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Research Article

Terminalia arjuna flowers: Secondary metabolites and antifungal activity

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ABSTRACT

Fungal pathogens are able to cause serious infections to human. Candidiasis caused by *Candida* species and pulmonary aspergillosis caused by *Aspergillus fumigatus* may occur as secondary complication in immunocompromised patients. Meanwhile, biologically guided isolation of bioactive constituents from natural sources represents a promising strategy in drug discovery process. In this concern, the antimicrobial screening of 11 plants collected from Egypt was performed against several microbes. *Terminalia arjuna* flower extract was among the most active extracts against *C. albicans*. The antifungal potential of n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) fractions of *T. arjuna* flower were tested against *C. albicans*, *A. fumigatus* and *Fusarium avenaceum*. Results revealed a potent activity of EtOAc extract against *C. albicans* (100% inhibition, MIC=7.81 µg/mL). Chromatographic investigation of *T. arjuna* flower afforded nine compounds; β-sitosterol (**1**), stigmasterol (**2**), β-sitosterol-3-*O*-β-D-glucopyranoside (**3**), rosamultin (**4**), niga-ichigoside Fl (**5**), luteolin (**6**), apigenin (**7**), gallic acid (**8**) and methyl gallate (**9**). The structures of the isolated compounds were elucidated using different spectroscopic techniques especially 1D and 2D NMR. Among the isolated compounds, the two triterpenoid glycosides (**4**) and (**5**) have not been previously tested for antifungal potential. The current study indicated moderate activity of rosamultin (**4**) and niga-ichigoside Fl (**5**) (MIC 250 µg/mL) against *C. albicans*.

Keywords:
Terminalia arjuna flower, *Candida*, *Aspergillus fumigatus*, Rosamultin, Niga-ichigoside Fl

1. INTRODUCTION

The current increased incidence of fungal infections could be attributed to the increased number of immunocompromised patients who usually suffer from fungal infection. Accordingly, the development of antifungal agents with wide spectrum of activity or new mechanism of action is a crucial need. Yeasts such as *Candida* sp., filamentous fungi such as; *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp., as well as the dermatophytes *Trycophyton* sp., *Microsporum* sp. and *Epidermophyton* sp. are considered as the most frequent fungal pathogens. Among all invasive fungal infections, *Candida* represents 70-90% while *Aspergillus* represents 10-20%[1]. In immunosuppressed patients receiving chronic corticosteroid treatment, *A. fumigatus* is responsible for over 70% of all cases of invasive pulmonary aspergillosis. Recently, pulmonary aspergillosis was also noted as a secondary complication of coronavirus disease 2019 (COVID-19), especially among critically ill patients in the intensive care unit (ICU)[2].

Candidiasis is the infection with overgrowth of *Candida* species, especially *C. albicans*, in the mucous membranes such as oral cavity and vagina[3]. Oral candidiasis occurs in children, patient on antibiotic or steroid therapy, or immunodeficient patients, while in women *Candida* infection causes vaginitis, white vaginal discharge and itching[3].

Plant kingdom offers a wide diversity of species with different enzyme systems and biochemical patterns. This provides a wide and undepletable source of chemical chemical moieties with variable biological activities. Testing the antifungal activity of plant extracts, using

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diffusion method, is a fast way for screening a large number of extracts. Moreover, adopting bio-guided isolation technique adds further advantages in terms of reducing the effort, cost and time spent during fractionation and structural elucidation processes.

Terminalia genus, the second largest genus of family *Combretaceae*, constitutes many species that were widely employed in traditional medicine for the treatment of various diseases such as: abdominal disorders, bacterial infections and skin diseases. Several pharmacological activities were also stated for *Terminalia* species e.g. cardioprotective[4], antioxidant, anti-inflammatory and hepatoprotective activity[5]. Terças et al. (2017)[6] reported the antifungal potential of *T. catappa* extracts against *C. albicans* and detected tannins and flavonoid glycoside in the most active subfraction. In addition, Sitapha et al, (2013)[7] concluded that extracts from bark of *T. ivorensis* A. Chev. exhibited antifungal activity against *A. fumigatus* more than against *C. albicans.* Similarly, *T. mantaly* H. Perrier was found active against *A. fumigatus*[8]. Moreover, *T. arjuna* leaves and bark were proved to be effective against ear pathogens especially *S. aureus*[9].

Many useful secondary metabolites e.g. flavonoids [10-12], phenolic acids[11-12], triterpene glycosides[11,13-14], sterols[10-11], gallotannins[11-13], ellagitannins[11,13] and proanthocyanidins[12] were isolated from *T. arjuna* leaves, bark, root and fruits. Among them, tannins and flavonoids were found responsible for the recorded anticancer activities while the cardiovascular effects were stated to be due to the triterpenoids content of this species[11]. Notably, no single report was traced concerning *T. arjuna* flowers.

The current research was designed to test the antimicrobial potential of eleven plants collected from the Egyptian flora. Afterwards, extract exhibiting promising activity was subjected to phytochemical exploration using different chromatographic and spectroscopic procedures.

2. MATERIALS AND METHODS

2.1. Plant materials

Different plants: *Araucaria columnaris* leaves, *Cupressus sempervirens* fruits, *Cycas revoluta* leaves, *Dioon edule* leaves, *Hordeum vulgare* leaves, *Pisum sativum* fruit pericarp, *Reaumuria hirtella* jaub. Et herb, *Fragaria ananassa* calyx, *Terminalia arjuna* flowers, *Thevetia peruviana* fruit, *Thuja orientails* leaves were collected from Beni-Suef, Matrouh, and Giza governorates. The identity of these plants was confirmed by Dr. Abdelhalim Mohamed (Plant Taxonomy Department, Agricultural Research Institute, Egypt). The Latin names, families, part-used, voucher numbers and area of collection were listed in Table 1.

2.2. General instruments and chemicals

NMR spectra were recorded on a Bruker NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR except for luteolin which was run at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. Column chromatography was carried out with Fluka silica gel (70-230 mesh size), Sephadex LH-20 (Sigma-Aldrich-USA) and RP C-18 (40-63 µm, Sorbent technologies, USA). Solvents were purchased from El Nasr for Intermediate Chemicals, Egypt. Microorganisms tested were *Staphylococcus aureus* (ATCC 9027), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9022) *Aspergillus fumigatus* ATCC 1022, *Fusarium avenaceum* ATCC24362, and *Candida albicans* ATCC14053. All strains were American type culture collection (ATCC). XTT [2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] was obtained from Sigma-Aldrich-chemicals, USA. Sabouraud dextrose agar and Muller Hinton agar were obtained from Oxoid Ltd., Basingstoke, Hampshire, UK. The BioTek 800 TS Microtiter plate reader was used (BioTek, Santa Clara, CA, USA).

2.3. Extraction and isolation

Powdered plants were extracted with 70% ethanol for the primary screening. The prepared extracts were dried under reduced pressure and used in the primary antimicrobial screening (Table 1). Extract with promising activity in the primary assay was subjected to solvent-solvent fractionation using solvents of different polarities; *n*-hexane, DCM and EtOAc. This fractionation step was used as a preliminary separation to simplify complex chemical compounds into broad groups based on their polarities. The prepared fractions were dried under reduced pressure and the residues were screened for their antifungal activity (Tables 2-3). *T. arjuna* flower extract was among the most active extracts against *C. albicans* while its chemical composition was not previously explored so, it was selected to the phytochemical investigation. One kg dried powdered *T. arjuna* flower was exhaustively extracted with 70% ethanol (5Lx 3) by percolation and concentrated under reduced pressure. The total ethanol extract was fractionated using the aforementioned solvents. The three fractions were subjected to variable chromatographic methods in order to isolate the major secondary metabolites (Figure 1).

2.3.1. n-Hexane fraction

n-hexane fraction was saponified using the method of Elghondakly et al. (2020)[15]. Unsaponifiable fraction (1.1 g) was chromatographed on silica gel column (50 g) and eluted using *n*-hexane with 2% increasing increments of EtOAc, to afford a mixture of two compounds **1** and **2** (100 mg).

Table 1. Primary sensitivity tests using agar diffusion method against some microorganisms using ethanolic extract of collected plant sample from different regions in Egypt.

Botanical names	Family	Part used	Voucher NO.	Inhibition zone (mm)				Area of collection
				Cacdida albicans	*Staphylococcus aureus*	*E. coli*	*Pseudomonas aeruginosa*	
Araucaria columnaris	Araucariaceae	Leaves	BUPD-79	17	12	11	12	M
Cupressus sempervirens	Cupressaceae	Seeds	BUPD-80	17	14	12	12	M
Cycas revoluta	Cycadaceae	Leaves	BUPD-43	11	13	11	12	BS
Dioon edule	Zamiaceae	Leaves	BUPD-81	16	11	11	NA	G
Hordeum vulgare	Graminae	Leaves	BUPD-82	NA	11	11	13	BS
Pisum sativum	Leguminosae	Pericarps	BUPD-83	17	11	12	14	BS
***Reaumuria hirtella* jaub. Et sp.**	Tamaricaceae	Herb	BUPD-84	18	21	17	11	M
Fragaria ananassa	Rosaceae	Calyx	BUPD-85	19	18	14	13	BS
Terminalia arjuna	Combretaceae	Flower	BUPD-63	19	22	14	16	BS
Thevetia peruviana	Apocynaceae	Fruits	BUPD-86	13	11	NA	13	M
Thuja orientails	Cupressaceae	Fruits	BUPD-64	14	13	12	11	BS

* NA: No Activity at the used concentration, M; Matrouh, BS; Beni-Suef, G; Giza Governrates

Table 2. Inhibitory percentage (%) of *Terminalia arjuna* extracts at 15.63 µg/mL against fungal strains using XTT assay.

	Mean of inhibitory percentage (%)±SD		
	***Aspergillus fumigatus* ATCC 1022**	***Fusarium avenaceum* ATCC24362**	***Candida albicans* ATCC14053**
***n*-hexane**	NA	51.36±1.30	18.35±0.85
DCM	22.85±0.96	69.32±0.74	72.14±1.30
EtOAc	54.96±1.80	76.35±1.20	100
Amphotricine B	100	100	100

*NA: No activity, ATCC: American type culture collection

Table 3. The minimal inhibitory concentrations (MICS) of different fractions of *Terminalia arjuna* required to inhibit 100% of tested fungi using XTT assay.

	MIC (µg/mL)		
	***Aspergillus fumigatus* ATCC 1022**	***Fusarium avenaceum* TCC24362**	***Candida albicans* ATCC14053**
***n*-hexane**	NA	125	500
DCM	500	62.50	62.50
EtOAc	125	31.25	7.81
Rosamultin (4)	NT	NT	250
Niga-ichigoside Fl (5)	NT	NT	250
Amphotricine B	1.95	0.98	0.49

*NA: No activity, NT; Not Tested, ATCC: American type culture collection

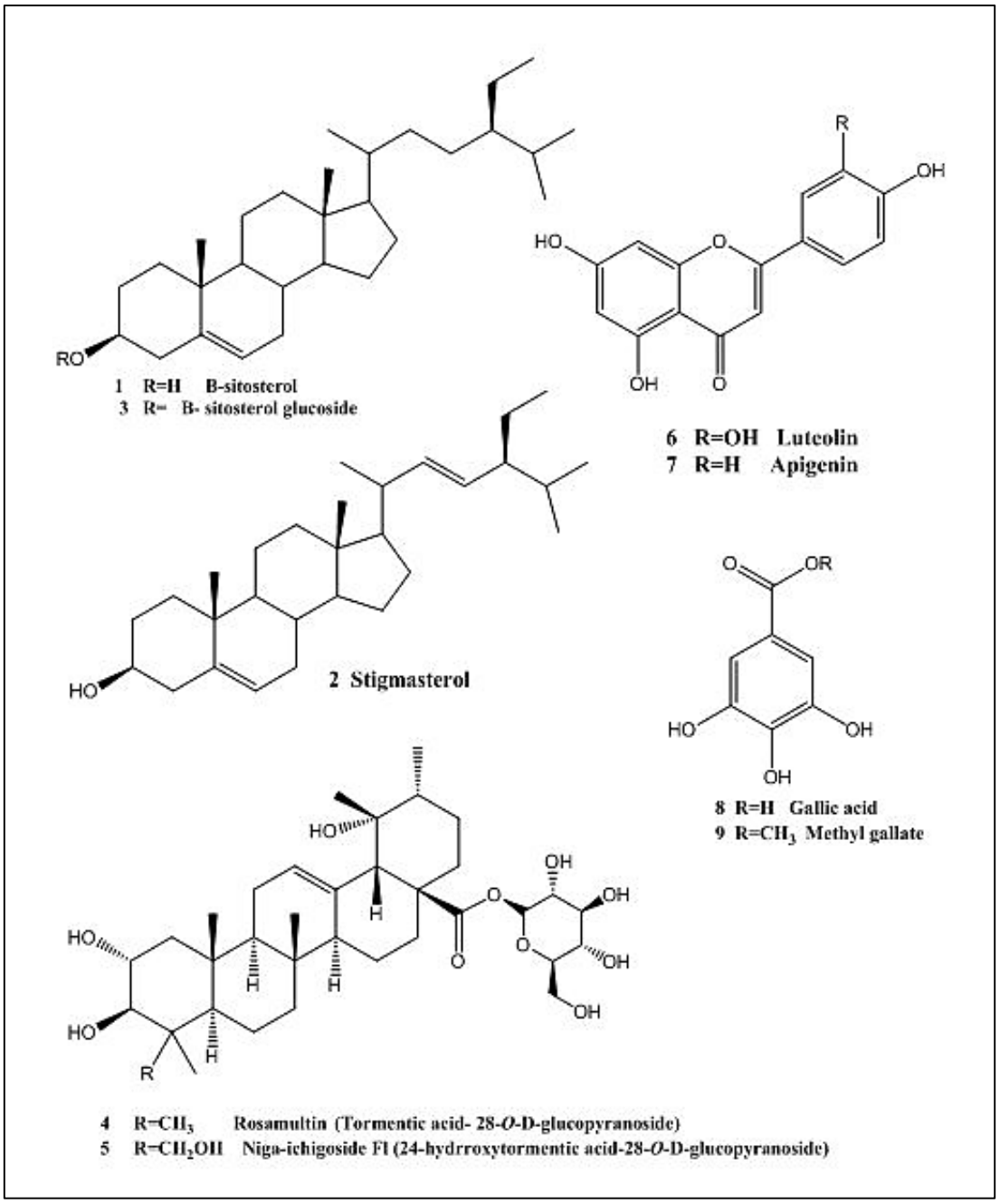

Figure 1. Chemical structure of compounds isolated from *T. arjuna* flowers extracts.

2.3.2. DCM fraction (0.6 g)

DCM fraction was applied onto silica gel column (30 g) eluting with DCM with increasing increments of methanol (MeOH) to give three main subfractions (DCM 1-3).

Subfraction DCM-1 was further chromatographed using reversed phase column (Si, C_{18}); eluting with water with increasing percentage of methanol to give compound **3** (24 mg). Sub fraction DCM-2 contains one major spot that was isolated in pure state (compound **4**, 90 mg); using normal phase silica gel column and DCM-MeOH as mobile phase. Similarly, compound **5** was purified from subfraction DCM-3 (18 mg).

2.3.3. Ethyl acetate fraction (1.5 g)

EtOAc fraction was chromatographed over silica gel column (100 g) eluting with DCM-MeOH; increasing polarity by 5% to give two main subfractions (EA-1-3). Subfraction EA-1 (300 mg) was found to contain the same spots previously isolated from sub fractions DCM-1 and 2. Subfraction EA-2 (400 mg) was chromatographed on Sephadex LH-20 eluted with methanol to afford compounds **6** (10 mg) and compound **7** (22 mg). Similarly, fraction EA-3 was applied onto Sephadex LH-20 column to give compounds **8** (50 mg) and **9** (10 mg).

2.4. Spectral data of the isolated compounds

β-sitosterol (**1**)

^{1}H-NMR (400 MHz, $CDCl_3$): Major signals; δ_H 5.38 (d, *J*=8 Hz, H-6), 3.54 (m, H-3), 1.03, 0.94, 0.87, 0.85, 0.83 and 0.70 (6×3H). DEPT-Q-NMR (100 MHz, $CDCl_3$): δ_C 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.30 (C-4), 140.7 (C-5), 121.7 (C-6), 31.92 (C-7), 31.91 (C-8), 50.1 (C-9), 36.5 (C-10), 21.0 (C-11), 39.7 (C-12), 42.33 (C-13), 56.7 (C-14), 24.3(C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.7 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.0 (C-28), 11.9 (C-29). Data were consistent with reported data for β-sitosterol[16].

Stigmasterol (**2**)

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 5.38 (d, *J*=8 Hz, H-6), 5.01-5.07 (m, H-23), 5.15-5.20 (m, H-22), 3.54 (m, H-3), 1.03, 0.94, 0.87, 0.85, 0.83 and 0.70 (6×3H). DEPT-Q-NMR (100 MHz, $CDCl_3$): δ_C 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.30 (C-4), 140.7 (C-5), 121.7 (C-6), 31.92 (C-7), 31.91 (C-8), 50.1 (C-9), 36.5 (C-10), 21.0 (C-11), 39.7 (C-12), 42.33 (C-13), 56.7 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.7 (C-21), 138.3 (C-22), 129.2 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.0 (C-28), 11.9 (C-29). NMR data were consistent with reported data for stigmasterol[17].

β-sitosterol-3-*O*-β-D-glucopyranoside (**3**)

^{1}H-NMR (400 MHz, DMSO); major signals δ_H 5.38 (br s, H-6), 4.43 (t, H-3), 4.21 (d, *J*=8 Hz, anomeric proton), 0.96, 0.89, 0.82, 0.80, 0.79 and 0.64 (6×3H). DEPT-Q NMR(101 MHz, DMSO); δ_C 37.3 (C-1), 31.8 (C-2), 78.4 (C-3), 42.3 (C-4), 140.9 (C-5), 121.6 (C-6), 31.92 (C-7), 31.8 (C-8), 50.0 (C-9), 35.9 (C-10), 21.0 (C-11), 39.2 (C-12), 42.3 (C-13), 55.9 (C-14), 24.3 (C-15), 28.2 (C-16), 56.6 (C-17), 12.1 (C-18), 19.4 (C-19), 36.6 (C-20), 19.0 (C-21), 33.8 (C-22), 25.9 (C-23), 45.6 (C-24), 29.1(C-25), 20.1 (C-26), 19.5 (C-27), 23.0 (C-28), 12.2 (C-29), sugar moiety; 101.3 (C-1'), 73.9 (C-2'), 77.2 (C-3'), 70.5 (C-4'), 77.1 (C-5'), 61.5 (C-6'). Data were consistent with reported data for β-sitosterol-3-*O*-β-D-glucopyranoside[18].

Rosamultin (**4**)

^{1}H-NMR(400 MHz, CD_3OD); δ_H 5.33 (2H, anomeric proton, H-12), 3.65 (1H, m, H-2), 2.92 (1H, d, *J*=12 Hz, H-3), 0.80 (3H,s, H-26), 0.83 (3H,d, H-24), 0.95 (3H,d, H-30), 1.03 (6H, s, H-23, H-25), 1.22 (3H,d, H-29), 1.35 (3H,d, H-27), the remaining proton signals are overlapped. DEPT-Q NMR(100 MHz, CD_3OD); δ_C 46.82 (C-1), 68.13 (C-2), 83.13 (C-3), 37.8 (C-4), 55.29 (C-5), 18.31 (C-6), 32.67 (C-7), 41.29 (C-8), 47.11 (C-9), 39.13 (C-10), 23.78 (C-11), 128.50 (C-12), 138.29 (C-13), 43.6 (C-14), 28.75 (C-15), 25.11 (C-16), 47.72 (C-17), 53.54 (C-18), 72.25 (C-19), 41.54 (C-20), 22.60 (C-21), 36.91 (C-22), 27.96 (C-23), 13.07 (C-24), 15.27 (C-25), 16.12 (C-26), 23.33 (C-27), 177.16 (C-28), 25.70 (C-29), 15.80 (C-30), sugar moiety; 94.37 (C-1'), 72.43 (C-2'), 77.17 (C-3'), 69.69 (C-4'), 76.87 (C-5'), 61.01 (C-6'). Data were consistent with reported data for rosamultin[19]

Niga-ichigoside Fl (**5**)

^{1}H-NMR (400 MHz, CD_3OD) δ_H: 5.33 (2H, anomeric proton, H-12), 3.65 (1H, m, H-2), 2.92 (1H, d, *J*=12 Hz, H-3), 0.80 (3H,s, H-26), 0.83 (3H,d, H-24), 0.95 (3H,d, H-30), 1.03 (6H,s, H-25), 1.22 (3H,d, H-29), 1.35 (3H,d, H-27). the remaining proton signals are overlapped. DEPT-Q NMR (100 MHz, CD_3OD) δ_C: 39.87 (C-1), 68.13 (C-2), 83.14 (C-3), 37.79 (C-4), 55.28 (C-5), 18.29 (C-6), 32.66 (C-7), 41.28 (C-8), 47.6 (C-9), 39.11 (C-10), 23.6 (C-11), 128.11 (C-12), 138.3 (C-13), ND (C-14), 28.23 (C-15), 25.1 (C-16), ND (C-17), 53.55 (C-18), 72.23 (C-19), 41.54 (C-20), 22.35 (C-21), 36.91 (C-22), 62.99 (C-23), 13.05 (C-24), 15.22 (C-25), 16.22 (C-26), 23.28 (C-27), 177.16 (C-28), 23.28 (C-29), 16.07 (C-30), sugar moiety 94.37 (C-1'), 72.41 (C-2'), 77.18 (C-3'), 69.7 (C-4'), 76.88 (C-5'), 61.0 (C-6'). Data were consistent with reported data for niga-ichigoside Fl[19-20].

Luteolin (**6**)

^{1}H NMR (500 MHz, DMSO); δ_H 7.42 (dd, *J*=8.3, 2.2 Hz, 1H), 7.40 (d, *J*=2.1 Hz, 1H), 6.89 (d, *J*=8.3 Hz, 1H), 6.68 (s, 1H), 6.44 (d, *J*=1.9 Hz, 1H), 6.19 (d, *J*=1.9 Hz, 1H). ^{13}C NMR (125 MHz, DMSO); δ_C 163.88 (C-2), 102.84 (C-3), 181.66 (C-4), 161.49 (C-5), 98.88 (C-6), 164.33 (C-7), 93.88 (C-8), 157.32 (C-9), 103.64 (C-10), 121.45 (C-1'), 113.33 (C-2'), 145.77 (C-3'), 149.79 (C-4'), 116.02 (C-5'), 119.02 (C-6'). Data were consistent with reported data for luteolin[21].

Apigenin (**7**)

^{1}H NMR (400 MHz, DMSO); δ_H 6.19 (s, H-6), 6.48 (s, H-8), 6.78 (s, H-3), 6.92 (d, *J*=8.4 Hz, H-3', H-5'), 7.91 (d, *J*=8.4 Hz, H-2', H-6'). DEPT-Q NMR (100 MHz, DMSO); δ_C 164.18 (C-2), 103.28 (C-3), 182.20 (C-4), 161.65 (C-5), 99.31 (C-6), 164.69 (C-7), 94.43 (C-8), 157.77 (C-9), 104.12 (C-10), 121.62 (C-1'), 128.92 (C-2', 6'), 116.42 (C-3', 5'), 161.91 (C-4'). The data was in agreement with the reported data of apigenin[22].

Gallic acid (**8**)

^{1}H NMR (400 MHz, CD_3OD); δ_H 7.08 (s, 2H, H-2, H-6). ^{13}C NMR (100 MHz, CD_3OD); δ_C 120.58 (C-1), 108.92 (C-2, C-6), 144.97 (C-3, C-5), 138.07 (C-4), 169.10 (C-7, COOH). The data was in agreement with the reported data of gallic acid[23].

Methyl gallate (**9**)

^{1}H NMR (400 MHz, CD_3OD); δ_H 3.71 (s, 3H), 6.93 (s, 2H, H-2, H-6). ^{13}C NMR (100 MHz, CD_3OD); δ_C 120.01 (C-1), 108.60 (C-2, C-6), 145.09 (C-3, C-5), 138.35 (C-4), 167.62 (C-7, COO-), 50.87 (O-CH_3). The data was in

agreement with the reported data of methyl gallate[23].

2.5. Biological study

2.5.1. Agar diffusion method

Agar diffusion method was used for primary screening of antimicrobial activity of the extracts according to Jacobs and Appelbaum (1995)[24]. The microbes used for screening were; *C. albicans*, *S. aureus*, *E. coli* and *P. aeruginosa*. Stock solution of the extracts was prepared in concentration 10 mg/mL DMSO and 100 µL of the test samples were applied using Muller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI, 2013). Cup diameter was 10 mm and starting culture of microorganism 1% (0.5 McFarland). DMSO was used as positive control and all plates were incubated at 35 C for 48 h.

2.5.2. XTT assay of antifungal activity

Colorimetric broth micro-dilution method using XTT reduction assay was adopted to determine the inhibitory percentages and the minimum inhibitory concentrations (MICs) of tested extracts[25]. All fungal strains (*C. albicans*, *A. fumigatus* and *F. avenaceum*) were cultured at 37°C on Sabouraud dextrose agar for 3-5 days. Fifty µL of adjusted microbial inoculum 5×10^3 CFU/mL of RPMI-1640 medium was added to each well, and then the Microtiter plates were incubated in the dark at 37°C for 24 h to promote germination of hyphae. The extracts were serially diluted in DMSO, and then 50 µL of each dilution at final concentrations of (1000-0.24 µg/mL) were added to wells in Microtiter plate (96 wells). Amphotericin B was used as standard antifungal. After incubation, 100 µL of freshly prepared XTT were added, incubated again for 1 h at 37°C. Colorimetric variation in the XTT assay was measured using a Microtiter plate reader at 492 nm. The inhibition percentage was calculated using the given formula:

$$\% \text{ Inhibition} = [1-(Abs_{(f+s)}-Abs_s / Abs_f-Abs_b)] \times 100.$$

$Abs_{(f+s)}$: Absorbance of wells containing fungi with extract, Abs_s: Absorbance of wells containing extract alone, Abs_f: Absorbance of wells containing fungi alone and Abs_b: Absorbance of wells containing media alone.

The MIC was specified as the extract concentration that produced a 100% decrease in optical density compared with control growth results[25].

3. RESULTS AND DISCUSSION

The antimicrobial activity of the ethanolic extract of eleven plant species, representing nine families, collected from three different regions in Egypt was investigated against selected examples of Gram positive, Gram negative bacterial as well as fungal microorganisms. Results of the primary sensitivity tests using agar diffusion method (Table 1) revealed significant antifungal activity of the alcohol extract of *T. arjuna* flowers against *C. albicans*. Hence *T. arjuna* flower extract was chosen for further investigation. Flower extract was fractionated using solvents with varying polarities; *n*-hexane, DCM and EtOAc. The activity of the three successive fractions, was tested against *C. albicans* in addition to two more fungal species: *A. fumigatus* and *F. avenaceum*, using XTT assay (Table 2). The three successive fractions displayed activity against *F. avenaceum* and *C. albicans* with inhibitory percentage ranging from 18.35-100%. While only DCM and EtOAc fractions exhibited activity against *A. fumigatus* with inhibitory percentages 22.85±0.96% and 54.96±1.8%, respectively. MIC was determined for the tested extracts against the three tested species and the results showed variable activities among the three extracts with *n*-hexane fraction being inactive against *A. fumigatus*. Interestingly was the result of ethyl acetate fraction against *C. albicans*, MIC=7.81 µg/mL compared to 0.49 µg/mL of Amphotericin-B (Table 3). Based upon the previous results; *T. arjuna* extract was selected for further phytochemical study. Chromatographic investigation, via a combination of silica gel and Sephadex LH-20 column chromatography methods, afforded nine compounds that were identified using different spectroscopic techniques as; β-sitosterol (**1**)[16], stigmasterol (**2**)[17], β-sitosterol-3-*O*-β-D-glucopyranoside (**3**)[18], rosamultin (**4**)[19], niga-ichigoside Fl (**5**)[19-20], luteolin (**6**)[21], apigenin (**7**)[22], gallic acid (**8**)[23] and methyl gallate (**9**)[23] (Figure 1). All compounds except the two triterpenoidal saponins; rosamultin (**4**) and niga-ichigoside Fl (**5**) were previously reported from *T. arjuna* leaves[10].

Compounds (**1**) and (**2**) were isolated from the *n*-hexane extract as a mixture in a proportion of 5:1 and were identified by comparison of their ^{1}H- and ^{13}C NMR spectra with reported values[17].

Fractionation of DCM extract using different chromatographic methods afforded 3 compounds (**3-5**). Compound **4** was obtained as a white amorphous powder, which gave a positive result in the Liebermann-Burchard test. The ^{13}C NMR spectrum of compound **4** showed 36 carbon resonances, confirmed the presence of a sugar moiety and a triterpene aglycone. The aglycone was confirmed as an urs-12-ene skeleton since the chemical shift of the olefinic carbons C-12 and C-13 at δ 128.1 and 138.3, respectively[26-27]. ^{13}C NMR showed 6 resonances [δC1'-C6'] 94.4 (d), 72.4 (d), 76.92 (d), 69.6 (d), 77.2 (d), and 61.1 (t)] ascribed to a typical glucopyranosyl ester unit[28]. The C-28 appeared at δ_c177.1 ppm indicating ester formation with the sugar unit which was also confirmed by HMBC correlation between the anomeric proton (δ_H 5.40 (d, *J*=7.9 Hz) and C-28 (δ_c 177.1 ppm), the large coupling constant of the anomeric proton indicated β-glucopyranosyl residue. The aglycone contain two oxy-

methine carbons at δc 68.1 and 83.1 ppm which were assigned to C2 and C3 respectively and a quaternary oxygenated carbon at δc 72.3 ppm assigned to C19. The large coupling constant of H-3 at δ_H 2.94 (d, *J*=9.5 Hz, 1H) indicated trans configuration with H-2. The structure was thus confirmed as 2α,3β,19α-trihydroxyurs-12-en-28-oic acid-28-*O*-D-glucopyranoside (tormentic acid ester glucoside; rosamultin) previously reported in the trunk bark of *T. argentea*[29]. Carbon assignment was done using HMQC and HMBC correlations. NMR data come in great accordance with the saponin isolated from leaves of *Aphloia theiformis*[26]. It is noteworthy that, compound 2α,3β,19α-trihydroxyurs-12-en-28-oic acid-28-*O*-D-glucopyranoside was previously isolated from bark of *T. arjuna*[14], however it was wrongly named as its epimer; Kaji-Ichigoside F1 which is the 3α-isomer of rosamultin[30-31]. Acid hydrolysis of compound **4** with 2 mol/L HCl/1,4-dioxane (1:1, v/v) furnished glucose, identified using TLC by comparison with an authentic sample. The sugar identity was further confirmed by the chemical shifts and coupling constants in the ^{1}H- and ^{13}C-NMR spectra[32]. Compound **5** showed ^{13}C NMR resonances close to compound **4** (Table 3); except for the appearance of oxygenated methylene carbon at δc 62.9 ppm and disappearance of one of the methyl signals indicating its hydroxylation. The NMR data was consistent with 23-hydroxy tormentic acid-28-*O*-D-glucopyranoside,(2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid-28-*O*-D-glucopyranoside (niga-ichigoside Fl)[19-20]. Fractionation of ethyl acetate fraction afforded luteolin (**6**), apigenin (**7**), gallic acid (**8**) and methyl gallate (**9**).

Results of the antifungal activity testing (Tables 2-3) indicated moderate activity (51.36% inhibition and MIC=125 µg/mL) of the n-hexane fraction against *F. avenaceum*. The observed activity was supported by the results of Kiprono et al., who reported activity of sitosterol against *Fusarium* sp.[33]. Another study confirmed the previous findings, where it stated the activity of both sitosterol and stigmasterol against *F. verticilloides* and *C. albican*[34].

Additionally, DCM fraction exhibited mild activity against the three tested fungal species, where *C. albicans* was the most sensitive. Considering compounds **4-5**, isolated from this fraction; the recoded antifungal activity results were supported by the previous findings of Yuan et al.[35], who reported modest antifungal activity of triterpene glycosides against *C. albicans* and *C. krusi* and stated that the hydroxylation of methyl group at C-23 in the ursane structure contribute to the observed antifungal activity[35]. For more confirmation, the two compounds: rosamultin (**4**) and niga-ichigoside Fl (**5**) were tested for antifungal potential against *C. albicans*. Results indicated that both compounds exhibited moderate activity against *C. albicans* with MIC=250 µg/mL (Table-2). It is noteworthy that this is the first report for testing the antifungal activity of both compounds.

Most interesting was the potent activity (100% inhibition and MIC=7.81 µg/mL) of the ethyl acetate extract against *C. albicans*. This activity might be attributed to the phytochemical content of this fraction. Gallic acid (**8**), isolated from the EtOAc extract was previously reported to have wide spectrum antifungal activity *in vitro* and *in vivo*, where *C. albicans* was the most sensitive species with MIC=12.5 µg/mL[36]. Moreover, Nguyen et al. (2013)[37] reported the antifungal potential of gallic acid isolated from *T. nigrovenulosa* bark extract against *F. solani*. Both studies came in great accordance with our results and accounted for the recorded activity of EtOAc extract against the three tested fungal species where *C. albicans* was the most sensitive. Additionally, methyl gallate (**9**) isolated from EtOAc fraction was also, proved to possess potent antifungal activity and was suggested to be used as a naturally occurring agent to control plant pathogens[38]. Furthermore, apigenin, the common plant flavone, was reported to exhibit antifungal activity against *C. albicans* that was proved to be due to induction of membrane disturbance of the fungal species that leads to shrinkage and leakage of intracellular components[39]. While, on the other hand, Alves, et al, reported weak activity of luteolin against four *Candida* species[40]. Based upon the aforementioned data, the observed antifungal activity of EtOAc fraction might be due to its content of gallic acid, methyl gallate and apigenin.

4. CONCLUSION

Testing the antifungal activity of *T. arjuna* flowers indicated its potent antifungal activity against *C. albicans*. The observed activity could be attributable to the synergistic effect of gallic acid, methyl gallate, together with other steroidal, triterpenoidal and flavonoidal constituents. These results support the possible use of *T. arjuna* flowers as adjunct therapy for external use in oral and vaginal candidiasis. This report represents the first phytochemical and biological study on *T. arjuna* flowers.

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