). This elevated incidence of brain tumors was confirmed by a recent study from China where there is an increased rate occurring before the age of 40, and slowing thereafter. However, an increase in the incidence and mortality is expected during the next 20 years (Ke *et al*., 2012). Gliomas account for about 70% of all brain tumors of which glioblastomas account for the majority (65%). The incidence rate is stable and tends to higher rates in the developed and industrialized countries owing to good diagnostic and therapeutic capabilities, and more people have access to the medical care (Ohgaki & Kleihes, 2005).

Plants are a valuable source of pharmacotherapeutic agents that have been employed in treatment of diverse ailments, including cancer (Graham *et al*., 2000; Mans *et al*., 2000). The *Asteraceae* and *Fabaceae* are plants with diverse biological activities (including anti-cancer activity). For example, from the *Asteraceae*, 25 aqueous and organic extracts were found to possess cytotoxic activity *in vitro* (at least 50%) against one or more cell

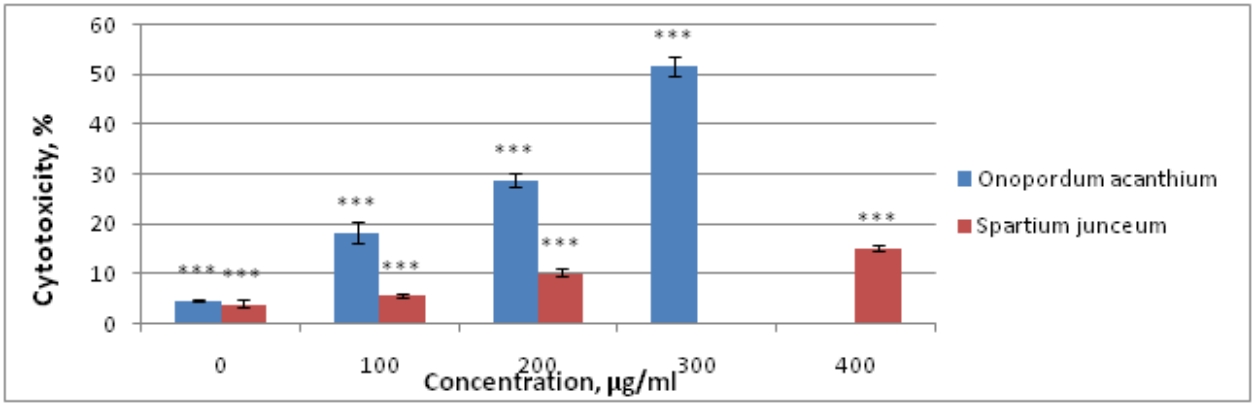

Fig. 3: The cytotoxic effects of OACE and SJCE in glioblastoma U-373 cells quantified using the trypan blue exclusion test of cell viability. Each bar represents the average from three trials. The results were significant for both plants. *** = P<0.001 (one-way ANOVA test).

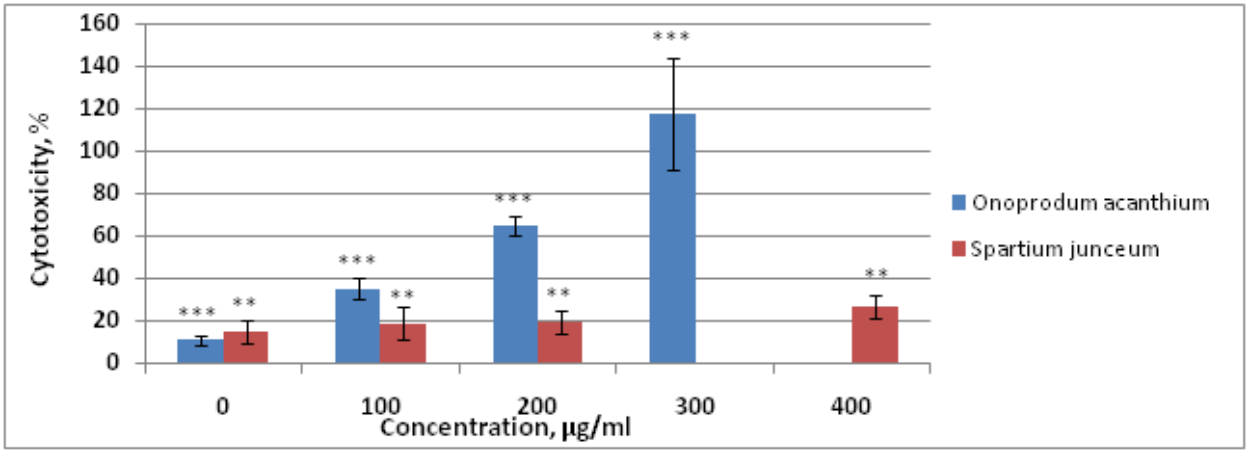

Fig. 4: Caspase-3 levels in glioblastoma U-373 cells treated with several concentrations of OACE and SJCE, and incubated for 24 hours. Each bar represents the average from 3 trials.
*** = p< 0.05, **= p>0.05 (one-way ANOVA test).

lines (Rethy *et al*., 2007). In another study, silibinin, a flavonoid from the milk thistle (*Sylibum marianum*, related to *O. acanthium*) demonstrated a dose-and time-dependent inhibitory effect on the invasion and motility, but not adhesion, of highly metastatic A549 cells in the absence of observable cytotoxicity (Chu *et al*., 2004). Nuclease from the mung bean sprout (*Phaseolus aureus,* a member of the *Fabaceae*) showed significant antitumor effects against human melanoma tumours following intratumoural or intraperitoneal administration (Soucek *et al*., 2006). Moreover, an aqueous extract of flowers from *Butea monosperma* (*Fabaceae*) had hepatoprotective and anti-cancer properties in a mouse model of hepatocellular carcinoma (Choedon *et al*., 2010).

Considered invasive plant species, neither *O. acanthium* nor *S. junceum* have been studied extensively for any putative biological activity. In our estimation, *O. acanthium*is a promising plant containing natural ingredients with biological activities (Auld BA, 1988; Briese DT *et al*., 2010). *In vivo* and *in vitro* studies showed that an aqueous extract of the plant stem and leaves exerted a stimulatory action on natural killer cells against YAC tumour cells derived from a virus-induced murine T cell lymphoma (Abuharfeil NM *et al*., 2001; Abuharfeil NM *et al*., 2000). In addition, OA flowers contain proteolytic and clotting activities useful in cheese making (Brutti *et al*., 2012). Potentially, such proteolytic activity may be utilized in future biochemical studies.

*S. junceum*has numerous uses in the folk medicine and was also studied for a variety of putative biological activities. This plant is one part of a Unani multicomponent herbal tea widely consumed in Syria (Carmona *et al*., 2005). In the folk veterinary medicine practiced in Maratea area of Basilicata, Italy, the plant is for the treatment of limb fractures of domestic animals (sheep, goats, dogs, cats, excluding cows) where bark is tied tightly to the fractured limb for 21 days (Guarrera *et al*., 2005). Butanol and aqueous fractions from the

methanolic flower extract have anti-ulcerogenic and protective effects in rats, with the final H_2O-fraction having the highest protective effect on the stomach. This activity was ascribed to a novel saponin known as spartitrioside (Yesilada *et al.*, 2000a). Potent antioxidant activity of the flavonoid-rich fractions from the flowers is also documented (Yesilada *et al.*, 2000b). In addition, a hexane extract fraction from the flowers demonstrated an anti-inflammatory effect in rats, while the methanolic extract and hexane fractions also had an analgesic activity in the test animals (Menghini *et al.*, 2006). Furthermore, the plant had a contraceptive activity in male rabbits and rats, presumably by affecting the acrosomal protease system (Baccetti *et al.*, 1993).

Different concentrations of *O. acanthium* crude extract (OACE) and *S. junceum* crude extract (SJCE) were cytotoxic, though the OACE was several folds more potent than SJCE. Condensed chromatin and nuclear fragmentation were seen within the affected cells, two important apoptotic, or more correctly, programmed cell death (PCD) hallmarks. PCD occurs by several different mechanisms, of which apoptosis is one mechanism. For cells affected by the cytotoxic component(s), apoptosis could be distinguished from other forms of PCD by the presence of condensed chromatin in more compact and/or geometric shapes (crescent-like or globular). However, more than one type of PCD may be ongoing at the same time (Leistand Jaattela, 2001; Wlodkowic *et al.*, 2012). Confirming apoptosis or/and the other forms of PCD by visual examination is not an easy task, as it is neither quantitative nor conclusive. While there was a high probability of error when determining the shape of speckles of the condensed chromatin; whether they were more or less compact, globular, crescent-shaped or had other arched and lumpy shapes; examining such images still provided a preliminary impression of the effect of the extract tested. However, the need for a more definitive test was necessary, and the caspase-3 assay test would confirm whether the cytotoxic effect was apoptotic or not.

The cytotoxic effect of both plants was quantified employing the trypan blue exclusion technique, where the viable cells (which possess intact cell membranes) exclude the dye (trypan blue) and were unstained, while dead cells were stained. The technique gave regression values (r^2) of 0.9714 and 0.9794 for *O. acanthium* and *S. junceum*, respectively. However, there are some cases where the result of cytotoxicity can either be overestimated or underestimated. Cytotoxicity can be overestimated when there is a delay in counting greater than 10 minutes, and when some cells are considered dead though they may be capable of repairing their cell membranes. On the other hand, underestimation occurs when the cells have transient membrane integrity, as small amounts of the dye are unnoticed (Strober, 2001).

O. acanthium was found to be about 5 times as cytotoxic as *S. junceum*, which was confirmed by the calculated IC_{50} for both plants (309µg/ml for *O. acanthium* and 1602 µg/ml for *S. junceum*). Accordingly, *O. acanthium* and *S. junceum* can be rated as having intermediate and weak cytotoxic activity, respectively. However, testing extracts from other parts of the plants, in addition to using different solvent (s), may give more favourable results. Future research should focus on pure components of the plants (especially *O. acanthium*), as it is very similar to *Sylibum marianum*, as plant proven to contain components with promising antitumour activity (Chu *et al*., 2004; Thongphasuk *et al.,* 2008).

Activation of caspases appears indispensible for apoptosis (Leistand Jaattela, 2001; Hengartner, 2000). Caspase activation is responsible for the typical morphological changes that take place in the apoptotic cells, primarily, the chromatin condensation and the cleavage of the internucleosomal DNA fragments (Kitanaka and Kuchino, 1999). Evaluation of caspase-3 levels in glioblastoma cells treated with various concentrations of OACE and SJCE suggested the *O. acanthium* extract exerted its cytotoxic effect through apoptosis. This was seen as a concentration-dependent increase in caspase-3 and was supported by the abundant chromatin speckles seen microscopically. These data lend weigh to the idea that *O. acanthium* was pro-apoptotic in treated tumour cells, and like most chemotherapeutic drugs, it exerted its effect through the intrinsic pathway. However, other caspase-independent forms of programmed cell death (PCD) cannot be excluded (Kitanaka and Kuchino, 1999).

On the other hand, caspase-3 levels stimulated by *S. junceum* extract were insignificant, supporting the idea that *S. junceum*acts by acaspase-independent, necrotic-like, non-apoptotic PCD. It is known that non-apoptotic, necrotic-like and caspase-independent forms PCD exist (Xiang *et al*., 1996); this form of PCD is common and characterized by less pronounced pyknosis with an abundance of autophagic vacuoles (Kitanakaand Kuchino, 1999). However, it is noteworthy that this necrotic-like cell death may also be caspase-dependent (Tsujimoto Y, 1997) and accordingly is divided into two subcategories, caspase-dependent and caspase-independent.

CONCLUSIONS

Even though the research on *O. acanthium* and *S. junceum* is minimal, they are considered promising plants with the potential biological activities. *O. acanthium* crude extract (OACE) and *S. junceum* crude extract (SJCE) possessed intermediate and weak cytotoxic effects against glioblastoma U-373 cells, respectively and OACE was ~5 times as cytotoxic as SJCE. OACE was pro-apoptotic in tumour cells (caspase-dependent cell death), while SJCE was thought to exert its cytotoxicity via a necrotic-like,

non-apoptotic and caspase-independent form of programmed cell death. *O. acanthium*, being pro-apoptotic was thought to exert its effect in a similar manner to most chemotherapeutic agents, via the intrinsic pathway of apoptosis. Future research should focus on purified components of *O. acanthium* and further investigate their particular antitumor activity.

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