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Bioassay-guided Isolation of Ursolic Acid as the Major Cytotoxic Compound Present in the Methanolic Extract of the Leaves of *Arbutus pavarii* Pamp.

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ABSTRACT: *Arbutus pavarii* Pamp. (Fam. Ericaceae), an endemic Libyan medicinal plant, is commonly known as "Shmeri", "Shmar" or "Libyan Strawberry", and almost exclusively found in the Al-Jabel Al-Akhdar mountainous region in Libya. This plant is a forage species for honeybees and thus, is important for honey production. *A. pavarii* has long been used in Libyan traditional medicine to treat both gastritis and kidney diseases. Previous limited phytochemical studies on this plant furnished the presence of simple phenolics like arbutin and gallic acid, and polyphenolics including flavonoids and tannins, for example, apigenin, epicatechin, hesperidin, kaempferol, naringin, quercetin and rutin, as well as some triterpenes and sterols. This paper describes, for the very first time, a cytotoxicity assay-guided isolation of the major active compound from the methanol extract of the leaves of *A. pavarii*, and its identification as ursolic acid (**1**) by comprehensive spectroscopic analyses.

Key words: *Arbutus pavarii*, Ericaceae, ursolic acid, prostate cancer, anticancer

INTRODUCTION

Arbutus pavarii Pamp., an endemic Libyan medicinal plant, belongs to the family Ericaceae, and its trivial names include "Shmeri", "Shmar" and "Libyan Strawberry".[1,2] The natural habitat of this evergreen shrub is almost exclusively confined to the Al-Jabel Al-Akhdar mountainous region in Libya. *A. pavarii*, an important forage species of plant for honeybees to produce a specific honey type, is important in honey production and has long been used in the Libyan traditional medicine for the treatment of both gastritis and kidney diseases.[3] While berries of this plant are a good source of minerals, nutrients, carbohydrates and vitamin C, the aerial parts of this plant are used in the tanning process.[4] Only a handful of phytochemical studies have been performed with this species to date, revealing the presence of simple phenolic and polyphenolic compounds, triterpenoids and plant sterols, with rutin and arbutin being the most abundant compounds within the aerial parts of this plant.[1,3-6] Similarly, a few published reports have depicted some preliminary antioxidant, antimicrobial and cytotoxic activities of the crude extracts of this species, but not with any purified compounds from this plant.[1,3,4,7,8] In fact, there has been no attempt made to date to perform bioassay-guided isolation of active compounds, and subsequent assessment of bioactivity of purified compounds. In this paper, we now report, for the very first time, a cytotoxicity assay-guided isolation of the major active compound from the methanol extract of the leaves of *A. pavarii*,

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and its structural elucidation as ursolic acid (**1**) by comprehensive spectroscopic analyses.

MATERIALS AND METHODS

General experimental procedure. 1D and 2D NMR spectroscopic analyses were carried out on a Bruker 600 MHz AMX Ultrashield NMR spectrometer using the deuterium locking. Chemical shifts are in δ ppm and coupling constants *J* in Hz. Solid-phase extraction (SPE) was performed on a Strata C_{18} (20 g) cartridge (Phenomenex, UK) using a step gradient comprising solvent mixtures of decreasing polarity (water-MeOH). Solvents for extraction and chromatographic work were of analytical grade, obtained from Fisher Scientific, UK, and used without further purification.

Plant materials and extraction. The leaves of *A. pavarii* Pamp. (Figure 1) were collected from the Al-Jabal Al-Akhdar region in Libya in 2016 (Figure 2), and a voucher specimen for this collection (D6854201) has been retained at the Herbarium of the Faculty of Science, Tripoli University, Libya. Shed-dried leaves were ground to a fine powder using a coffee grinder, and a portion (150 g) of this powder was Soxhlet-extracted sequentially with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), 900 mL each. All extracts were filtered using Whatman filter paper, evaporated to dryness using a rotary evaporator (Cole-Parmer, UK) and stored at 4°C.

Figure 1. *A. pavarii*, a Libyan medicinal plant.

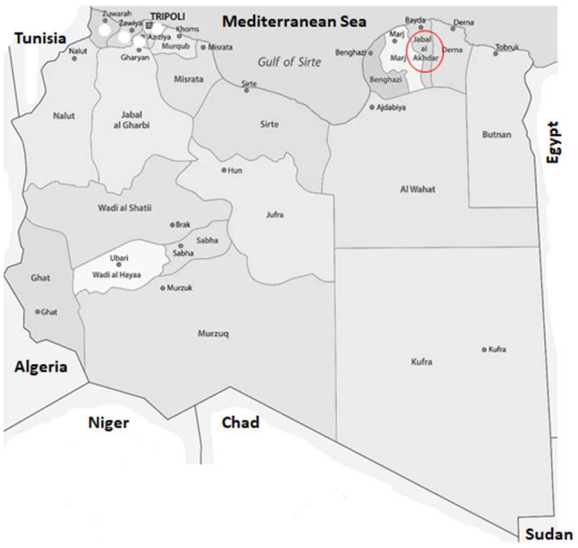

Figure 2. Map of Libya showing the plant collection area in red circle.

Cytotoxicity assay with crude extracts. Human cancer cell lines A549 (human lung carcinoma), EJ138 (human bladder carcinoma), HepG2 (human liver hepatocellular carcinoma), MCF7 (human breast adenocarcinoma) and PC3 (prostate cancer) were used in this study. These cell lines were obtained from the European Collection of Authenticated Cell Cultures. All these cancer cell lines were adherent epithelial cells derived from human carcinoma or adenocarcinoma. Cytotoxicity of the DCM and MeOH extracts of *A. pavarii* at different concentrations (0.0, 0.005, 0.01, 0.05, 0.1 and 0.5 mg/mL) was assessed against these cell lines using the MTT assay.[9,10] The IC_{50} values of these extracts against the tested cell lines were determined following the method described by Patel *et al.*[11] Any IC_{50} value of greater than 100 mg/mL was considered non-cytotoxic. The cytotoxicity of the *n*-hexane extract could not be assessed because of its insolubility in DMSO.

Fractionation of the active MeOH extract – solid-phase extraction (SPE). SPE fractionation of the active MeOH extract (2 g) was performed according to the procedure described by Sarker and Nahar[12]. A step-gradient elution was applied using mixtures of water-MeOH (200 mL) of decreasing polarity (20, 50, 80 and 100% MeOH in water) to obtain fractions SPE-1, SPE-2, SPE-3 and SPE-4. All

fractions were evaporated to dryness using a rotary evaporator and stored at 4°C.

Cytotoxicity assay with the SPE fractions. Four SPE fractions of the active MeOH extract were subjected to the MTT assay as outlined above, but using different concentrations (0.0, 0.0004, 0.002, 0.01, 0.05 and 0.25 mg/ml) against the prostate cancer cell line PC3, and the IC_{50} values were determined.

HPLC analysis of the active SPE fraction. The active SPE fraction (SPE-4) obtained from the active MeOH extract was subjected to reversed-phase HPLC analysis to develop an optimum method for separating and isolating compounds using the preparative HPLC. A Dionex Ultimate 3000 UHPLC, coupled with an autosampler, degasser, a photodiode array detector, and a computer with control and data analysis software (Chromeleon 7) and a Thermo Scientific™ Hypersil GOLD™ C_{18} column (150 mm x 4.6 mm, 5 mm), equipped with a guard column, were used. A gradient elution with 50-100% MeOH in water (both containing 0.1% trifluoro acetic acid, TFA), at a flow rate of 1 mL/min was used. The volume of injection used was 20 µL from each fraction solution of 1 mg/mL. The chromatogram was monitored at four different wavelengths, *i.e.,* 220, 254, 280 and 320 nm during the run, and then UV-Vis spectra of each separated peak after the run were analysed.

Isolation of the active compound by preparative HPLC. SPE-4 (10 mg/ml) was subjected to reversed-phase preparative HPLC separation on an Agilent 1200 prep-HPLC system comprising a binary gradient pump, a photodiode array detector and a computer with control and data analysis software. In the preparative isolation of compounds, an ACE prep-column [150 × 21.2 mm, 5 µm, Hichrom Ltd., UK; MeOH-water linear gradient: 50-100% MeOH in water (both containing 0.1% 0.1% trifluoro acetic acid, TFA) in 40 min, flow rate: 10 ml/min, monitored simultaneously at 215, 254, 280 and 320 nm] was used with a volume of injection of 200 µl.

Identification of the active compound. The identity of the isolated active compound was confirmed by spectroscopic means, particularly, by MS and 1D and 2D NMR data analyses.

Assessment of cytotoxicity of the active compound, ursolic acid (1). The cytotoxicity of ursolic acid (**1**) was assessed by the MTT assay against the prostate cancer cell line PC3 and the IC_{50} value was determined following the method outlined above.

Lactate dehydrogenase (LDH) assay of cell membrane integrity (LDH release assay) for ursolic acid (1). LDH assay is a useful method for detection of necrosis. Necrosis is one of the mechanisms by which cytotoxic compounds kill cells. This assay was performed with ursolic acid (**1**) to understand its possible mechanism of action. The protocol was based on the methods described previously.[10,13] The LDH activity was measured at two absorbances: 490 and 690 nm. The LDH assay was performed using a commercial cytotoxicity assay kit produced by Roche (REF 11644793001).

RESULTS AND DISCUSSION

Extraction. The Soxhlet extraction of the shed-dried ground leaves of *A. pavarii* afforded three different extracts, *i.e., n*-hexane, DCM ad MeOH, with good % yields (4.4, 1.8 and 36.0%, respectively). The highest % yield of the MeOH extract was possibly due to the presence of large amounts of polar phenolic and polyphenolic compounds and their glycosides in the leaves.

Cytotoxicity of the extracts. The cytotoxic activity of both DCM, and MeOH extracts of *A. pavarii* leaves was assessed using the MTT assay against five human cancer cell lines: A549, EJ138, HepG2, MCF7 and PC3. The *n*-hexane extract was not tested for cytotoxicity due to its insolubility in DMSO. Although the DCM extract showed the most prominent cytotoxicity against the PC3 cell line (IC_{50} = 26 µg/mL), the MeOH extract was active against the (MCF7 IC_{50} = 60 µg/ml) and PC3 (IC_{50} = 98 µg/ml) cells (Table 1). As the MeOH extract was the highest yielding extract (36.0%) but the DCM extract

was the least (1.8%), the MeOH extract was chosen for subsequent chromatographic analyses to obtain active compounds in sufficient quantities to allow further relevant spectroscopic and cytotoxicity analyses. The human prostate cancer cell line PC3 was chosen for assessing cytotoxicity of chromatographic fractions and purified compounds, because both DCM and MeOH extracts were active against this cell line. Besides, the cytotoxic properties of the MeOH extract of the aerial parts of *A. pavarii* were previously demonstrated against both HepG2 hepatic carcinoma and T47D breast cancer cell lines, and the IC_{50} values were determined as 19.7 and 19.0 mg/ml, respectively.[3] Earlier, Alsabri *et al.*[4] showed the cytotoxicity of the MeOH extract of the aerial parts of this plant against lung (A549) and breast (MCF7) cancer cell lines with IC_{50} values of below 30 mg/ml. Moreover, in the present study the MeOH extract of the leaves were inactive against either HepG2 or A549 cell lines. Notably, this is the first report on the assessment of cytotoxicity of *A. pavarii* against the bladder (EJ138) and prostate cancer (PC3) cell lines.

Table 1. The IC_{50} values of DCM and MeOH extracts of *A. pavarii* leaves against human cancer cell lines.

Cancer cell lines	IC_{50} values (mg/ml)	
	DCM extract	MeOH extract
EJ138	90	>100
HepG2	>100	>100
A549	>100	>100
MCF7	>100	60
PC3	26	98

SPE fractionation of the active MeOH extract. The MeOH extract (2 g) of *A. pavarii* was subjected to SPE fractionation resulting in four fractions of different weights (Table 2), with a recovery rate of over 96% (total recovery from seven fractions = 1.922 g). The fraction SPE-1, which was obtained by eluting with 20% MeOH in water, was the highest yielding fraction with a weight of 1627.2 mg. It indicated that the compounds present in the MeOH extract could be predominantly highly polar compounds like tannins, polyphenolic glycosides or even sugars. All four fractions were subjected to the MTT assay using the prostate cancer cell line PC3.

Table 2. SPE fractions of the MeOH extract, and their weights.

SPE fractions	Solvent composition of the gradient	Weights (mg)
SPE-1	20% MeOH in water	1627.2
SPE-2	50% MeOH in water	230.7
SPE-3	80% MeOH in water	19.0
SPE-4	100% MeOH in water	45.6

Cytotoxicity assay with the SPE fractions. The SPE fractions (SPE-1, SPE-2, SPE-3 and SPE-4) of the active MeOH extract were assessed for cytotoxicity against PC3 cell line using the MTT assay at different concentrations (0.0, 0.0004, 0.002, 0.01, 0.05 and 0.25 mg/ml). Among the fractions, the fraction eluted with 100% MeOH in water (SPE-4) displayed the highest level of cytotoxicity toward the PC3 cells. None of the other SPE fractions was active. This suggested that almost all cytotoxic compounds should be present in fraction SPE-4. SPE-4 was subjected to HPLC-based separation and isolation of compounds.

HPLC analyses of the SPE fraction SPE-4. Reversed-phases HPLC analysis of SPE-4 optimised the separation of compounds and identified the solvent system comprising a gradient elution using 50-100% MeOH in water (both containing 0.1% TFA) over 40 min as the best system for separation of compounds present in this fraction. This method was transferred to the preparative reversed-phase HPLC to purify the major active compound (**1**) of SPE-4 (t_R = 33.0 min, 3.6 mg) (Figure 3).

Identification of the active compound as ursolic acid (1). Compound **1** (Figure 4) was isolated as a white amorphous powder. The UV (MeOH) λ_{max} of this compound was 204 nm. The ESIMS exhibited the sodiated molecular ion peak at *m/z* 479 $[M+Na]^+$ indicating the molecular formula $C_{30}H_{48}O_3$. The ^1H NMR (600 MHz, CD_3OD) (Table 3) showed the methyl signals at δ_H 0.79, 0.86, 0.89, 0.90, 0.91, 0.99 and 1.02 for seven methyl groups, a broad doublet of a doublet at δ_H 3.17 assignable to an oxymethine and

a triplet at δ_H 5.14 for an olefinic methine proton. All these signals were characteristic to the known triterpene acid, ursolic acid (**1**), which is known for its various biological activities.[14] A ^{13}C NMR (150 MHz, CD_3OD) spectrum revealed signals for all 30 carbon atoms required for the structure of ursolic acid (**1**). The characteristic ^{13}C NMR signals for ursolic acid (**1**) included signals at δ_C 180.3 assignable to the carboxylic acid functionality at C-28, the signals for two olefinic carbons at δ_C 125.5 (C-12) and 138.3 (C-13), and the oxymethines signal at δ_C 78.4 for C-3. While the ^1H-^1H COSY spectrum of compound **1** displayed all ^1H-^1H scaler couplings, the ^1H-^{13}C HSQC experiment showed the attachment of protons to carbon atoms (^1H-^{13}C direct coupling). The ^1H-^{13}C HMBC exhibited all ^2J and ^3J long-range ^1H-^{13}C correlations (Table 3) and thus, established the structure as ursolic acid (**1**). All spectroscopic data were comparable to those published for the pentacyclic triterpene acid, ursolic acid (**1**).[14] This is the first report on the occurrence of ursolic acid (**1**) in the leaves of *A. pavarii*. However, related pentacyclic triterpenes were previously isolated from the fruits of another species of this genus, *A. unedo*, for example, α- and β-amyrin, lupeol olean-12-en-3*b*,23-diol.[15]

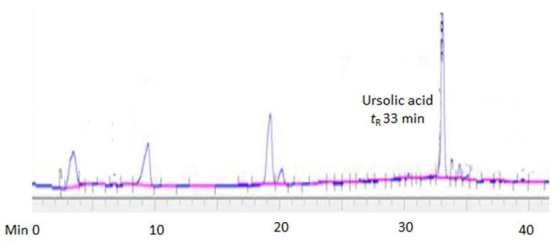

Figure 3. Prep-HPLC chromatogram of SPE-4.

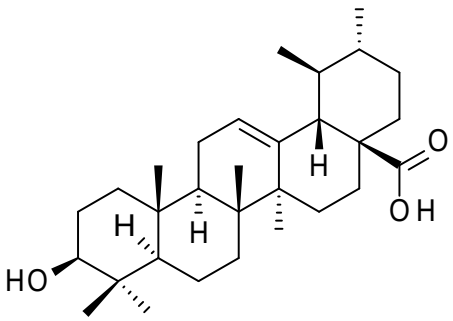

Figure 4. Structure of ursolic acid (**1**).

Table 3. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data together with ^1H-^{13}C HMBC long-range correlations for ursolic acid (1).

Carbon number	Chemical shifts δ in ppm		^1H-^{13}C HMBC correlations	
	^1H (coupling constant *J* in Hz)	^{13}C	^2J	^3J
1	1.56 t (3.6), 2H	38.5	C-10	
2	1.73 m, 2H	27.4	C-1	
3	3.17 dd (4.8 and 11.5), 1H	78.4	C-2	C-1, C-5
4	-	38.6	-	-
5	0.77 d (10.5), 1H	55.4	C-4	C-9
6	1.63 m, 2H	18.1	C-5, C-7	C-4, C-8
7	1.35 m, 2H	33.0	C-8	C-9, C-14
8	-	39.4	-	-
9	1.09 t (3.2), 1H	47.4	C-10	C-12, C-14
10	-	36.7	-	-
11	1.95 dd (3.7 and 8.8), 2H	23.9	C-9, C-12	
12	5.24 t (3.5), 1H	125.5	C-11, C-13	C-9, C-18
13	-	138.3	-	-
14	-	41.9	-	-
15	2.06 t (4.6), 2H	30.4	C-14, C-16	C-17
16	1.74 m, 2H	24.0	C-15, C-17	C-18
17	-	48.0	-	-
18	2.23 d (11.0), 1H	53.0	C-13, C-17	
19	1.51 t (3.2), 1H	39.1	C-29	C-13, C-17, C-30
20	1.44 m, 1H	38.6	C-30	C-29
21	1.63 m, 2H	30.4	C-20, C-22	
22	2.21 dt (3.7 and 8.8)	36.7	C-21, C-23	
23	0.91 s, 3H	23.0	C-4	C-3, C-5, C-24
24	0.89 s, 3H	23.9	C-4	C-3, C-5, C-23
25	0.86 s, 3H	16.3	C-10	C-1, C-5, C-9
26	0.89 s, 3H	18.1	C-8	C-7, C-9
27	1.02 s, 3H	26.5	C-14	C-8, C-13, C-15
28	-	180.3	-	-
29	0.90 d (6.5), 3H	16.4	C-19	C-18, C-20
30	0.79 d (5.2), 3H	20.2	C-20	C-19, C-21

Spectra obtained in CD_3OD

Cytotoxicity of ursolic acid (1). Ursolic acid (**1**) demonstrated significant cytotoxicity against the prostate cancer cell line PC3 in the MTT assay with an IC_{50} value of 8.22 mM (Figure 5). The cytotoxic activity of ursolic acid (**1**) observed in the present study was more potent than any previous reports.

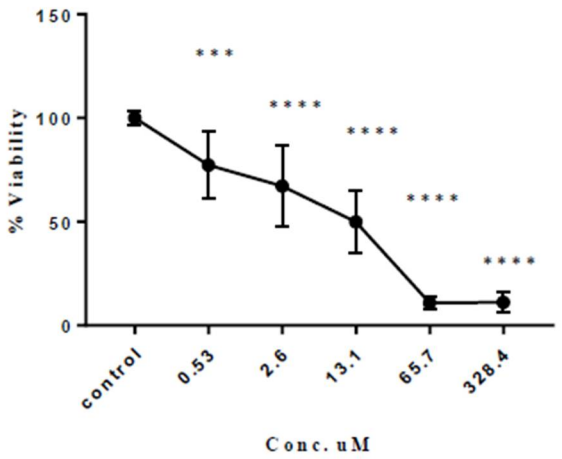

Figure 5. Cytotoxicity of ursolic acid (**1**) against PC3 cells in the MTT assay [(the results are mean values SEM derived from n12 from three separate occasions, and the asterisk indicate significant difference control (***P<0.01, ****P<0.001)].

The LDH assay was employed to try to understand whether ursolic acid (**1**) could damage the plasma membrane to render cytotoxic effects. LDH is a soluble cytoplasmic enzyme that is released into extracellular space, when the plasma membrane is damaged. The leakage level can be determined by the conversion of a substrate into a product that is then quantified spectrophotometrically.[10,13] In this assay, the leakage of LDH into cell culture medium is detected by the presence of a substrate, a tetrazolium salt. Firstly, the LDH enzymes catalyse the oxidation of lactate to pyruvate, which then leads to the release of reduced nicotinamide adenine dinucleotide (NADH) and, secondly, the formation of a coloured formazan product, which can be quantified using a spectrophotometer.[13] The LDH assay with ursolic acid (**1**) revealed that this compound could cause cytotoxicity to the prostate cancer cell line PC3, through at least, in part, by initiating cell membrane damage. The necrotic percentage was 53.4%, which was expressed using the formula: (sample value/maximal release) × 100%.[13]

Pentacyclic triterpenes and terpene acids, including ursolic acid (**1**), have previously been reported to possess several biological activities. Ursolic acid (**1**) has been widely used in the Chinese herbal medicines,[16] and its cytotoxicity was previously reported against PC3, LNCaP and DU145 prostate cancer cells with IC_{50} values of 35 μM, 47 μM and 80 μM, respectively,[17] and the cytotoxicity was found selective, i.e., it was more toxic to cancer cells than to non-cancerous cells. The selectivity index (SI) was determined by comparing cytotoxicity against cancer cell lines and the normal human cell lines.[18] In fact, the SI is the IC_{50} (μg/mL) against the normal cells divided by the IC_{50} (μg/mL) against the cancer cells, where IC_{50} is the concentration required to kill 50% of the cell population. Ursolic acid (**1**) is known to possess anti-inflammatory, antioxidant, anti-apoptotic and anticancer properties, and the isolation of ursolic acid (**1)** from *A. pavarii* in the present study as the major cytotoxic component in this plant has reiterated its anticancer potential. Ursolic acid (**1**) has been widely used in many health supplements and traditional medicinal products, which are available over the counter. It is popular as a weight loss and muscle building supplement, albeit, without any proper scientific data that could support these uses; on the contrary, a study has just shown that ursolic acid (**1**) does not offer any additional effects on muscle strength and mass in active men.[19] In a recent study, Zhang *et al.*[20] have shown that ursolic acid could be useful in immunomodulatory therapies for multiple sclerosis, while it was found to improve intestinal damage and bacterial dysbiosis in liver fibrosis mice.[21] Although in the present study it has been shown that the cytotoxicity of ursolic acid (**1**) against various cancer cells could be mediated through the damage of plasma membrane, there are several other mechanisms implicated to its anticancer potential. For example, it was suggested that the anticancer effect of ursolic acid (**1**) could involve mitochondria-dependent pathways such as via reduction of mitochondrial oxidative stress and p53-modulated mitochondrial pathway.[22]

CONCLUSIONS

Bioassay-guided approach afforded isolation of the triterpene acid, ursolic acid (**1**), as the major compound responsible for the cytotoxicity of the MeOH extract of the leaves of *A. pavarii* against the prostate cancer cell line PC3. From the LDH assay, it was clear that ursolic acid (**1**), among other mechanisms of action, could exert its cytotoxicity by damaging plasma membrane. This is the first report on the isolation of ursolic acid (**1**) from the leaves of *A. pavarii*.

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REFERENCES

1. Nahar, L., Al Groshi, A. and Sarker, S. D. 2021. *Arbutus pavarii* Pamp. – An updated profile. *Trends Phytochem. Res.* **5**, 45-46.
2. Kabiel, H. F., Hegazy, A. K., Lovett-Doust, L., Al-Rowaily, S. L. and El-Nasser, A. 2016. Demography of the threatened endemic shrub, *Arbutus pavarii*, in the Al-Akhdar mountainous landscape of Libya. *J. For. Res.* **27**, 1295-1303.
3. El Hawary, S. S., El Shabrawy, A. E. R., Ezzat, S. M. and El-Shibani, F. A. A. 2016. Evaluation of the phenolic and flavonoid contents, antimicrobial and cytotoxic activities of some plants growing in Al Jabal Al-Akhdar in Libya. *Int. J. Pharmacogn. Phytochem. Res.* **8**, 1083-1087.
4. Alsabri, S. G., El-Basir, H. M., Rmeli, N. B., Mohamed, S. B., Allafi, A. A., Zetrini, A. A., Salem, A. A., Mohamed, S.S., Gbaj, A. and El-Baseir M. M. 2013. Phytochemical screening, antioxidant, antimicrobial and antiproliferative activities study of *Arbutus pavarii* plant. *Journal of Chemical and Pharmaceutical Research* **5**, 32-36.
5. Asheg, A. A., El-Nyhom, S. M., Ben Naser, K. M., Kanoun, A. H. and Abouzeed, Y. M. 2014. Effect of *Arbutus pavarii*, *Salvia officinalis* and *Zizyphus vulgaris* on growth performance and intestinal bacterial count of boiler chickens. *Int. J. Veterin. Sci. Med.* **2**, 151-155.
6. Buzgaia, N., Awin, T., Elabbar, F., Abdusalam, K., Lee, S. Y., Rukayadi, Y. and Abas, F., Shaari, K. 2020. Antibacterial activity of *Arbutus pavarii* Pamp. Against methicillin-resistant *Staphylococcus aureus* (MRSA) and UHPLC-MS/MS profile of the bioactive fraction. *Plants* **9**, article number: 1539.
7. Hussain, H. and Tobji, R. S. 1997. Antibacterial screening of some medicinal plants. *Fitoterapia* **68**, 467-470.
8. Hasan, H. H. Habib, I. H., Gonaid, M. H. and Islam, M. 2011. Comparative phytochemical and antimicrobial investigation of some plants growing in Al Jabal Al-Akhdar. *J. Nat. Prod. Plant Resou.* **1**, 15-23.
9. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**, 55-63.
10. Evans, A. 2003. *In vitro* and *ex vivo* studies on the toxicity and efficacy of a selection of non-viral transfection reagents. Thesis (PhD)-Liverpool John Moores University, Liverpool, pp. 32-34.
11. Patel, S., Geewala, N., Suthar, A. and Shah, A. 2009. *In-vitro* cytotoxicity activity of *Solanum nigrum* extract against HELA cell line and VERO cell line. *Int. J. Pharm. Pharmaceu. Sci.***1**, 38-46.
12. Sarker, S. D. and Nahar, L. 2012. Natural Products Isolation, 3rd edition, Humana Press-Springer-Verlag, New Jersey, USA.
13. Chan, F. K. M., Moriwaki, K. and De Rosa, M. J. 2013. Detection of necrosis by the release of lactate dehydrogenase (LDH) activity. *Meth. Mol. Biol.* **979**, 65-70.
14. Mishra, T., Arya, R. K., Meena, S., Joshi, P., Pal, M., Meena, B., Upreti D. K., Rana T. S. and Datta, D. 2016. Isolation, characterization and anticancer potential of cytotoxic triterpenes from *Betula utilis* bark. *PLoS One* **11**, article number: e0159430.
15. Miguel, M. G., Faleiro, M. L., Guerreiro, A. C. and Antunes, M. D. 2014. *Arbutus unedo* L, chemical and biological properties. *Molecules* **19**, 15799-15823.
16. Wu, Y. J., Shi, Q. Y., Lei, H. L., Jin, Y., Liu, X. S. and Luan, L. J. 2014. Simple and efficient preparation of 3,6′-disinapoylsucrose from Polygalae Radix via column chromatographic extraction and reversed-phase flash chromatography. *Sep. Purif. Technol.* **135**, 7-13.
17. Park, J. H., Kwon, H. Y., Sohn, E. J., Kim, K. A., Kim, B., Jeong, S. J., Song, J. H., Koo, J. S., Kim, S. H. 2013. Inhibition of Wnt.b-catenin signaling mediates ursolic acid-induced apoptosis in PC-3 prostate cancer cells. *Pharmacol. Rep.* **65**, 1366-1374.
18. Badisa, R. B., Darling-reed, S. F., Joseph, P., Cooperwood, J. S., Latinwo L. M. and Goodman. C. 2009. Selective cytotoxic activities of two novel synthetic drugs on human breast carcinoma MCF-7 cells. *Anticancer Res.* **29**, 2993–2996.

19. Lobo, P. C. B., Vieira, I. P., Pichard, C., Gentil, P. and da Silva, E. L. 2020. Ursolic acid has no additional effect on muscle strength and mass in active men undergoing a high-protein diet and resistance training: A double-blind and placebo-controlled trial. *Clin. Nutr.* **40**, 581-589.
20. Zhang, Y., Li, X., Ciric, B., Curtis, M. T., Chen, W-J., Rostami, A. and Zhang, G-X. 2020. A dual effect of ursolic acid to the treatment of multiple sclerosis through both immunomodulation and direct remyelination. *PNAS* **117**, 9082-9093.
21. Wan, S-Z., Liu, C., Huang, C-K., Luo, F-Y. and Zhu, X. 2019. Ursolic acid improves intestinal damage and bacterial dysbiosis on fibrosis mice. *Frontiers in Pharmacology* **10**, article number: 1321.
22. Feng, X-M. and Su, X-L. 2019. Anticancer effect of ursolic acid via mitochondria-dependent pathways. *Oncology Letters* **17**, 4761-4767.