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# Comparative study of two *Lotus* species: Phytochemistry, cytotoxicity and antioxidant capacity

[Estudio comparativo de dos especies de *Lotus*: Fitoquímica, citotoxicidad y capacidad antioxidante]

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**Abstract**

*Context*: Phenolic and flavonoid compounds present in *Lotus arabicus* and *L. glaber* have not been identified yet. Also, no reports are demonstrating the cytotoxic and antioxidant activities of both *lotus* species. Therefore, it is interesting to study the anticancer effects and antioxidant capacity of them.

*Aims*: To identify and evaluate the potential cytotoxic and antioxidant activities of phenolic and flavonoid compounds of methanol extracts of two *Lotus* species.

*Methods*: High-Performance Liquid Chromatography (HPLC) was used to identify and quantify the potential compounds of the methanol extracts of both *Lotus* species. The cytotoxic activity of each methanol extract of the *Lotus arabicus* and *L. glaber* was investigated against seven cancer cell lines and one normal cell line. The cell viability was quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the half-maximal inhibitory concentration ($IC_{50}$) was estimated and compared with doxorubicin. Moreover, the antioxidant capacity of them was evaluated by 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPHH) assay, and the scavenging percentage was calculated and compared with ascorbic acid.

*Results*: The identified and quantitated phenolic compounds of the methanol extracts of both *Lotus species* were 17 phenolic acids and 16 flavonoids. The results showed that the active constituents of *L. arabicus* had cytotoxic activities against prostate cancer ($IC_{50}$ = 401 ± 5 µg/mL), colon cancer ($IC_{50}$ = 511 ± 181 µg/mL) and breast cancer ($IC_{50}$ = 978 ± 54 µg/mL) greater than *L. glaber* which showed cytotoxic activities against prostate cancer ($IC_{50}$ = 5094 ± 531 µg/mL), colon cancer ($IC_{50}$ = 6522 ± 272 µg/mL) and breast cancer ($IC_{50}$ = 2964 ± 404 µg/mL) in comparison with doxorubicin.

*Conclusions*: *L. arabicus* may have active constituents that could be used for the treatment of prostate, colon or lung cancer. Therefore, isolation of phenolic and flavonoid compounds exist in *L. arabicus* may be required in the future.

*Keywords*: cancer; flavonoid; HPLC; *Lotus arabicus*; *Lotus glaber*; phenolic compound.

**Resumen**

*Contexto*: Aún no se han identificado compuestos fenólicos y flavonoides presentes en *Lotus arabicus* y *L. glaber*. Además, no hay informes que demuestren las actividades citotóxicas y antioxidantes de ambas especies de loto. Por tanto, es interesante estudiar los efectos anticancerígenos y la capacidad antioxidante de los mismos.

*Objetivos*: Identificar y evaluar las potenciales actividades citotóxicas y antioxidantes de compuestos fenólicos y flavonoides de extractos metanólicos de dos especies de *Lotus*.

*Métodos:* Se utilizó cromatografía líquida de alta resolución (HPLC) para identificar y cuantificar los compuestos potenciales de los extractos de metanol de ambas especies de *Lotus*. Se investigó la actividad citotóxica de cada extracto metanólico de *Lotus arabicus* y *L. glaber* frente a siete líneas celulares cancerosas y una línea celular normal. La viabilidad celular se cuantificó mediante el ensayo de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT), y se estimó la concentración inhibidora cincuenta ($IC_{50}$) y se comparó con la doxorrubicina. Además, se evaluó la capacidad antioxidante de los mismos mediante ensayo de 2,2-difenil-1-picril-hidrazil-hidrato (DPHH), y se calculó el porcentaje de captación y se comparó con el ácido ascórbico.

*Resultados*: Los compuestos fenólicos identificados y cuantificados de los extractos de metanol de ambas especies de *Lotus* fueron 17 ácidos fenólicos y 16 flavonoides. Los resultados mostraron que los componentes activos de *L. arabicus* tenían actividades citotóxicas contra el cáncer de próstata ($IC_{50}$ = 401 ± 5 µg/mL), cáncer de colon ($IC_{50}$ = 511 ± 181 µg/mL) y cáncer de mama ($IC_{50}$ = 978 ± 54 µg/mL) mayor que *L. glaber* que mostró actividades citotóxicas contra cáncer de próstata ($IC_{50}$ = 5094 ± 531 µg/mL), cáncer de colon ($IC_{50}$ = 6522 ± 272 µg/mL) y cáncer de mama ($IC_{50}$ = 2964 ± 404 µg/mL) en comparación con la doxorrubicina.

*Conclusiones*: *L. arabicus* puede tener componentes activos que podrían usarse para el tratamiento del cáncer de próstata, colon o pulmón. Por lo tanto, es posible que en el futuro sea necesario aislar los compuestos fenólicos y flavonoides existentes en *L. arabicus*.

*Palabras Clave*: cáncer; compuesto fenólico; flavonoide; HPLC; *Lotus arabicus*; *Lotus glaber*.

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## INTRODUCTION

In Egypt, the medicinal herbs *Lotus arabicus* L. and *Lotus glaber* Mill. (*L. tenuis.* Willd) are two of the eighteen species of genus *Lotus* (*Fabaceae*). They are growing wild in the Mediterranean region, Nile banks, cultivated ground and wetlands (Boulos, 1999). The most important trait of them is tolerance to different climatic and soil conditions (Girardi et al., 2014).

In folk medicine, plants of genus *Lotus* are used as contraceptives, for the treatment of sexually transmitted diseases and treatment of peptic ulcers (El Mousallami et al., 2002). *L. corniculatu* (birdsfoot trefoil) is used externally for local skin inflammation (Koelzer et al., 2009), its flowers are used as antispasmodic, cardiotonic and sedative. Also, the root of *L. corniculatu* is used as carminative, febrifuge and restorative (Chiej, 1984). Previous phytochemical studies on aerial parts of genus *Lotus* reported the presence of condensed tannins that prevented meteorism in ruminants (Acuña et al., 2008, Naumann et al., 2013). A decade later, the presence of these compounds was confirmed in *Lotus* sp. and other forage legumes (Acuña et al., 2008; Naumann et al., 2013). Besides, *Lotus* species contain flavonoids of flavonol type, like quercetin and kaempferol (Reynaud and Lussignol, 2005), rutin and vitexin (Moro et al., 2010). Flavones and flavonols are the most common constituents of the aerial parts of *Lotus* species (El Mousallami et al., 2002; El-Ghani et al., 2001; Abdel-Kader et al., 2007). Nevertheless, there are few studies for anticancer and antioxidant effects of some species of genus *Lotus* such as *L. corniculatus* (Khalighi-Sigaroodi et al., 2012). roanthocyanidins, tannins, flavonoids, oleanolic acid, and saponins were identified in these species (Foo et al., 1996). These species have close affiliations with *L. arabicus* and *L. glaber*. Furthermore, the aerial parts of *L. corniculatus* contained kaempferitrin, oleanolic acid, and β-sitosterol. These compounds showed important anti-inflammatory activity in a mouse model (Koelzer et al., 2009). Also, its aerial parts exhibited significant antioxidant, immune stimulant and anticancer activities (Alqasoumi et al., 2013). Since up to now no pharmacological evaluation or any scientific reports have been done on *L. arabicus* and *L. glaber* for their cytotoxic and antioxidant activities.

This work aimed to evaluate the cytotoxic and antioxidant activity of methanol extracts of two *Lotus* species, *L. arabicus* and *L. glaber* growing in Egypt.

## MATERIAL AND METHODS

### Plant material

*Lotus arabicus* and *L. glaber* aerial parts were collected from Al-Azhar gardens (30.0408°N, 31.2647°E), Egypt, during the fruiting stage between May to July 2018. They were identified by Dr. Iman Al-Gohary from Herbarium Unit, Desert Research Centre (DRC), and Cairo, Egypt. Voucher specimens were deposited at the Herbarium of the Centre (CAIH). The plants were washed, dried in shad, and subsequently powdered.

### Preparation of plant extract

The air-dried aerial parts of two *Lotus* sp. (200 g) were extracted with slight modifications (Moustafa et al., 2014). The extract was prepared by percolation of material with 500 mL methanol (70%), and then fully extracted by percolation at ambient temperature on a shaker for 72 hours. The extracts were filtered using Whatman No.1 paper and concentrated under reduced pressure at 40°C, dried by high vacuum and the crude extracts were kept in the refrigerator at 4°C until used for the experiment. Yields were 24.3 g for *L. arabicus* and 19.7 g for *L. glaber*.

### Determination of total phenolic and flavonoid contents

Quantification of phenolic compounds was carried out using Folin–Ciocalteau's method (Siger et al., 2008). The methanolic extract 80% (0.2 mL) was placed in a volumetric flask (10 mL), and 0.5 mL Folin–Ciocalteu reagent (2 N) was added. After 3 min, saturated sodium carbonate (1 mL) (20% in distilled water) was added, and the volume was

completed with distilled water. After 1 h, the absorbance of blue color was measured at $\lambda_{max}$ 725 nm against a blank (distilled water) using Unicam UV-visible Spectrometer. The standard calibration curve was obtained by gallic acid. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight.

Total flavonoid content was determined by an aluminum chloride colorimetric method (Liu et al., 2002). The methanolic extract (0.25 mL) was diluted with 1.25 mL of distilled water. Then, 75 µL of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 µL of a 10% $AlCl_3.6H_2O$ solution were added, and the mixture was allowed to stand for another 5 min, 0.5 mL of 1 M NaOH solution was added, and the total was made up to 2.5 mL with distilled water. The solution was mixed, and the absorbance was measured immediately against the prepared blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results were expressed as milligrams of catechin equivalents (CE) per gram dry weight.

## Determination of phenolic compounds

### *Chemicals*

Reagents for HPLC analysis of phenolic compounds, methanol, and acetonitrile (HPLC grade) were purchased from SDS (Peypin, France), phosphoric acid from Probus (Badalona, Spain) and acetic acid from Merck (Darmstadt, Germany). Pure water was obtained from Milli- Q (Millipore, MA, USA). Phenolic standards: protocatechuic acid, coumaric acid, quercetin, β- hydroxyl benzoic acid, alloevodionol, 5-hydroxyverotric acid, chlorogenic acid, neptein,3,4,'-dimethoxychrysin, 3,4'-dimethoxychrysin, epicatechin, catechin, gallic acid, ferulic acid, caffeic acid, chlorogenic acid, trimethoxy quercetin, apigenin hyperoside, kaempferol, kaempferol-3-O-glucoside, keampferol-3-rutinoside, kaempferol-7-O-neohesperidoside, quercitrin, isoquercitrin, iso-rhamnetin, luteolin, luteolin-6-C-glucoside, luteolin-8-C-glucoside, luteolin-7-O-glucoside, apigenin, apigenin-7-O-glucoside, apigenin 6-arabinose, apigenin-8-C-glucoside, amentoflavone, naringin, naringenin, naringenin-7-O-glucoside, isorhoifolin and rutin were purchased from Sigma Co. (St. Louis, MO, USA). The purity of the standards was 98%.

### *HPLC apparatus*

Reverse-phase HPLC apparatus, Agilent Series 1200 apparatus (Agilent, USA) equipped with auto sampling injector, solvent degasser, quaternary HP pump (series 1200), ultraviolet (UV) detector and a Zobrax ODS C18 column (particle size 5 µm, 250 mm × 4.6 mm Ø) was used for phenolic compounds analysis.

### *Quantitative determination of phenolic compounds by HPLC*

The previously prepared methanolic extract of the two studied *Lotus* spp. (100 mg) were dissolved in HPLC grade acetonitrile (2 mL). Separation of phenolic acids was carried out with the mobile phase consisted of three solvent A ($CH_3COOH$ 2.5%) in water, B ($CH_3COOH$ 8%) in water and C (acetonitrile). Gradient profile was as follows: at 0 min, A:B:C, 95: 5:0; at 20 min, A:B:C 90:10: 0; at 50 min, A:B:C, 70:30:0; at 55 min, A:B:C, 50:50:0; at 60 min, A:B:C, 0:100:0; at 100 min, A:B:C, 50:50:50; at 110 min, A:B:C, 0:0:100 until 120 min (Pascale et al., 1999). Chromatography was performed at 35°C with a flow-rate of 1 mL/min. The injection volumes were 10 µL. UV traces were measured at 280 nm (Pascale et al., 1999). Flavonoid separation was done using a mobile phase consisting of 50 mM $H_3PO_4$, pH 2.5 (solution A) and acetonitrile (solution B) acetic acid (40:60, v/v) in the following gradient: isocratic elution 95% A: 5% B, 0–5 min; linear gradient from 95% A:5% B to 50% A:50 % B, 5–55 min; isocratic elution 50% A:50% B, 55–65 min; linear gradient from 50% A:50% B to 95% A:5% B, 65–67 min (Mattila et al., 2000). The temperature of the column was maintained at 35°C. The flow rate of the mobile phase was 0.7 mL/min. The volume injected was 5 µL. UV traces were measured at 330 nm (Mattila et al., 2000). UV spectra were recorded between 200 and 600 nm for peak characterization. Standard flavonoids and phenolic acids were prepared as 10 mg/50 mL solutions in methanol and they were diluted to make concentrations (20–40 µg/mL) and

injected into HPLC. Peak area of the external standards was used to quantify the phenolic compounds of the plant extracts and the concentrations of the identified compounds were expressed as mg/100g.

## Cytotoxic evaluation

### *Cell lines*

Hepatocellular carcinoma (HepG 2), human epithelioma cells (Hep-2), cervical carcinoma (Hela), prostate cancer (PC3), breast cancer (MCF 7), colon cancer (Caco 2) and normal human fetal lung fibroblast (WI 38) were obtained from tissue culture laboratory, Vacsera, Dokkey, Giza, Egypt.

### *Culturing of cell lines*

A laminar airflow cabinet was used to maintain the sterility of the procedure. The cell culture was maintained in a Roswell Park Memorial Institute medium (RPMI 1640). It contained a 1% antibiotic-antimycotic mixture (10,000 µg/mL streptomycin sulphate, 25 µg/mL amphotericin B and 10,000 U/mL potassium penicillin), 1% L-glutamine. Fetal bovine serum (10% heat-inactivated) was used to supplement the medium, and culturing and subculturing were performed (Thabrew et al., 1997).

### *MTT assay*

The cytotoxicity was evaluated by the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyl tetrazolium bromide). The yellow MTT undergoes a mitochondrial reduction to form a purple formazan (Mosmann, 1983). The 96-well tissue culture microplate was inoculated at a cell concentration of 1 × $10^5$ cells per well in 100 µL of growth medium. The microplate was incubated at 37°C in 5% $CO_2$ for 24 h to develop a complete monolayer sheet. The growth medium was decanted from 96 well microplates after the formation of the confluent layer of cells. The extract of the seeds was dissolved in dimethyl sulfoxide (DMSO). Serial dilutions of the dissolved extract were prepared by RPMI–1640 medium to give a final concentration of 156.25; 312.5; 625; 1250; 2500; 5000; 10,000 and µg/mL. Each concentration of the extract (0.1 mL) was added to confluent cell monolayers dispensed into 96-well microplate using a multichannel pipette. The treated cells were incubated at 37°C in 5% $CO_2$ for 24 h. Three wells were used for each concentration of the extract. Control cells were incubated without the seeds extract. MTT powder was dissolved in PBS (Bio Basic Canada Inc.) to give a solution at the concentration of 5 mg/mL. After the end of the incubation period, 20 µL of the MTT solution was added to each well. The mixing was allowed at 150 rpm for 5 min using the shaker (MPS-1, Biosan, London, UK). Then, the 96- well microplate was incubated at 37°C in 5% $CO_2$ for 4 h to allow the MTT metabolism. Formazan (MTT metabolic product) was resuspended in 200 µL of DMSO and placed on a shaker at 150 rpm for 5 min for a thorough mixing (Mosmann, 1983). The optical density was recorded using a microplate reader (Mindray-96A, Mindray, Nanshan, Shenzhen, China) at 560 nm. The results were corrected using a reference wavelength of 620 nm as a background (Pang et al., 2010). All experiments were carried out in triplicate.

### *Microscope*

Inverted microscope (Nikon, 118811) with objective 8× was used to observe the morphological structures of cell lines at varied concentrations of methanol *Lotus* species extracts.

### *Determination of $IC_{50}$ values*

GraphPad Prism version 7 software, Inc., California, US A was used to calculate the half-maximal inhibitory concentration ($IC_{50}$) values of different concentration of methanol extract of two studied *Lotus* species, and doxorubicin (as a positive control) against HepG-2, Hep-2, Hela, PC-3, MCF-7, Caco-2, A549 and WI-38 cell lines. The percentage of growth inhibition was calculated using equation [1]:

Growth inhibition (%) = 100 – [Mean OD of individual test group/Mean OD of control group] × 100 [1]

## Antioxidant capacity

The free radical scavenging capacity of methanol extract of both studied *Lotus* species was car-

ried out (Braca et al., 2001). Methanol solution (0.004%) of the stable free radical 2,2-diphenyl-1 picrylhydrazyl (DPPH) was freshly prepared. An aliquot of 1 mL of each methanolic extract at different concentrations (10 - 1280 µg/mL) was added to 3 mL of DPPH solution and allowed to react at 37°C in the dark. After thirty minutes, the absorbance was measured at 517 nm. The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated as [2]:

$$PI = (A\ control - A\ sample / A\ control) \times 100 \quad [2]$$

Afterward, a curve of PI (% DPPH bleaching activity) *versus* concentration was plotted, and $IC_{50}$ values were calculated. $IC_{50}$ denotes the concentration of the sample required to scavenge 50% of DPPH free radicals (Braca et al., 2001).

### Statistical analysis

The parameters of experiments were conducted, and results are presented as mean ± standard deviation. The statistical significance of the data obtained from *in vitro* studies was evaluated by the one-way analysis of variance (ANOVA), followed by the Dunnett's multiple comparisons test at $p<0.05$, $p<0.001$ and $p<0.0001$ using Statistical Software GraphPad Prism version 7 software, Inc., California, USA.

## RESULTS

Total phenolic contents of *L. arabicus* and *L. glaber* were 45.62 mg and 27.73 mg GAE)/g of dry weight (DW), and that of the total flavonoids were 36.58 mg and 17.13 mg CE/g DW, respectively. A total of thirty-four phenolic compounds has been identified by using standards and MS data of the detected peaks with that reported in the literature and by searching the phytochemical dictionary of natural products database (CRC), as shown in Fig. 1. Identification compounds belonged to various classes (Table 1), including 17 phenolic acids and 16 flavonoids in both *Lotus* species. The major phenolics of *L. arabicus* and *L. glaber* were hydrocarbon compound, pyrogallol (1.5 mg /100 g and 1.05 mg/100 g), caffeine (0.8 mg/100 g and 1.15 mg/100 g), benzoic acid (0.59 mg /100 g and 0.71 mg /100 g ), catechin (0.81 mg/100 g and 0.64 mg/100 g) and 3,4,5,methoxy cinnamic (0.67 mg/100 g and 0.69 mg/100 g) respectively. The hydroxycinnamic acid derivative, P-coumaric (1.57 mg /100 g), was identified only in *L. glaber*. The other phenolic acids showed small amounts in the two *Lotus* spp.

Flavonoids were present mostly in the two studied *Lotus* sp. as flavones (including both O- and C-glycosides with apigenin or luteolin as aglycone). The highest concentration of flavonoids in methanolic extracts of *L. arabicus* and *L. glaber* were apigenin 6-arabinose (34.8 mg/100 g and 50.2 mg/100 g) and luteolin-7-glucoside (5.30 mg/100 g and 3.78 mg/100 g), respectively (Fig. 2). Flavonols derived from aglycones; quercitrin, quercetin, rutin and kaempferol were 4.88 mg/100 g, 1.32 mg/100 g, 1.98 mg/100 g and 0.14 mg/100 g in *L. arabicus*, respectively. Whereas the concentrations of these flavonoids were 3.08 mg/100 g, 0.31 mg/100 g, 0.80 mg/100 g and 0.023 mg/100 g, respectively in *L. glaber*. Flavanones, naringin and hesperidin and their glycosides were 2.22 mg/100 g and 22.3 mg/100 g in *L. arabicus*, respectively. While in *L. glaber* were 1.63 mg/100 g and 10.06 mg/100 g, respectively are shown in Table 2.

The anticancer effects of *L. arabicus* and *L. glaber* against hepatocellular carcinoma (HepG-2), human epithelioma cells (Hep-2), cervical carcinoma (Hela), prostate cancer (PC-3), breast cancer (MCF-7), colon cancer (Caco-2) and normal human fetal lung fibroblast (WI 38) cell lines were investigated using MTT assay as shown in Table 3. All the $IC_{50}$ values of both plant extracts were compared to the $IC_{50}$ values of the positive control, doxorubicin, using the statistical analysis of Dunnett's test. Both plants demonstrated various anticancer effects against the studied cancer cell lines. The cytotoxic activities of *L. arabicus* against prostate cancer (PC3) ($IC_{50}$ = 401 ± 5 µg/mL), colon cancer (Caco-2) ($IC_{50}$ = 511 ± 181 µg/mL) and breast cancer (MCF-7) ($IC_{50}$ = 978 ± 54 µg/mL) were higher than *L. glaber* in comparing with a positive control as

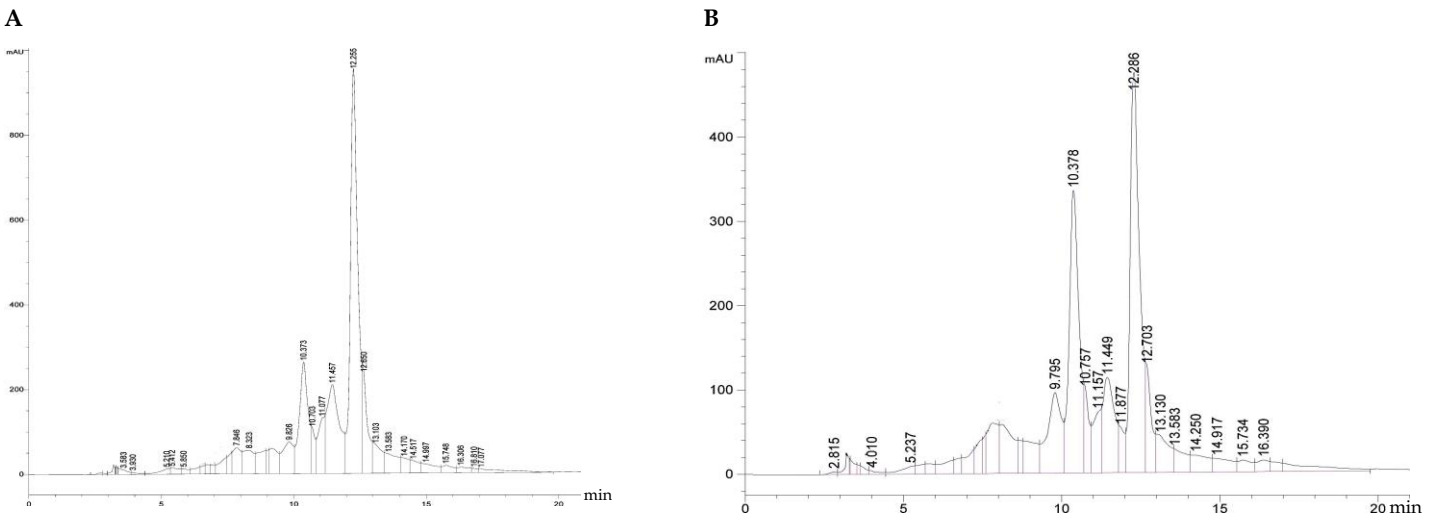


**Figure 1.** HPLC spectra of *Lotus arabicus* and *Lotus glaber*. **(A)** Phenolics of *L. arabicus*; **(B)** Phenolics of *L. glaber*.

**Table 1.** Phenolic compounds of *L. arabicus* and *L. glaber* using HPLC.

| No. | Phenolic compounds | *Lotus arabicus* | | *Lotus glaber* | |
|---|---|---|---|---|---|
| | | **RT** | **mg/100 g** | **RT** | **mg/100 g** |
| 1 | Pyrogallol | 5.41 | 1.50 | 5.48 | 1.05 |
| 2 | Gallic acid | 5.89 | 0.19 | 5.80 | 0.23 |
| 3 | Protocatechuic acid | 7.02 | 0.04 | 7.00 | 0.05 |
| 4 | Catechol | 7.32 | 0.08 | 7.24 | 0.22 |
| 5 | 4-Aminobenzoic acid | 7.44 | 0.03 | 7.39 | 0.08 |
| 6 | Catechin | 7.58 | 0.81 | 7.56 | 0.64 |
| 7 | Chlorogenic acid | 7.96 | 0.26 | 7.95 | 0.26 |
| 8 | P-OH-benzoic acid | 8.02 | 0.10 | 8.05 | 0.10 |
| 9 | Benzoic acid | 8.14 | 0.59 | 8.13 | 0.71 |
| 10 | Caffeic acid | 8.63 | 0.06 | ---- | ------- |
| 11 | Vanillic acid | 8.76 | 0.11 | 8.67 | 0.23 |
| 12 | Caffeine | 9.82 | 0.81 | 9.79 | 1.15 |
| 13 | P-Coumaric acid | ---- | ------ | 9.95 | 1.57 |
| 14 | Ferulic acid | 10.22 | 0.24 | 10.37 | 0.04 |
| 15 | Iso-ferulic acid | 10.70 | 0.26 | 10.75 | 0.34 |
| 16 | α-Coumaric acid | 11.24 | 0.08 | 11.28 | 0.04 |
| 17 | Coumarin | 11.95 | 0.10 | 11.89 | 0.21 |
| 18 | 3,4,5-trimethoxycinnamic acid | 12.47 | 0.67 | 12.48 | 0.69 |

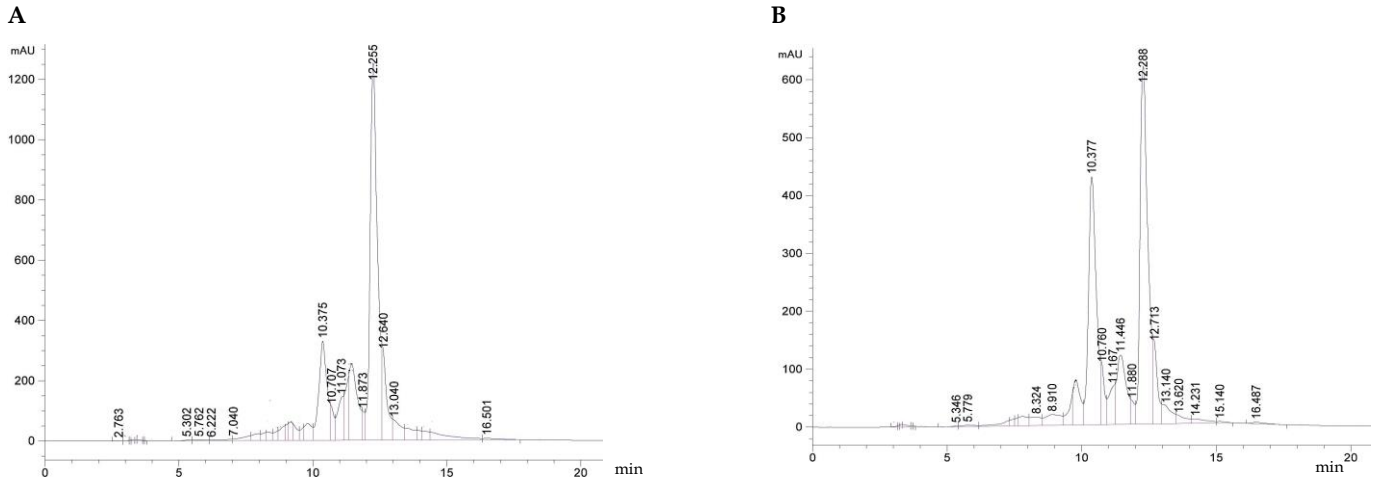


**Figure 2.** HPLC spectra of *Lotus arabicus* and *Lotus glaber*. **(A)** Flavonoids of *L. arabicus*; **(B)** Flavonoids of *L. glaber*.

**Table 2.** Flavonoids compounds of *L. arabicus* and *L. glaber* using HPLC.

| No. | Flavonoids | *Lotus arabicus* | | *Lotus glaber* | |
|---|---|---|---|---|---|
| | | RT | mg/100g | RT | mg/100g |
| 1 | Apigenin 6-arabinose | 10.56 | 34.87 | 10.60 | 50.23 |
| 2 | Naringin | 10.88 | 2.22 | 10.85 | 1.63 |
| 3 | Rosmarinic | 10.97 | 0.45 | 10.96 | 0.11 |
| 4 | Luteolin-7-O-glucoside | 11.16 | 5.30 | 11.09 | 3.78 |
| 5 | Hesperidin | 11.21 | 22.37 | 11.24 | 10.06 |
| 6 | Rutin | 11.30 | 1.98 | 11.29 | 0.80 |
| 7 | Apigenin-7-O-glucoside | 11.45 | 0.95 | 11.44 | 0.63 |
| 8 | Apigenin 7-O-neohesperidoside | 11.98 | 0.99 | 11.88 | 0.77 |
| 9 | Quercitrin | 12.11 | 4.88 | 12.09 | 3.08 |
| 10 | Naringenin | 13.20 | 1.27 | 13.29 | 0.22 |
| 11 | Quercetin | 13.43 | 1.32 | 13.43 | 0.31 |
| 12 | Hesperetin | 13.80 | 0.17 | 13.75 | 0.07 |
| 13 | Kaempferol 3-2-p-comaroyl | 13.86 | 0.24 | 13.79 | 0.20 |
| 14 | Luteolin 7-rutinoside | 13.94 | 0.91 | 13.90 | 0.55 |
| 15 | Kaempferol | 14.62 | 0.14 | 14.69 | 0.02 |
| 16 | Apigenin | 14.89 | 0.40 | 14.89 | 0.03 |

shown in Table 3. However, the anticancer effects of both *lotus* species were similar against HepG-2, Hep-2, and Hela cancer cell lines compared to the positive control. The cytotoxic effects on normal human fetal lung fibroblast (WI 38) cell lines of *L. arabicus* ($IC_{50}$ = 1156 ± 104 µg/mL) and *L. glaber* ($IC_{50}$ = 850 ± 38 µg/mL) were very low. A microscopic examination of the different cell lines treat-

ed with 1000 µg/mL of plant extracts for 72 h was done. The analysis revealed that treatment of methanol extract of *L. arabicus* caused Hela, PC-3, Caco-2 and MCF-7 cells to shrink, became rounded and detached in comparison with untreated control cells; however, HepG-2 and Hep-2 cell lines exhibited no change in morphology at all. In contrast, all cells treated with methanol extract of *L. glaber* did not show morphological changes in comparison with untreated control cells except on HepG-2 and MCF-7 cell lines, as shown in Fig. 3. As a result, *L. arabicus* may possess anticancer effects greater than *L. glaber* against prostate, colon, and breast cancers without adversely affecting the normal cells.

The DPPH assay is known to provide reliable information regarding the antioxidant capacity of specific extracts or compounds within a short time scale (Huang et al., 2012). Hydrogen atoms or the ability of electrons donation of the plant extract and some pure compounds were recorded from the bleaching of a purple-colored methanol solution of DPPH (Kubola and Siriamornpun, 2008). The methanolic extracts of the two studied *Lotus* species were tested for the antioxidant activity (*in vitro*). The results of this study showed that, in general, the tested extracts have low antioxidant activity compared to ascorbic acid. The $IC_{50}$ of methanol extract of *L. arabicus* reached to value of ($IC_{50}$ = 714.71 ± 2.9 µg/mL), whereas $IC_{50}$ of methanol extract of *L. glaber* had ($IC_{50}$ = 834.12 ± 2.3 µg/mL). Furthermore, the increase of concentrations of methanolic extract of *L. arabicus* reached to 1280 µg/mL attained 72.55% of DPPH scavenging, while methanolic extract of *L. glaber* attained 65.73% DPPH scavenging shown in Table 4. Also, the bioactive compounds (phenolic and others) are in the form of crude, not pure, and present in low amounts in the two extracts. Therefore, as mentioned previously, the small amount of total phenolics of *L. arabicus* (45.62 mg) and *L. glaber* (27.73 mg), and the small amount of total flavonoids of *L. arabicus* (36.58 mg) and *L. glaber* (17.13 mg) produced a low antioxidant activity as compared with ascorbic acid. Furthermore, phenolic acids and flavonoids components are very low, as shown in Tables 1 and 2.

**Table 3.** Cytotoxic effects of methanol extract of *L. arabicus* and *L. glaber* on cancer cell lines.

| | **$IC_{50}$ (µg/mL)** | | |
|---|---|---|---|
| **Cell Lines** | ***L. arabicus*** | ***L. glaber*** | **Doxorubicin** |
| HepG-2 | 3308 ± 491*** | 2107 ± 491*** | 30 ± 2 |
| Hep-2 | 846 ± 8*** | 1191 ± 47*** | 40 ± 4 |
| Hela | 4228 ± 452*** | 4063 ± 399*** | 66 ± 3 |
| PC3 | 401 ± 5$^{ns}$ | 5094 ± 531*** | 40 ± 3 |
| MCF-7 | 978 ± 54** | 2964 ± 404*** | 67 ± 9 |
| Caco-2 | 511 ± 181* | 6522 ± 272*** | 34 ± 3 |
| WI38 | 1156 ± 104*** | 850 ± 38*** | 137 ± 10 |

$IC_{50}$: The half-maximal inhibitory concentration; HepG-2: human hepatocellular carcinoma; Hep-2: Human epithelioma cells; Hela: cervical carcinoma; PC3: prostate cancer; MCF-7: human breast cancer; Caco-2: human colon cancer; and WI38: normal human fetal lung fibroblast. The results are presented as mean ± SD (n = 3). $^{ns}p>0.05$, *p< 0.05, **p<0.001 and ***p<0.0001 indicate significant differences with respect to a positive control (doxorubicin). The one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test between *Lotus* species extracts and doxorubicin was performed. Doxorubicin was used as a positive control.

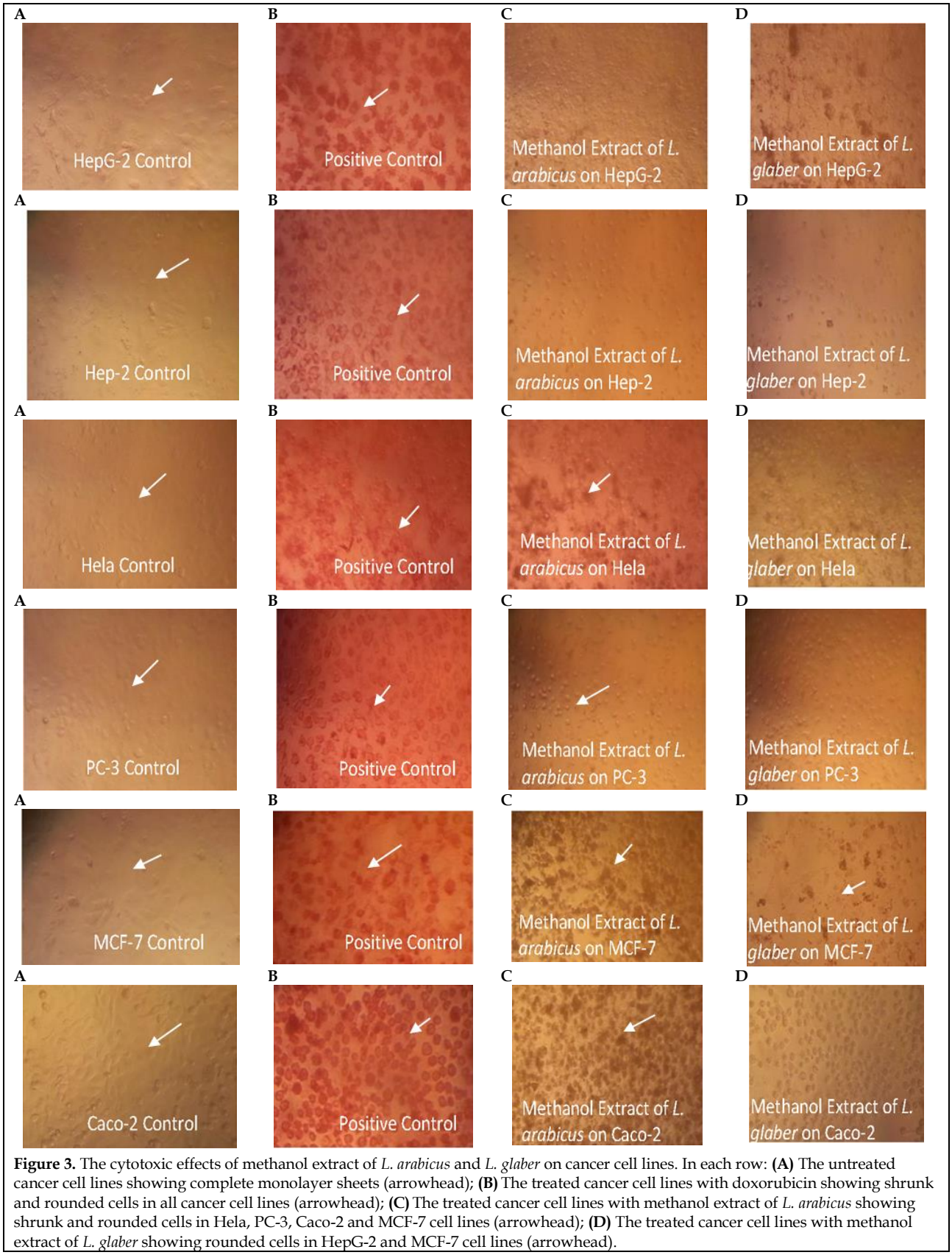


**Figure 3.** The cytotoxic effects of methanol extract of *L. arabicus* and *L. glaber* on cancer cell lines. In each row: **(A)** The untreated cancer cell lines showing complete monolayer sheets (arrowhead); **(B)** The treated cancer cell lines with doxorubicin showing shrunk and rounded cells in all cancer cell lines (arrowhead); **(C)** The treated cancer cell lines with methanol extract of *L. arabicus* showing shrunk and rounded cells in Hela, PC-3, Caco-2 and MCF-7 cell lines (arrowhead); **(D)** The treated cancer cell lines with methanol extract of *L. glaber* showing rounded cells in HepG-2 and MCF-7 cell lines (arrowhead).

**Table 4.** DPPH radical scavenging of *L. arabicus* and *L. glaber* extracts and ascorbic acid.

| Sample conc. (µg/mL) | DPPH scavenging % | | | |
|---|---|---|---|---|
| | *L. arabicus* | *L. glaber* | Sample conc. (µg/mL) | Ascorbic acid |
| 10 | 2.61 ± 0.58*** | 0.76 ± 0.17*** | 5 | 11.34 ± 2.81 |
| 20 | 4.10 ± 0.22*** | 1.93 ± 0.47*** | 10 | 16.89 ± 3.76 |
| 40 | 6.26 ± 0.83*** | 3.34 ± 0.25*** | 15 | 54.40 ± 3.81 |
| 80 | 8.41 ± 1.45*** | 5.82 ± 0.95*** | 20 | 70.51 ± 2.10 |
| 160 | 15.98 ± 1.96*** | 12.82 ± 2.10*** | 25 | 76.90 ± 0.88 |
| 320 | 27.92 ± 3.64*** | 24.51 ± 2.53*** | 30 | 81.67 ± 1.16 |
| 640 | 43.38 ± 3.32*** | 37.77 ± 3.66*** | 35 | 87.16 ± 0.64 |
| 1280 | 72.55 ± 5.83*** | 65.73 ± 5.72*** | 40 | 92.98 ± 1.44 |

The results are presented as mean ± SD (n = 3). $^{ns}$ $p>0.05$, *$p<0.05$, **$p<0.001$ and ***$p<0.0001$ indicate significant differences with respect to ascorbic acid. The one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test between *lotus* species extracts and ascorbic acids.

## DISCUSSION

The results obtained pointed out that the preliminary phytochemical screening of the two *Lotus* species, *L. arabicus* and *L. glaber* showed the presence of important bioactive compounds such as alkaloids, phenolics, flavonoids, sterols, saponins, tannins, cardiac glycosides, and carbohydrates. Our results were in accordance with other species of genus *Lotus* (Foo et al., 1996; Acuña et al., 2008; Moro et al., 2010; Girardi et al., 2014). The cytotoxic effects of methanol extracts of the two *Lotus* species were evaluated against the HepG-2, Hep-2, Hela, PC-3, MCF-7, Caco-2 and W138. The *L. arabicus* has a higher cytotoxic effect against PC3, Caco-2 and MCF-7 cell lines as compared with *L. glaber*. As far as no report of antioxidant and cytotoxic activities of *L. arabicus* were seen from an extensive literature search. However, other species that have close affiliations with this plant have been investigated and reported. Recent studies with *L. corniculatus* showed important anti-inflammatory activity in a mouse model of pleurisy induced by carrageenan, its constituent's, kaempferitrin, oleanolic acid, and β-sitosterol may be responsible for this (Koelzer et al., 2009). Its aerial parts exhibited significant antioxidant, immune stimulant and anticancer activities (Alqasoumi et al., 2013). The antiproliferative activity of n-BuOH and chloroform extracts of *L. corniculatus* on three continuous murine and human culture cell lines J774 A- 1, HEK 293 and WEHI-164 were investigated. The n-BuOH showed moderate cytotoxic activity (Abderrahmane et al., 2014). A new galloyl glycoside from *L. corniculatus* was isolated and assessed for its cytotoxicity against five human cancer cell lines, and the results showed that it had no activity (Zhao et al., 2019).

The cytotoxic effects of compound saponins were dependent on the number of sugar units: the ones having less sugar moieties were more intense in activity as compared with those having more sugar moieties (Abderrahmane et al., 2014). A possible explanation is that the number of the sugar moieties determines the hydrophilic properties of a compound; the hydrophilic compounds are less able to pass through the cell membrane of mammalian cells, which is reflected in a lower cytotoxicity (Abderrahmane et al., 2014). Furthermore, the antioxidant activity of the two *Lotus* species showed low inhibition of DPPH, where they were found to be high $IC_{50}$ values of 797 ± 79.2 µg/mL and 924 ± 87.9 µg/mL for *L. arabicus* and *L. glaber*, respectively. Our results exactly in accordance with the literature (Abderrahmane et al., 2014; El-Sharkawy et al., 2017; Zhao et al., 2019), where the low inhibition of DPPH may be due to low concen-

tration of phenolic compounds in the methanolic extracts and also, their crudes were not pure. However, the methanolic extract concentration of the sample (1280 µg/mL) of *L. arabicus* and *L. glaber* attained antioxidant activity of 70.44 ± 5.83% and 65.36 ± 5.72% scavenging of DPPH, respectively. In the present study, the crude extracts of the two *Lotus* species have been studied and further investigation using single components from these extracts may explore potent antioxidant and anticancer properties.

## CONCLUSIONS

The two *Lotus* species, *L. arabicus* and *L. glaber* showed the presence of bioactive compounds that may have potential anticancer effects. Both plants demonstrated various anticancer effects against the studied cancer cell lines. The highest anticancer effect was observed by *L. arabicus* against prostate, colon and breast cancers. Therefore, the active constituents in *L. arabicus* may be isolated and evaluated for their anticancer effect on prostate, colon and breast cancers.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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|---|---|---|---|---|
| Concepts or ideas | x | x | x | x |
| Design | x | x | x | x |
| Definition of intellectual content | x | x | x | x |
| Literature search | x | x | x | x |
| Experimental studies | x | x | x | x |
| Data acquisition | x | x | x | x |
| Data analysis | x | x | x | x |
| Statistical analysis | x | | | |
| Manuscript preparation | x | | | x |
| Manuscript editing | x | | | x |
| Manuscript review | x | x | x | x |

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