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Phytochemistry and Antiviral Properties of Two *Lotus* Species Growing in Egypt

Fitoquímica y propiedades antivirales de dos especies de *Lotus* que crecen en Egipto

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ABSTRACT

Background: *Lotus arabicus* L and *Lotus glaber* Mill. belong to the family Fabaceae, and they grow in the wild in Egypt and have different therapeutic uses in folk medicine. **Objectives:** This study aimed to evaluate the phytochemical profile, antimicrobial and antiviral properties of the methanolic extracts of two Lotus spp. growing in Egypt, *L. arabicus* and *L. glaber*. **Material and methods:** Gas chromatography-mass spectrometry was used to identify the compounds of the extracts of two *Lotus* species. An MTT colorimetric assay and the disc diffusion method were performed to investigate the antiviral and antimicrobial activities of two *lotus* species, respectively. **Results:** The n-hexane and methanol extracts of *L. arabicus* contained high percentages of alkane hydrocarbons, such as 5-methyloctadecane, while *L. glaber* contained dodecane. The major compounds in the methanol extract of *L. arabicus* were hexadecanoic acid methyl ester and dodecanoic acid,2,3-bis(acetyloxy)propyl ester. The major compounds in the methanol extract of *L. glaber* were palmitic acid and lucenin 2. The indole alkaloid ditaine was found only in *L. arabicus*. This alkaloid was identified for the first time in the genus Lotus. The antimicrobial properties of the extracts of the two *Lotus* species showed that the n-hexane extract of both *Lotus* species may have potential antifungal activity against *Candida parapsilosis* and *Aspergillus flavus*. Moreover, the methanolic extracts of both *Lotus* species have potential antiviral activity against the coxsackie B virus, but only the *L. arabicus* extract showed activity against the hepatitis A virus. **Conclusion:** *Lotus arabicus* might have potential antifungal or antiviral activity greater than *L. glaber*.

Keywords: Antimicrobial activity, Antiviral Effects, *Lotus arabicus*, *Lotus glaber*, Phytochemistry.

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RESUMEN

Antecedentes: *Lotus arabicus* L y *Lotus glaber* Mill. pertenecen a la familia de las fabáceas y crecen en estado silvestre en Egipto y tienen diferentes usos terapéuticos en la medicina popular. **Objetivos**: El objetivo de este estudio es evaluar el perfil fitoquímico y las propiedades antimicrobianas de los extractos metanólicos de dos especies de Lotus que crecen en Egipto, *L. arabicus* y *L. glaber.* **Material y métodos:** Se utilizó la cromatografía de gases-espectrometría de masas para identificar los compuestos de los extractos de las dos especies de *Lotus.* Se realizó un ensayo colorimétrico MTT y el método de difusión en disco para investigar las actividades antiviral y antimicrobiana de las dos especies de Lotus, respectivamente. **Resultados**: Los extractos de n-hexano y metanol de *L. arabicus* contenían altos porcentajes de hidrocarburos alcanos, como el 5-metiloctadecano, mientras que *L. glaber* contenía dodecano. Los principales compuestos del extracto de metanol de *L. arabicus* eran el éster metílico del ácido hexadecanoico y el éster dodecanoico, 2,3-bis(acetiloxi)propilo. Los principales compuestos del extracto de metanol de *L. glaber* fueron el ácido palmítico y la lucenina 2. El alcaloide indólico ditaína sólo se encontró en *L. arabicus.* Este alcaloide fue identificado por primera vez en el género *Lotus.* Las propiedades antimicrobianas de los extractos de las dos especies de Lotus mostraron que el extracto n-hexano de ambas especies de Lotus puede tener una potencial actividad antifúngica contra *Candida parapsilosis* y *Aspergillus flavus.* Además, los extractos metanólicos de ambas especies de *Lotus* tienen una potencial actividad antiviral contra el virus coxsackie B, pero sólo el extracto de *L. arabicus* mostró actividad contra el virus de la hepatitis A. **Conclusión**: *L. arabicus* puede tener una potencial actividad antifúngica o antiviral mayor que *L. glaber.*

Palabras claves: Actividad Antimicrobiana, Efectos Antivirales *Lotus arabicus, Lotus glaber,* Fitoquímica

INTRODUCTION

The two legume forage species of the genus *Lotus* (Fabaceae) that grow in the wild in Egypt are an annual pubescent or pilose herb (*Lotus arabicus* L.) and a perennial glabrous herb (*Lotus glaber* Mill. [L. tenuis Willd.]). They are mainly distributed in the Mediterranean region, Nile banks, cultivated ground, and wetlands of Egypt (1) and have adapted to different climatic and soil conditions (2). Previous phytochemical investigations of this genus revealed the presence of condensed tannins (4, 5), flavonoids (2,3,6-9), sterols (10), anthocyanins (11), alkaloids (12), and cyanogenic glycosides (13).

Despite these bioactive compounds present in the genus *Lotus*, few reports have investigated the antibacterial activity of some species of this genus that have close affiliations with *L. arabicus* and *L. glaber* (2, 14, 15). However, extracts of *lotus uliginosus*, *L. tenuis*, and *L. corniculatus* demonstrated antifungal activities against *Alternaria sp.* and *Fusarium graminearum* (2). Also, some compounds were isolated from ethyl acetate and butanol fraction of methanol extract of *lotus creticus* that showed antiviral activity against Hepatitis A, Herpes Simplex-1, and Coxsackie viruses (29). Additionally, we can not find reports demonstrating the antiviral activities of *L. arabicus, L. glaber,* or other species of the genus *Lotus*. Thus, this is the first report to demonstrate the antifungal and antiviral effects of *L. arabicus* and *L. glaber* and determine their bioactive compounds that could be responsible for these activities.

MATERIALS AND METHODS

Plant material:

The *Lotus arabicus* and *L. glaber* aerial parts were collected from Al-Azhar University gardens, Egypt, during the fruiting stage from May to July 2018. The material was washed and dried for 10 days in a ventilated room in the shade. The loss weight was measured every 24 hours until it reached a constant weight and then grounded to a fine powder (26).

Plant extracts:

Two hundred grams of air-dried powder of each studied plant was extracted with methanol (500 ml × 3 times) by the cold percolation method for 72 hours. The methanolic extracts were filtered through a Buchner funnel. The filtrate was evaporated in a rotary evaporator at a temperature below 40 °C, and the residue was dried in a dissector, obtaining 24.33 g/100 g DW (dry weight) and 19.70 g/100 g DW for *Lotus arabicus* and *L. glaber,* respectively. The crude methanol extract was used for GC/MS analysis to determine bioactive compounds.

Gas chromatography-mass spectrometry (GC/MS):

A Thermo Scientific TRACE 1310 gas chromatograph was attached to an ISQLT single quadrupole mass spectrometer. Column, DB5-MS, 30 m, 0.25 mm ID (J & W Scientific); ionization mode, EI; ionization voltage, 70 EV. The temperature program was as follows: 40 °C (3 min.)-280 °C (5 min.), then to 290 °C at a rate of 5 °C/min. (held for 1 min), and then static at 7.5 °C/min. Detector temperature, 300 °C; injector temperature, 200 °C; carrier gas, helium;

flow, 1.0 ml/min. Searched Library: WILEY & NIST Mass Spectral Data Base.

Hexane extract of the lipids:

One hundred grams of the air-dried powder of each studied plant was extracted with n-hexane for 24 hours using a Soxhlet apparatus. The lipids, 2.6 g/100 g DW and 3.4 g/100 g DW for *L. arabicus* and *L. glaber,* respectively, were obtained by distillation of the solvent. The last traces of solvent were removed by heating each sample in a vacuum oven at 40 °C until each sample reached a constant weight.

GC-MS analysis for lipids:

Mass spectra were recorded using a Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with an Rtx-5MS fused bonded column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The initial column temperature was maintained at 45 °C for 2 min (isothermal), programmed to 300 °C at a rate of 5 °C/min, and remained at 300 °C for 5 min (isothermal). The injector temperature was 250 °C. The helium carrier gas flow rate was 1.41 ml/min. All mass spectra were recorded under the following conditions: (equipment current) filament emission current, 60 mA; ionization voltage, 70 EV; and ion source, 200 °C. Diluted samples (1 % v/v) were injected in split mode (split ratio 1:15).

Antimicrobial assay:

Microbial strains:

Microorganisms were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The three gram-positive bacteria were *Staphylococcus aureus* (RCMB 010010), *Bacillus subtilis* (RCMB 015 (1) NRRL B-543) and Bacillus cereus (RCMB 027 (1)). The three gram-negative bacteria were *Enterobacter cloacae* (ATCC 23355), *Escherichia coli* (ATCC- 25922), and *Proteus vulgaris* (ATCC 13315). The three fungi were *Candida parapsilosis* (ATCC-22019), *Candida albicans* (ATCC-10231), and *Aspergillus flavus* (RCMB 002002).

Culture medium and inoculums:

The stock cultures of microorganisms used in this study were maintained on plate count agar slants at 4 °C. The inoculum was prepared by suspending a loop full of bacterial cultures into 10 ml of nutrient agar broth followed by incubation at 37 °C for 24 hours. Approximately 60 µl of bacterial suspensions adjusted to 106-107 colony forming units (CFU)/ml were removed and poured into Petri plates containing 6 ml of sterilized nutrient agar medium. Bacterial suspensions were spread to obtain a uniform lawn culture.

Antimicrobial activity:

The disc diffusion method was employed to evaluate the antimicrobial activities using a range of microorganisms. Sterile discs (Whatman, 6 mm) were impregnated with 10 µl of each reconstituted crude extract (1 mg/ml) and placed on the surface of Muller-Hilton agar dispersion plates inoculated with microbes. Each extract was tested in triplicate. Control discs containing 10 µl of the solvent (DMSO) were used as a negative control. Standard antibiotics, 4 µg/ml gentamycin (antibacterial agent) and 100 µg/ml amphotericin B (antifungal agent), served as positive controls. Agar plates containing bacteria were incubated at 37 °C for 24-48 hours; the yeast Candida albicans was incubated at 30 °C for 24-48 hours; and the filamentous fungi *Aspergillus flavus* was incubated at 25 °C for 48 hours. Blank paper discs (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated with 10 µl of the tested concentration of the stock solution. Inhibition zones were measured and recorded as the diameter of growth-free zones (IZs), which included the diameters of the discs in mm, at the end of the incubation period (21, 22). For disc diffusion, the zone diameters were measured with slipping calipers from the National Committee for Clinical Laboratory Standards (NCCLS) (23).

Antiviral assay:

Plant extract:

The methanol extracts of the aerial parts of the two *Lotus* species were prepared as described previously (16). The crude extracts were resuspended in methanol to 50 mg/ml concentration and stored in the dark at 4oC. For the antiviral tests, each extract was diluted with Dulbecco's modified Eagle medium (DMEM) with 0.1 % serum to give a 1 mg/ml final concentration of the extract, which was filtered through a sterile 0.2 µm pore cellulose acetate filter (16).

Cells and viruses:

The Vero cell line (Cercopithecus aethiops kidney epithelial cells) was maintained in RPMI 1640 medium (Gibco, Tunisia) supplemented with fetal

bovine serum (FBS) (10 % v/v) plus L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were incubated in a 5 % CO2 humidified atmosphere at 37 °C. Hepatitis A (HAV) and coxsackie (COXB4) adenoviruses were kindly provided by Dr. Mohammed Ali, Laboratory of Virology, Science Way for scientific research and consultations, Faculty of Medicine, Al-Azhar University, Egypt.

Determination of the maximum non-toxic concentration:

Vero cells were seeded into 96-well microliter plates (100 µl/well, 1×104 cells/well). When cell confluence reached approximately 100 %, the growth medium was decanted. The cell monolayer was washed twice with wash media and then treated with different concentrations of the extracts (starting at 10 mg/ml; 312.5 µg/ml/well). Double-fold dilutions of the tested sample were made in DMEM, and 0.1 ml of each dilution was tested in different wells; 3 wells were used as a control and received only the maintenance medium. The plate was incubated in an atmosphere of 5 % CO2 at 37 °C and frequently examined for up to 2 days. Cells were checked for any physical signs of toxicity, e.g., partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was performed as described previously (17). In brief, MTT solution was prepared (5 mg/ml in PBS) (B10 BASIC CANADA INC.), and 20 µl of MTT solution was added to each well. The plate was then placed on a shaking table at 150 rpm for 5 min to thoroughly mix the MTT solution into the media followed by incubation (37 °C, 5 % CO2) for 4 hours to allow the MTT to be metabolized. The media was decanted, and the plate was dried on paper towels to remove any residue if necessary. The formazan (MTT metabolic product) was resuspended in the 200 µl of DMSO (dimethyl sulfoxide) that was added to each well. The plate was placed on a shaking table at 150 rpm for 5 min to mix the formazan into the solvent thoroughly. The optical density was then measured at 560 nm, and the background at 620 nm was subtracted. The optical density should directly correlate with the cell quantity. The maximum non-toxic concentration (MNTC) of the extract was determined. The experiment was conducted in triplicate, and averaging was performed.

Antiviral activity:

The hepatitis A (HAV) and coxsackie (COXB4) viruses were separately propagated in Vero cells, and the infective titer of the stock solution was 10-7 TCID 50/ml (50 % tissue culture infective dose). The maximum non-toxic concentration (MNTC) of each extract of the two studied *Lotus* species was evaluated for their antiviral properties with a cytopathic effect (CPE) inhibition assay (18). The non-lethal dilution of the tested sample (MNTC) was dissolved in 1 ml of distilled dimethyl sulfoxide (DMSO). The volume was made up to 10 ml with a maintenance medium to obtain a stock solution with a concentration of 1 mg/ml, sterilized by filtration, and further dilutions were made from the stock solution. The cytotoxicity assays were carried out using 0.1 ml of cell suspension (TCID50) containing 10,000 cells seeded in each well of a 96-well microliter plate. Each test sample (0.1 ml) was added to different wells, leaving 3 wells as the control without the test sample but with DMSO. The microliter plates were incubated at 37 °C in a humidified incubator with 5 % CO2 for two days. Cells were checked daily for any physical signs of toxicity, e.g., partial or complete loss of the monolayer, rounding, shrinkage, cell granulation under an inverted microscope, and the cytopathogenic effect (CPE) was determined. Evaluation of the viability of the infected and non-infected cells was performed using the absorbance values of the formazan used in the MTT inclusion assay, as described for the cytotoxicity assay. Anti-HAV and anti- COXB4 activities were determined by the inhibition of cytopathic effects compared with the control, i.e., the protection offered by the test samples to the cells was scored. The reduction in the yield of the virus after the cells were treated with the plant extracts was determined.

Determination of CC50 values of *Lotus* extracts on Vero cells:

GraphPad Prism version 7 software, California, USA, was used to calculate the 50 % cytotoxic concentration (CC50) values of the different concentrations of the methanol extracts of the two studied *Lotus* species on Vero cells. The percent cell viability was calculated using Equation [1] (19):

$$\text{Cell viability}(\%) = \left[\frac{\text{Mean OD of extract treated cells}}{\text{Mean OD of control cells}}\right] \text{x}100 \quad (1)$$

Determination of the maximum non-toxic concentration [MNTC]:

The cell toxicity percentage was obtained as the [cell viability percentage – 100] and plotted on the Y-axis; the concentration of the *Lotus* species extract was plotted on the X-axis, which allowed to obtain the maximum non-toxic concentration (MNTC). The MNTC for both *Lotus* species was 625±7 µg/ml, which was used for antiviral studies (Fig. 1)

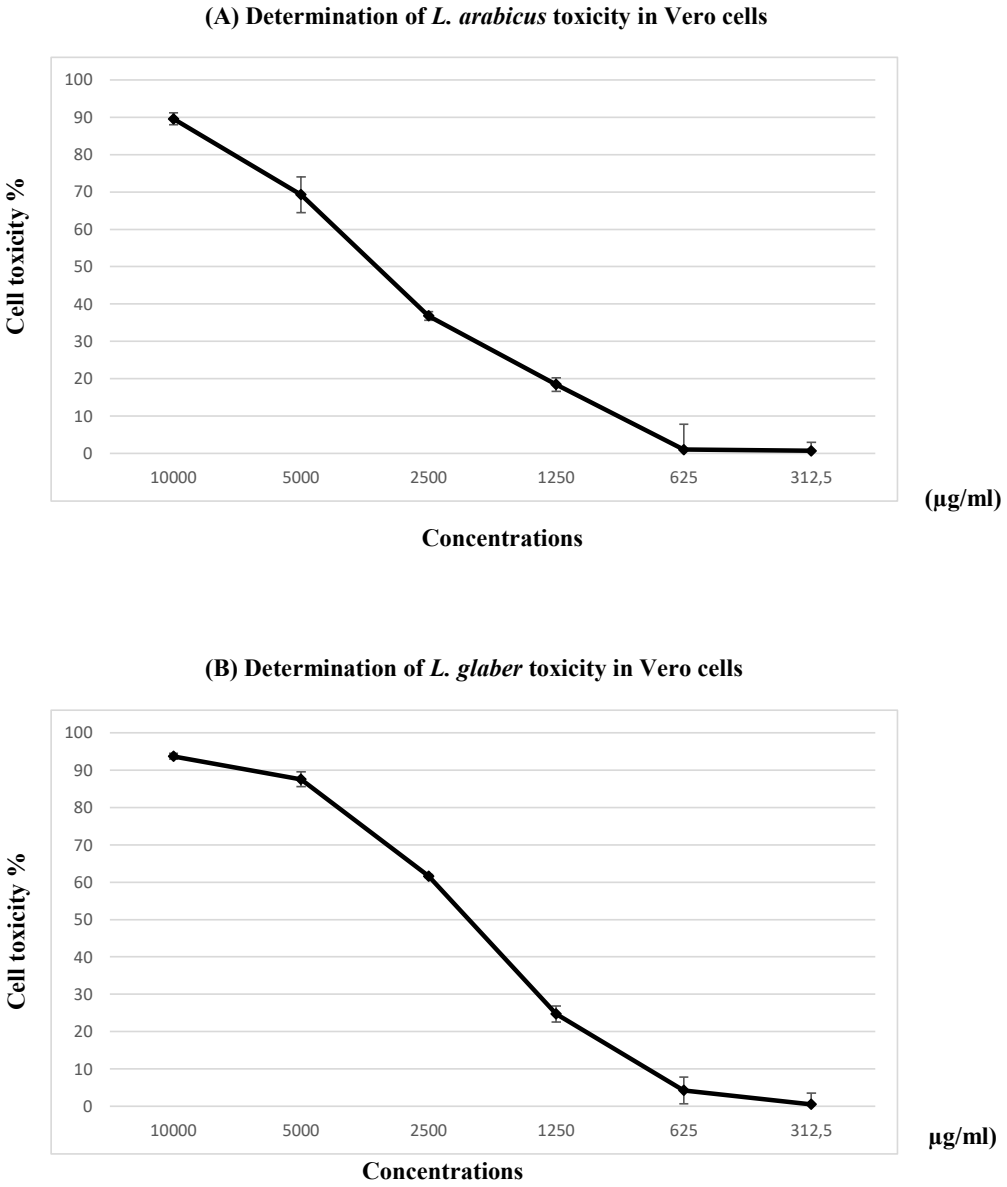

Figure 1: Determination of the maximum non-toxic concentration (MNTC) of (A) *L. arabicus* (B) *L. glaber* and the MNTC is 625 ±7 µg/ml for both *lotus* species

Determination of the cell viabilities of Vero cells infected with viruses:

The cell viabilities of Vero cells infected with viruses were determined after treating the cells with the *Lotus* extracts at their MNTCs, each 625±7 µg/ml, using Equation [2] (20)

$$\text{Cell viability \%} = \left[\frac{\text{OD treatment}}{\text{OD Vero control}}\right] \text{x100} \qquad (2)$$

Determination of the cell protection rate (CPR) of the *Lotus* extracts against Vero cells infected with viruses

The maximum non-toxic concentration (MNTC), 625±7 µg/ml, was used to determine the cell protection rate, which is the protection offered by the test samples to the cells, of the two *Lotus* species extracts in Vero cells infected with the HAV and COXB4 viruses using Equation [3] (20).

$$\text{CPR } \% = \left[\frac{\text{OD treatemnt} - \text{OD virus control}}{\text{OD Vero control} - \text{OD virus control}} \right] \text{x}100 \quad (3)$$

Determination of the virus inhibition rate (I) of the Lotus extracts on virus-infected Vero cells

The virus inhibition rates (I %) of the *Lotus* extracts at the MNTC against the Vero cells infected with the HAV, and COXB4 viruses were calculated using Equation [4] (20).

$$\text{I } \% = \left[\frac{\text{OD treatment} - \text{OD virus control}}{\text{OD virus control}} \right] \text{x}100 \quad (4)$$

Determination of the inhibition rate of cell growth (IR):

The inhibition rate of cell growth (IR %) was determined using Equation [5] (20).

$$\text{IR } \% = \left[\frac{\text{OD control} - \text{OD treatment}}{\text{OD control}} \right] \text{x}100 \quad (5)$$

Statistical analysis:

Statistical analyses were undertaken using GraphPad Prism version 7 software, California, USA. Analysis of variance and t-tests were applied. $^{ns}p > 0.05$; $^{**}p < 0.001$ and $^{***}p < 0.0001$ indicate statistically significant differences. Values are expressed as the mean ± standard deviation.

Microscopy:

An inverted microscope (Nikon, 118811) with an 8× objective was used to observe the morphological structures of Vero cells infected with the HAV and COXB4 viruses and Vero cells infected with the viruses treated with the methanol *Lotus* species extracts at the concentration of 625±7 µg/ml.

RESULTS

The GC/MS results of the lipid contents of *L. arabicus* and *L. glaber* species are illustrated in Fig. 2. The spectrum shows that the main m/z peaks found in the lipid extracts of aerial parts of the two *Lotus* species were alkane hydrocarbons: 5-methyloctadecane had a relative abundance (R.A.) 33.66 % in *L. arabicus*. In contrast dodecane had an R.A. 33.85 % in *L. glaber*. Three fatty acids were identified in *L. arabicus*: methyl stearate (R.A. 0.15 %), linoleic acid ethyl ester (R.A. 0.15 %), and hexadecanoic acid ethyl ester (R.A. 2.48 %). In *L. glaber*, three fatty acids were identified, hexadecanoic acid ethyl ester (R.A. 3.76 %), methyl stearate (R.A. 0.49 %), and linoleic acid ethyl ester (R.A. 0.27 %). Seven sterols were identified in *L. arabicus*, with gamma sitosterol showing the highest content (R.A. 1.41 %), whereas this sterol had an R.A. 0.35 % in *L. glaber*. 1,3,4,6-Tetrakis-O-(trimethylsilyl)-β-D-*fructofuranosyl* -α-D-glucopyranoside (4.21 %) were present only in *L. arabicus*.

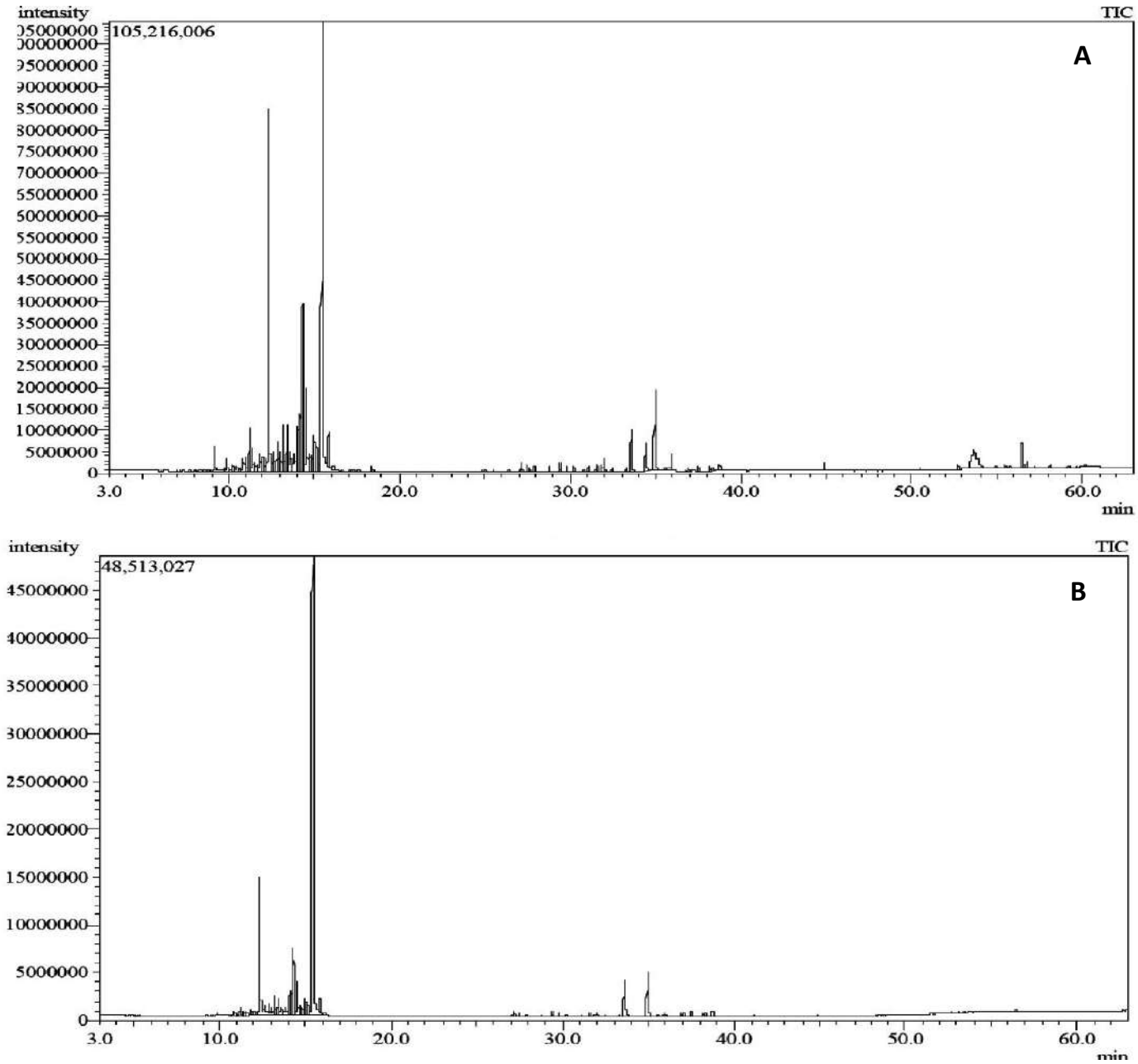

Figure 2. GC/MS of the n-hexane extracts of (A) *L. arabicus* and (B) *L. glaber*.

The GC/MS analysis of the methanol extracts revealed that the aerial parts of the two studied *Lotus* species contained different classes of bioactive secondary metabolites, including fatty acids, sterols, flavonoids, terpenes, and alkaloids (Table 1 and Fig.3). The major compounds in the methanolic extract of *L. arabicus* were hexadecanoic acid methyl ester (R.A. 21.50 %), dodecanoic acid,2,3-bis(acetyloxy)propyl ester (R.A. 6.35 %), octadecanoic acid (R.A. 4.00 %), dihydroxanthin (R.A. 3.97 %), eicosanoic acid (R.A. 3.34 %); flavonoids, lucenin 2 (R.A. 0.53 %); and one carbazole indole alkaloid, ditaine (R.A. 2.57 %). The major compounds in *L. glaber* were palmitic acid (R.A. 6.59 %), methyl stearate (R.A. 1.76 %), hexadecanoic acid methyl ester (R.A. 1.28 %), dodecanoic acid,2,3-bis(acetyloxy)propyl ester (R.A. 1.28 %), flavonoids, lucenin 2 (R.A. 1.35 %), (5α) pregnane-3,20α-diol, 14α,18α-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-diacetate (R.A. 3.09 %).

Table 1. Chemical constituent identification of extracts of *L. arabicus* and *L.glaber* by gas chromatography/mass spectrometry

N°	COMPOUNDS	M.F.	M.W.	*L. arabicus*		*L. glaber*	
				RT.	R.A.%	RT.	R.A. %
1	Octadecenoic acid,(2-phenyl-1,3-dioxolan-4-yl) methyl ester	$C_{28}H_{44}O_4$	444	20.70	0.71		
2	3,5-Heptadienal, 2-ethylidene-6-methyl	$C_{10}H_{14}O$	150	21.46	2.26		
3	2-Hydroxymethyl-9-[-d-ribofuranosyl]hypoxanthine	$C_{11}H_{14}N_4O_6$	298	22.61	0.22		
4	2-Carbamyl-9-[-d-ribofuranosyl]hypoxanthine	$C_{11}H_{13}N_5O_5$	295	22.79	0.63		
5	Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	$C_{19}H_{34}O_6$	358	30.18	6.35	40.16	1.28
6	Oxindole, 1,3-dimethyl-3-[3-[N,N-dimethylamino]propyl]-5-methoxy	$C_{16}H_{24}N_2O_2$	276	30.93	2.62		
7	Dihydroxanthin	$C_{17}H_{24}O_5$	308	31.06	3.97		
8	Myristic acid (Tetradecanoic acid)	$C_{14}H_{28}O_2$	228	37.48	0.05		
9	2-Pentadecanone, 6,10,14-trimethyl- (Myristic acid Trimethyl)	$C_{18}H_{36}O$	268	37.97	0.99		
10	Lucenin 2	$C_{27}H_{30}O_{16}$	610	38.94	0.53	51.02	1.35
12	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	40.94	21.50	42.99	1.28
13	Ditaine	$C_{22}H_{29}N_2O_4$	385	41.97	2.57		
14	Stevioside	$C_{38}H_{60}O_{18}$	804	42.70	2.83		
15	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	284	42.76	4.00		
16	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256	43.30	0.21	45.53	6.59
17	Eicosanoic acid	$C_{20}H_{40}O_2$	312	43.37	3.34		
18	Methyl stearate	$C_{19}H_{38}O_2$	298	46.92	0.67	49.10	1.76
19	cholestane 3,5,6triol	$C_{27}H_{49}O_3$	420	65.97	0.29		
20	2-Hexadecanol	$C_{16}H_{34}O$	242			40.16	1.28
21	Octadecanoic acid 9,10- dichloro-methyl ester	$C_{19}H_{36}Cl_2O_2$	366			48.19	1.14
22	(5α)Pregnane-3,20α-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	$C_{28}H_{43}NO_6$	489			48.44	3.08
23	(5α)- Cholestan-3-one, cyclic 1,2-ethanediyl acetal,	$C_{29}H_{50}O_2$	430			51.02	1.35

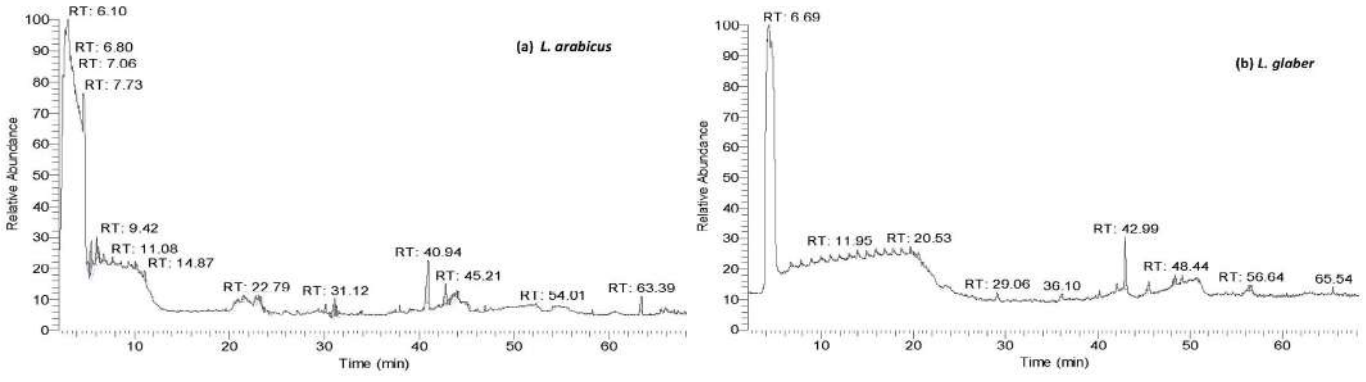

Figure 3. Gas chromatography-mass spectrometry spectra of the methanol extracts of (a) *L. arabicus* and (b) *L. glaber*.

The methanolic extracts from the two studied *Lotus* species showed no inhibitory effects on the growth of any of the tested bacteria. However, the n-hexane extracts showed efficient inhibition of two fungi, *Candida parapsilosis*, and *Aspergillus flavus*. The growth of these fungi was inhibited by both n-hexane extracts and showed a higher range of inhibition diameters zones (IDZs), from 11.7±0.8 – 14±0.9 mm for *Candida parapsilosis* and 10.3±0.3 – 11.7±0.8 mm for *Aspergillus flavus*. The IDZ for amphotericin B ranged from 16±0.07 – 22±0.05 mm at 100 µg/zone (Table 2).

Table 2. Antimicrobial assay of n-hexane extracts of the studied two *Lotus* species.

Tested microorganisms	Sample code	*Lotus arabicus*	*Lotus glaber*	Amphotericin B
FUNGI		mm		
Candida parapsilosis	(ATCC-22019)	14 ± 0.9 **	11.7 ± 0.8 **	22 ± 0.05
Aspergillus flavus	(RCMB 002002)	11.8 ± 0.8^{ns}	10.3 ± 0.3^{ns}	16 ± 0.07
Candida albicans	RCMB005003(1) ATCC 10231	NA	NA	20

The test was done using the diffusion agar technique, Well diameter: 6.0 mm (100µl was tested), Inhibition zone diameter (mm / mg sample), RCMB: Regional Center for Mycology and Biotechnology, Positive control for fungi: Amphotericin B 100µg/ml. The sample was tested at 10 mg/ml concentration. The results are presented as mean ± SD. ns $p > 0.05$ and ** $p < 0.001$indicate the significant difference with respect to amphotericin B (a positive control). The t-test analysis was applied to compare the inhibition diameter zone (IDZ) of both n-hexane extracts of *lotus* species with amphotericin B. *NA: No antifungal activity.

The results of cytotoxicity assays are presented in Table 3, and the extracts of both studied *Lotus* species were found to be non-cytotoxic. The extract concentrations at which 50 % cytotoxicity (CC50) was observed were 4.68±0.171 mg/ml for *L. arabicus* and 2.11±0.08 mg/ml for *L. glaber* when compared to the untreated controls. The maximum non-toxic concentration (MNTC) of the extracts on Vero cells was estimated to be 625±7 µg/ml. This concentration was used while testing the antiviral potency of the plant. The methanol extract of the areal parts of *L. arabicus* exhibited detectable antiviral effects towards HAV and COXB4 with an inhibitory concentration of 625±7 µg/ml. However, the *L. glaber* extract failed to show significant antiviral properties against HAV, but it possessed an antiviral effect against COXB4 (Table 3). The results obtained by both the CPE inhibition assay and virus yield assay were comparable. The extracts from *L. arabicus* and *L. glaber* exhibited viral inhibitory activity in both assays against COXB4. However, the *L. glaber* extract failed to reduce the HAV virus yield compared to the virus yield reduction that was found in the *L. arabicus* extract. The data showed that the antiviral effects of *L. arabicus* in infected HAV-infected and COXB4 -infected Vero cells were significantly different from the untreated Vero cells. Meanwhile, the antiviral effects of *L. glaber* were only significant for the COXB4 -infected Vero cells compared to the untreated infected Vero cells (Fig.4).

Table 3. Cell viability and antiviral activity of two *Lotus* species methanol extracts at MNTC.

Virus	*Lotus* sp.	MNTC µg/ml	Cell viability %	IR %	CC_{50} (µg/ml)	CPR %	I %	Viral activity %
	Control (vero cells)		100	0				
HAV		625±7	49.57 ±5	50.43 ±5		0	_	
	L. arabicus		79.20±2.5 ***	20.79 ±2.5	4685.38 ±171	59 ± 10	59.8 ±7	41 ± 7
	L. glaber		53.41±3.4 ^{ns}	46.58 ±3.4	2110.53 ± 83	8 ± 5	7.5 ± 5	92 ± 5
	Control (vero cells)		100	0				
COXB4			46.15 ±3.5	53.84 ±3.5		0	_	
	L. arabicus		72.9±1.1 ***	27.06 ±1.1	4685.38 ±171	50 ± 4	58 ±10	50 ±10
	L. glaber		84.76±1.5 ***	15.24 ±1.5	2110.53 ± 83	72 ± 2	83.8 ± 6	28 ± 6

MNTC: maximun nontoxic concentration; Cell viability % = [Mean $OD_{extract\text{-}treated\ vero\ cells}$/ Mean $OD_{vero\ control\ cells}$] x 100; IR: inhibition rate of cell growth, IR % = [($OD_{control}$ - $OD_{treatment}$ / $OD_{control}$)] x 100; *CC*50: The 50 % cytotoxic concentration calculated by GraphPad Prism version 7 software; CPR : the cell protection rate, CPR % = [(ODextract -ODvirus control) / (ODcell control -ODvirus control)] x 100; I : virus inhibition rate, I % = [($OD_{treatment}$ - $OD_{virus\ control}$ / $OD_{virus\ control}$)] x 100; Viral activity %= (I %- 100). The results are presented as mean ± SD. ns $p > 0.05$ and ***$p < 0.0001$ indicate significant differences with respect to untreated cells (control). The t-test analysis was applied to compare the cell viability percentage of *lotus* extracts on vero-infected HAV and COXB4 viruses with untreated cells.

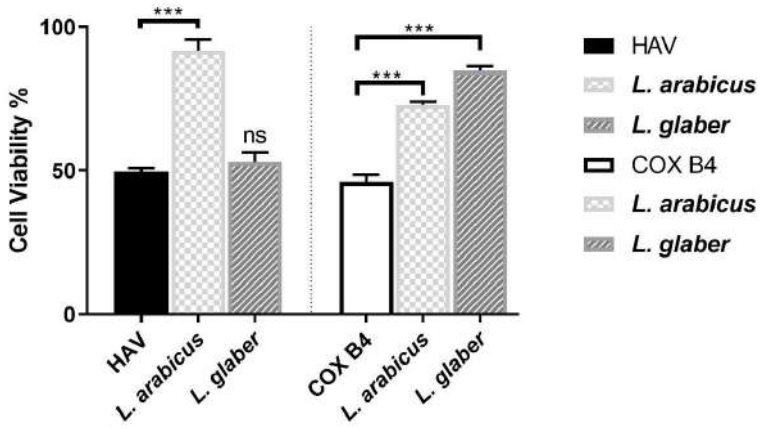

Figure 4. The antiviral effects of the methanol extracts of *L. arabicus* and *L. glaber* on Vero cells infected with HAV and COXB4. The results are presented as the mean ± SD. $^{ns}p > 0.05$ and $***p < 0.0001$ indicate significant differences with respect to untreated cells (control). The t-test analysis was applied to compare the cell viability percentages of the *Lotus* extracts on HAV- and COXB4 -infected Vero cells with untreated cells.

Microscopic examination of the HAV- and COXB4 -infected Vero cells treated with 625±7 µg/ml plant extracts was performed. The analysis revealed that the HAV and COXB4 viruses caused Vero cells to have irregular outlines and show cytoplasmic projections, intense cytoplasmic vacuolization, nuclear membrane disintegration, mottled cytoplasm, and lumps of diffuse mass distributed throughout the cytosol with dense lysosomes and myelin figures compared with infected cells treated COXB4 with the methanol extracts of the *Lotus* species (Fig. 5).

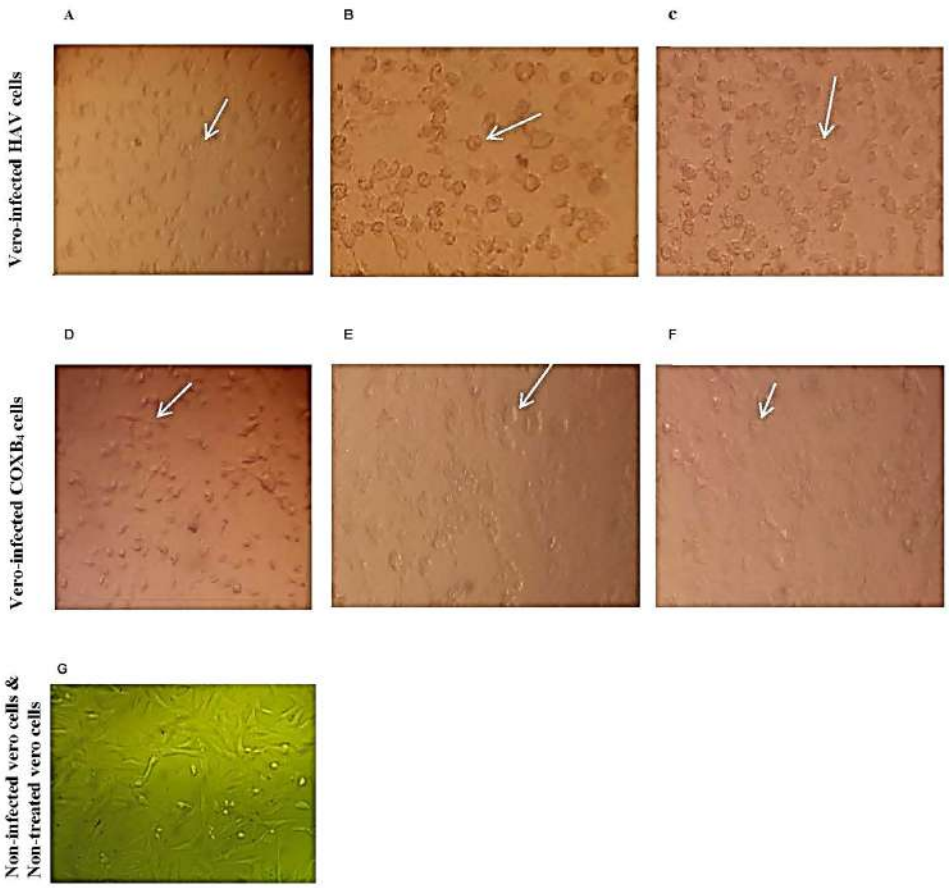

Figure 5. (A) The effects of the HAV virus on Vero cells. (A) The effects of *L. arabicus* on Vero-infected HAV-infected Vero cells. (C) The effects of *L. glaber* HAV-infected Vero cells. (D) The effects of the COXB4 virus on Vero cells. (E) The effects of *L. arabicus* on COXB4 -infected Vero cells. (F) The effects of *L. glaber* on COXB4 -infected Vero cells (G) Non-infected and non-treated vero cells (Negative control vero cells).

DISCUSSION

The GC/MS analysis of *L. arabicus* and *L. glaber* methanolic extract showed different phytochemical compounds such as eicosanoic acid, sitosterol, and one carbazole indole alkaloid. These findings are in line with those found in the literature, where the GC/MS analysis of ethanolic extract of leaf, stalk, and flower of *lotus* species, *Nelumbo nucifera, r*evealed also the presence of eicosanoic acid in the flower, sitosterol in the stalk and 1H-Indole, 5-methyl-2-phenyl- in the leaf (25). Generally, the tested organisms were more sensitive to the hexane extract of *L. arabicus* than the hexane extract of *L. glaber*. The extracts of *L. arabicus* and *L. glaber* did not show any antibacterial activities against the studied bacteria. Dalmarco et al. reported similar observations in their experiments where the crude extract, aqueous and n-butanol fractions of *L. corniculatus* var. São Gabriel, against all bacteria tested, were considered inactive (14). Also, Girardi et al. stated that the birdsfoot trefoil cv. São Gabriel (*L. corniculatus*) hydro-alcoholic extract and its fractions were inactive against gram-negative bacteria and displayed weak to moderate activity against gram-positive bacteria (2). They added that larger contents of coumarins, flavonoids, catechin, epicatechin, and rutin were directly related to the antifungal activity. However, Salman et al. showed that the ethanolic extract of *L. corniculatus* had remarkable activity against some gram-negative bacterial strains, and it also showed improved susceptibility against two fungal strains (15). Demirkol reported high antibacterial activity from the chloroform, ethanol, and ethyl acetate shoot extracts of *L. aegaeus* and *L. corniculatus* against *Clavibacter michiganensis* and the shoot extracts of all solvents of *L. angustissimus* against *Pseudomonas phaseolicola* (24). It was reported that 5-methyloctadecane, hexadecanoic acid methyl ester (palmitic acid, methyl ester), dodecanoic acid,2,3-bis(acetyloxy)propyl ester, and dodecane found in methanolic extract of *Colpomenia sinuosa, Padina pavonia, Cystoseira barbata,* and *Sargassum vulgare* showed potential antifungal activities against *Aspergillus niger, A. flavus, Penicillium parasiticus, Candida utilis,* and *Fusarium solani* (27). These compounds were present in high amounts in the methanol extracts of *L. arabicus* and *L. glaber.* Also, the free fatty acids hexadecanoic acid methyl ester (palmitic acid, methyl ester) found mainly in ethanol extract of *Alpinia eremochlamys,* and *Etlingera acanthoides* showed antiviral activity against HIV-infected MT-4 cells (28). Lucenin 2 found in both studied *lotus* species may possess antiviral activity. This finding is in line with those found in the literature, where the lucenin 2 was isolated from ethanolic extract of *bignonia binate* leaves showed antiviral activity against COVID-19 (30).

CONCLUSION

The methanolic extracts of both plants, *L. arabicus* and *L. glaber,* did not show antibacterial activity. However, the hexane extracts did show antifungal activity against two fungal strains, *Candida parapsilosis*, and *Aspergillus flavus*. Furthermore, the methanolic extracts of *L. arabicus* and *L. glaber* exhibited viral inhibitory activity against coxsackie (COXB4), but *L. glaber* failed to reduce the virus yield of hepatitis A (HAV) compared to the virus yield reduction found from *L. arabicus*. Therefore, the major compounds 5-methyloctadecane, palmitic acid and dodecanoic acid,2,3-bis(acetyloxy)propyl ester present in *L. arabicus* and dodecane, palmitic acid, and lucenin 2 found in *L. glaber* may have potential antifungal or antiviral activity from their hexane and methanol extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS´ CONTRIBUTIONS

AMMY, ZASE the data collection, conducted the data analysis, and manuscript revisions. AMMY, ZASE, and DAMM participated in the development of the study protocol and the study design, and conducted the data analysis, interpretation of the findings, manuscript writing, and manuscript revisions. MMY participated in the statistical analysis and manuscript revisions. All authors read and approved the final manuscript.

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