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Phytochemical Analysis and Antifungal Bioactivity of *Pulicaria undulata* (L.) Methanolic Extract and Essential Oil

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PULICARIA UNDULATA is one of the aromatic annual herbs used in folk medicine in Egypt. The aerial parts of *P. undulata* plants were collected from the Red sea coastal desert during the flowering stage and used for phytochemical analysis. It was revealed that *P. undulata* aerial portions contain phenols, flavonoids terpenoids, saponins, alkaloids, glucosinolates and cardiac glycosides highlighting its participation in drug development and using as an alternative strategy to the harmful synthetic fungicides. The greater accumulation of the bioactive compounds in the methanolic extract was comparable with its higher antioxidant capacity and IC50 value as compared with those of the petroleum ether extract. The GC-MS analysis revealed the occurrence of ten bioactive phytocomponents including cyclotrisiloxane, hexamethyl-, cyclotetrasiloxane, octamethyl-, cyclopentasiloxane, decamethyl, dodecanoic acid, methyl ester, tetrafluorophthalonitrile, gamma-Sitosterol, nonacosane, 4,4,6a,6b,8a,11,11,14b-Octamethyl ,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one, thiazolo[4,5-f]quinoline and 7-methyl-. In addition, the methanolic extract exhibited antifungal activity against the investigated six pathogenic fungal strains: *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Microsporum canis*, *Microsporum boulardii* and *Trichophyton mentagrophytes*. Based on the values of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC), the methanolic extract has demonstrated a fungicidal effect on *M. boulardii* IFM 56403, *T. mentagrophytes* AUMC 11661, *M. canis* AUMC 11663, and *C. albicans* AUMC 9142. The cytotoxicity evaluation of the methanolic extract showed safe levels in case of these four fungal strains. The petroleum ether extract, inhibited the tested fungi but with lower efficiency. Our results suggest the promising antifungal activity of *P. undulata* on medically important fungi.

Keywords: *Pulucaria undulata*, Phytochemicals, GC-MS, Antifungals.

Introduction

Nowdays, there has been an increasing demand for medicinal plants in the folk medicine and pharmaceutical industry all over the world. For instance, it is reported that various plants are effective against several pathogenic microorganisms (Adeniyi et al., 2009; Dejussi et al., 2013). Aromatic medicinal plants possess large varieties of chemical substances, which explain their biological activities in the treatment of various human diseases. These chemical substances include diverse range of secondary metabolites such as alkaloids, flavones, flavonoids, sesquiterpene, lactones, diterpenes, triterpenes, naphthoquinones, anthocyanin, coumarin, catechins, isocatechins, and others (Christaki et al., 2012; Chhetri et al., 2015).

Pulicaria undulata (L.) C.A. Myer, is a perennial aromatic herb belongs to family Asteraceae which has been used traditionally to treat diabetes, cardiac disorders, skin diseases, abscesses, inflammations, an insect repellent as well as an herbal tea and tonic

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(Elegami et al., 1994; Hammiche & Maiza, 2006; Hegazy et al., 2012). Several investigations have reported the chemical composition, antibacterial and antioxidant activities of the essential oil of *P. undulata* (El-Kamali et al., 2009; Mehdi et al., 2011; Ali et al., 2012; Ajaib et al., 2015; Boumaraf et al., 2016).

The prevalence of microbial diseases has currently expanded, particularly mycoses which cause high death rate in immune-compromised patients. Among these diseases, the offensive fungal infections produced by *Candida* and *Aspergillus* represent the substantial proportion (Brown et al., 2012). *Candida* spp. as a pathogen can cause both superficial and serious systemic disease (Nerurkar et al., 2012). Also, *Candida* spp. are the fourth most frequent microbial pathogens causing bloodstream infections, after common bacterial pathogens (Wisplinghoff et al., 2004). The predominant microbial infections in hematopoietic stem cell transplant recipients were caused by *Aspergillus* spp. (Kontoyiannis et al., 2010). Recent reports also recorded an elevation in the mortality rate from invasive aspergillosis and candidemia to be about 30-50% (Denning & Bromley, 2015). The incidence of superficial mycosis has been assessed to be more than 25% among the worldwide population. Dermatophytes, which belong to one of three genera, *Trichophyton, Microsporum* and *Epidermophyton*, are recognized to be the most frequently occurred agents of superficial mycoses (Havlickova et al., 2008). Dermatophytosis, is referred to as tinea infection, constitute a major public health problem in developing countries including Egypt (Zaki et al., 2009). Unfortunately, several investigations have pointed to the resistance of fungal pathogens to the available antimycotic agents (Andriole, 1994; Martinez-Rossi et al., 2008; Sanglard, 2016). This situation urges the need for novel safe antifungals with fungicidal effect as well as wide spectrum activities. In fact, there is limited data on the antimicrobial activities and antifungal properties of *Pulicaria undulata* (El-Kamali et al., 2009; Ali et al., 2012; Ajaib et al., 2015). The present study was therefore undertaken to investigate the antifungal activities of the crude extract and essential oil of *P. undulata* growing wild in Egypt, against six of the most common invasive and superficial fungal pathogens (*Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Microsporum canis, Microsporum boulardii* and *Trichophyton mentagrophytes*). The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were also assayed to explore their effectiveness in treatments of mycotic infections. In addition, the chemical composition and antioxidant activity of *P. undulata* extract were determined.

Materials and Methods

Collection and identification of plant materials

The samples of *Pulicaria undulate* were collected from Wadi Hagol arid habitat, Egypt, during the flowering season. The identification of the plant was confirmed by the Department of Botany, Faculty of science, Ain shams university. The aerial parts (leaves/stem) and flower heads were cleaned, dried at room temperature in the shade and then ground to a powder by mechanical mills.

Preparation of crude extracts

The dried powder (100g) was extracted with distilled water or ethyl alcohol, methyl alcohol, petroleum ether, ethyl acetate or hexane at 4ºC. After 72hr, the extracts were filtered, and the filtrates were concentrated on rotary evaporator under reduced pressure at 30°C. Then, the color of the crude concentrated extracts was discharged by using active charcoal, and next completed to final volume with water for phytochemical analysis **(**Harborne, 1973). The qualitative phytochemical analysis was carried out to determine the suitable solvent for the maximum quantitative estimation of nutraceuticals secondary metabolites as described below.

Qualitative and quantitative analysis of phytochemicals

Phytochemical screening was carried out to test the presence of tannins, flavonoids, terpenoids, saponins, alkaloids, quinones, cardiac glycosides, glycosinolates and anthroquinones in all plant extracts following the standard protocol of Makkar et al. (1993).

Carbohydrate determination

Total soluble carbohydrates were determined using anthrone reagent (Fairbairn, 1953).

Antioxidants

The water-soluble non-enzymatic antioxidants such as glutathione and ascorbic acid were determined according to Griffith (1980) and Kampfenkel et al. (1995), respectively.

Measurement of total phenolics and tannins

The total phenolics and tannins were measured by using Folin-Ciocalteu method as described by Makkar et al. (1993).

Measurement of condensed tannins (proanthocyanidins)

The total proanthocyanidins was determined by using vanillin reagent as described by Price et al. (1978) where the absorbance was measured at 500nm using spectrophotometer (Shimadzu UV-265, Japan).

Extraction and estimation of total flavonoids content

The aluminum chloride colorimetric method described by Harborne (1998**)** was used for determination of total flavonoids in the extracts. The total flavonoid contents were calculated as µg/g dry weight from a standard curve of quercetin.

Determination of alkaloid

The total alkaloids were quantified according to the method described by Harborne (1973).

Determination of saponins

The total saponins was calculated from the diosgenin standard solution by using vanillin reagent according to the method of Francis et al. (2002).

Determination of glucosinolate

Glucosinolate was determined by spectrophotometrically based on Makkar et al. (1993).

Determination of cardiac gylcosides

Cardiac glycoside content in the extracts was evaluated using Buljet‘s reagent as described by El-Olemy et al. (1994).

Total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. (1999).

DPPH radical scavenging activity

The antioxidant activity of the extracts was estimated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method (Yamaguchi et al., 1998). IC50, the half maximal effective concentration value was measured from % Inhibition *vs*. different concentrations of standard ascorbic acid or a plant extract (1-500µg/ml) and calculated according to the following equation:

$$\% \text{ DPPH radical scavenging} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the DPPH solution (blank), and A_1 is the absorbance of the sample.

Essential oil extraction

The powder sample was extracted with petroleum ether (PE 40-60ºC) for 48hr at room temperature. The extract was evaporated to least volume using a rotary evaporation at reduced pressure. The essential oil was passed over dark anhydrous sodium sulfate to remove moisture. The fraction obtained was stored in a refrigerator at 4 ºC in dark to identify the chemical constituents of oil (Adams, 2007).

GC-MS analysis

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted by using Agilent 7890B GC system coupled to an Agilent 5977A MSD with a capillary column (0.6m x 100µm x 0µm) (Agilent Technologies, Santa Clara, CA, USA). Helium gas was used as carrier gas at a constant flow rate of 1.5ml/min. The injector temperature was set at 250°C and the ion source temperature was set at 230°C. The initial oven temperature was set at 40°C for 2min, then 10°C/min to 180°C for 5min and then 10°C/min to 250°C for 10min. The total GC running time was 38 min. The GC-MS was run in Scan/SIM mode and the identification of sample’s components was performed using Agilent Mass Hunter software (NIST14 L).

Evaluation of antifungal activity

Tested fungi: The investigated human pathogenic fungi including clinical strains of *Aspergillus flavus* AUMC 10311, *Aspergillus fumigatus* AUMC 51, *Candida albicans* AUMC 9142 and three dermatophytes (*Trichophyton mentagrophytes* AUMC 11661, *Microsporum canis* AUMC 11663, and *Microsporum boulardii* IFM 56403). All strains were purchased from Assiut University mycological center except *M. boulardii* IFM 56403 was obtained from the Medical Mycology Research Center, Chiba University (Chiba, Japan). Sabouraud dextrose agar (SDA; Difco, Sparks, MD, USA) was used for the maintenance and sub-culturing of the fungal strains at 28ºC. All cultures were stored at 4ºC.

Preparation of fungal inocula: The inoculum size of each test strain was standardized according to clinical laboratory standard institute guidelines (CLSI 2008a, 2008b). Test strains were grown on potato dextrose agar slants (PDA; Difco, Sparks, MD, USA and SDA; for *C. albicans*) for 2-7 days or until good sporulation is obtained at 25°C. Inoculum count was adjusted to 1-5 $\times 10^6$CFU/ml using the method described by Johnson et al. (2015). This stock solution was then diluted to obtain 0.4-5$\times$ 10^4CFU/ml as a final spore concentration to be used for the experiment testing the minimum inhibitory concentration (MIC).

Screening of antifungal effect

Samples of the crude extract and essential oil extracted by petroleum ether recovered from *P. undulata* were separately dissolved in dimethyl sulfoxide (DMSO; 10% v/v) to obtain 200mg/mL working solutions. The agar-well diffusion assay (Cole, 1994) was performed to evaluate the antifungal activity of the methanolic extract and the essential oils, separately, against each of the selected test fungi. The inoculum size was adjusted to about 1-5$\times$ 10^6CFU/ml and plates were inoculated in the same way as for disk diffusion methodology of CLSI M44-A2 (CLSI, 2009) and CLSI M51-A (CLSI, 2010) for yeast and mold, respectively. Briefly, an inoculum was speared onto a surface of Mueller Hinton agar (MHA; Difco, Sparks, MD, USA) plates using a sterile cotton swab. An Aliquot of 100µL of the methanolic extract or the essential oil was applied to 9mm diameter wells. Negative control (100µL of DMSO 10% v/v) and positive control (100µL of 0.2mg/ml itraconazole; Sporanox, Janssen Pharmaceutica, Beerse, Belgium) were also included for the comparative purpose. The diameters of the growth inhibition zones around each well were measured after incubation at 28°C for 2-14d (according to the rate of growth of each species). For each extract, three replicate trials were conducted against each fungus and data were presented as mean± SD (standard deviation).

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of the methanolic extract and essential oil were determined using the macrodilution technique in accordance with the guidelines of the M27-A3 of the CLSI for the yeasts (CLSI, 2008a) and the M38-A2 CLSI for the filamentous fungi (CLSI, 2008b) as previously detailed by Johnson et al. (2015) with some modifications. Briefly, 100µL of spore suspension (~1.5$\times$ 10^4CFUml^{-1}) was added to 1ml Sabouraud dextrose broth containing serially two-fold diluted (at concentrations from 68 mg/ to 1.06 mg/ml) methanolic extract or essential oil. Tubes were incubated at 28 °C and observed for the presence or absence of visible growth. Controls for strain viability and medium sterility were used. MIC was defined as the lowest concentration that inhibited visible fungal growth after 14d (for dermatophytes).

Determination of the minimum fungicidal concentration (MFC) and MFC/MIC ratio

Based on the MIC assay, 200µL aliquots of the tube corresponding to the MIC and two concentrations above were plated onto Petri dishes containing SDA medium and incubated at 28°C. The reading was performed by visual observation of fungal growth on the solid medium. Each experiment was repeated three times and mean values were calculated for MICs and MFCs. The MFC was regarded as the lowest concentration that did not show any visible growth on solid medium (even after 14 days of incubation). The MFC/MIC ratio was calculated to determine whether the substance had a fungistatic (when MFC/MIC>4) or fungicidal effect (when MFC/MIC$\leq$ 4) (Rasooli & Abyaneh, 2004).

Evaluation of cytotoxicity against VERO cell line

According to Mosmann (1983) and Gomha et al. (2015), the cytotoxicity effect of methanolic crude extracts was evaluated against VERO cells (mammalian cells from African green monkey kidney). VERO cells were obtained from VACSERA Tissue Culture Unit in Egypt. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and trypan blue stain were purchased from Sigma (St. Louis, MO, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Louis, MO, USA) with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin (Lonza. Basel, Switzerland). These cells were kept up at 37ºC with 5% CO_2 and were subcultured two times each week. The survival curve of the cell line was plotted between the percentage of surviving cells and drug concentration. The 50% inhibitory concentration (IC50) was determined. IC50 is the concentration of test extract resulted in toxicity of a 50% of intact cells as recorded from the plotted curve using Graphpad Prism software (San Diego, CA, USA).

Statistical analysis

All results were expressed as mean value ± standard error (SE). Statistical significance was tested between control, APAP and ES treatments as well as between APAP and ES+APAP treatments using the one-way analysis of variance (ANOVA) test. Post hoc testing was performed for intergroup comparisons using the Least Significant Difference (LSD) test at P value <0.05.

Results

The bioactive constituents of P. undulate

The extracts of *P. undulate* aerial parts revealed the presence of nutraceuticals bioactive metabolites: Phenols, flavonoids, tannins, triterpenoids, steroids, alkaloids, coumarins, proanthocyanidines and cardiac glycosides (Table 1). Alcoholic extracts, particularly methanol, showed the greatest constituents relative to others (Table 1). The results thus justified quantitative assay of secondary metabolites in the *P. undulate* methanolic extract. It is revealed that the extract contains 1.46mg soluble sugar, 7.76µmole ascorbic acid (ASA), 27.0mmol reduced glutathione (GSH), 3.93mg total phenols, 1.35µg flavonoids, 0.18µg total tannins, 261.0µg total saponins, 2.08mg proanthocyanidines, 14.72µM cardic glycosides, 83.1µmol glucosinolates and 4.3mg total alkaloids per g dry matter (Table 2). However, the petroleum ether extracts containing oil assay exhibited 1.06mg soluble sugar, 6.16µmole ASA, 22.0mmol reduced glutathione (GSH), 3.09mg total phenols, 0.617µg flavonoids, 149.5µg total saponins, 1.67mg proanthocyanidines, 10.7µM cardic glycosides, 55.9µmol glucosinolates, 3.12mg total alkaloids and 0.19µg total tannins per g dry matter (Table 2).

TABLE 1. Qualitative phytochemical analysis of *P. undulate*.

Secondary metabolites	Aqueous	Ethanol	Methanol	Ethyl acetate	Hexane
Quinones	+++	+++	+++	Traces	Traces
Anthroquinone	-	-	-	-	-
Phlobatannins	-	-	-	-	-
Tannins	+	++	+++	-	-
Coumarins	++	++	+++	-	-
Saponins	++	+	+	+	+
Cardic glycoside	+	++	++	+	-
Flavonoids	+	++	+++	-	-
Alkaloids	-	++	+	+	+
Steroids	+	++	+++	+	+
Triterpenoids	+	++	++	Traces	Traces
Phenolic compounds	+	++	+++	-	-

(-): Absence, (+): Less presence, (++): Moderate presence, (+++): High presence.

TABLE 2. Quantitative phytochemical analysis of *P. undulate* methanolic extract and oil extracted by petroleum ether.

Parameters	Value	
	Methanolic extract	Petroleum ether oil extract
Soluble sugar (mg/g DW)	1.46±0.16	0.44±0.01
Ascorbic acid (µmole/g DW)	7.76±0.04	5.98±0.32
Glutathione (mmole/g DW)	27.0±0.15	15.2±0.22
Tannins (µg/g DW)	0.18±0.003	0.19±0.08
Proanthocyanidine (µg/g DW)	2.08±0.08	1.67±0.07
Saponins (µg/g DW)	261.0±1.00	149.5±3.4
Cardic glycoside (µM/g DW)	14.718±0.69	10.7±0.43
Flavonoids (µg/g DW)	1.53±0.003	0.617±0.09
Alkaloids (mg/g DW)	4.30±0.70	3.12±0.12
Glycosinolate (µmole/g DW)	83.1±0.61	55.9±1.32
Total phenols (µg/g DW)	3.93±0.03	2.02±0.25
DPPH %	28.1±0.15	24.0±0.11
FRAP (nmole/g DW)	117.4±1.77	82.6±0.73

As for DPPH radical-scavenging procedure, the methanolic extract and petroleum ether extracted oil showed potential free-radical activity (DPPH) reached about 28.1% and 24.0% and total antioxidant capacity (FRAP) about 117.4 and 82.6 nmole ascorbic acid/g, respectively (Table 2). The antioxidant activities of the tested extracts using DPPH radical-scavenging method showed that the methanolic extract exhibited the significant activity IC50 value with inhibition percentage of 67.2 ±0.31µg/ml whereas the petroleum ether extract was about 38.7±0.12µg/ml inhibition, indicating the potential antioxidant activity of both extracts (Figs. 1, 2).

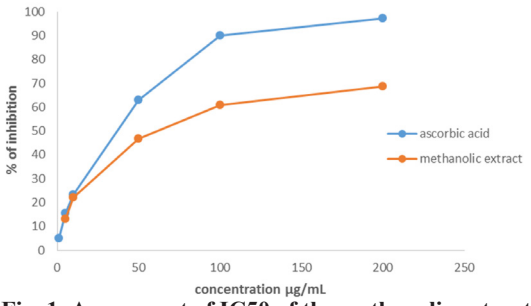

Fig. 1. Assessment of IC50 of the methanolic extract of *Pulicaria undulata* and standard ascorbic acid.

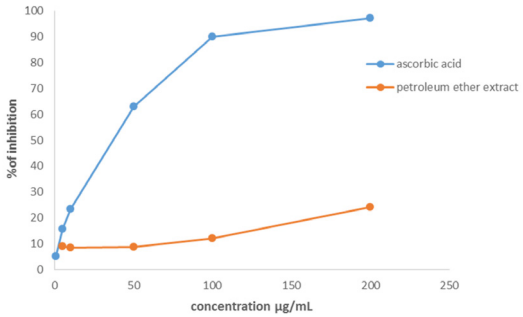

Fig. 2. Assessment of IC50 of the petroleum ether extract of *Pulicaria undulata* and standard ascorbic acid.

The GC-MS chromatogram analysis of the methanolic extract of *P. undulate* aerial portions (Fig. 3) revealed presence of 43 major phytocomponents. On comparison of the mass spectra of the constituents with the NIST library, the five phytocompounds were characterized and identified (Table 3). The major identified bioactive constituents are cyclotrisiloxane, hexamethyl-, cyclotetrasiloxane, octamethyl-, cyclopentasiloxane, decamethyl-, dodecanoic acid, methyl ester, tetrafluorophthalonitrile, gamma-sitosterol, nonacosane, 4,4,6a,6b,8a,11,11,14b-octamethyl 1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12 ,12a,14,14a,14b-octadecahydro-2H-picen-3-one, Thiazolo[4,5-f]quinoline and 7-methyl-. These compounds showing biological activities as indicated in Table 3. On the other hand, the GC-MS analysis revealed presence of 50 major phytocomponents in the petroleum ether extract (oil) of *P. undulate* aerial portions (Fig. 4 and Table 4). The major identified bioactive constituents are dodecanoic acid, methyl ester, hexadecanoic acid, methyl ester, n hexadecanoic acid, octadec-9-enoic acid, 9,12 octadecadienoic acid (Z,Z)-, methyl ester, 9,12-octadecadienoic acid (Z,Z) octadecenoic acid (Z) methyl ester, 9-octadecenamide, (Z), squalene, 11-octadecenoic acid, methyl ester, and hexacosane.

Evaluation of antifungal activity

The methanolic extract had a significantly higher inhibitory effect on the tested fungal pathogens than the essential oil (Table 5, Fig. 5). The tested strains of dermatophytes were more susceptible to this extract than the opportunistic pathogenic strains of *Candida* and *Aspergillus*. The inhibition zone diameter resulted due to the application of this extract (200mg/ml) against the tested dermatophytes was ranged from 29.3-31.7mm, while the inhibition zone diameter for the opportunistic pathogens exposed to this extract was in the range of 20.0-24.0mm (Table 5). The essential oil of *P. undulata* (200mg/ml) was shown to possess a little or no antifungal activity against the tested fungi with inhibition zone diameter ranged from 16.7-19.0mm, an exception was *Trichophyton mentagrophytes* AUMC 11661 which showed high sensitivity to this extract (inhibition zone diameter of 24.0mm, Table 5). No inhibitory effect of essential oil was observed against *Microsporum canis* AUMC 11663.

The MICs and MFCs of both the methanolic and essential oil of *P. undulata* are presented in Table 6. The methanolic extract exhibited the higher inhibitory activity against the six tested fungi than the essential oil (Table 6). The methanol extract consistently had fungicidal effect against four of the tested fungal isolates. The lowest MFC value was recorded against *M. boulardii* IFM 56403 followed by *T. mentagrophytes* AUMC 11661 and *M. canis* AUMC 11663, whereas *C. albicans* AUMC 9142 showed the highest MFC value (Table 6). Although growth of five of the six tested fungi was inhibited by the essential oil, no fungicidal effect was reported even at the highest concentration of 68mg/ml (Table 6).

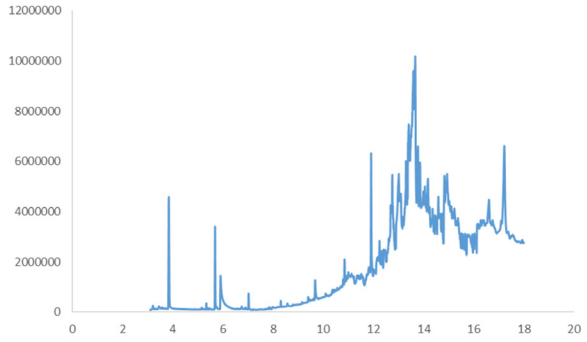

Fig. 3. GC-MS spectrum of phytoconstituents of methanolic extract of *Pulicaria undulata*.

TABLE 3. Phytoconstituents of *P. undulate* methanolic extract

Peak N.	R.T	Peak area (%)	Compound name	Formula	Biological activity
1	3.8363	1.4192	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	Antimicrobial (Keskin et al., 2012)
2	5.6844	0.6625	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	Antimicrobial activity (Mary & Giri, 2017)
3	7.0205	0.1361	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	Antimicrobial activity (Jasim et al., 2015)
4	9.674	0.2852	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	Antifungal (Zhang et al., 2016)
5	10.8471	0.2645	Methyl tetradecanoate	$C_{15}H_{30}O_2$	No pharmacological properties have as yet been assigned to these compounds (Teixeira et al., 2005)
6	11.0008	0.1709	6-Ethyl-3-methylthio-1,2,4-triazolo(3,4-b)-1,3,4-thiadiazole	$C_6H_8N_4S_2$	--
7	11.7363	0.2777	4-Penten-1-ol, 5-phenyl-3-piperidino	$C_5H_{10}O$	--
8	11.8201	0.6165	2,3-Dihydro-6-isopropyl-1,2,4-triazolo(3,4-b)-1,3,4-thiadiazole-3-thione	$C_6H_8N_4S_2$	--
9	11.9086	1.7516	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	--
10	12.1739	1.0675	1,4-Benzenedicarbonitrile, 2,3,5,6-tetrafluoro-	C8F4N2	--
11	12.2391	0.711	Hexadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	--
12	12.4067	0.6481	Tetracarbonyl-(diisopropylamino-ethoxycarben)-iron	$C_{13}H_{19}FeNO_5$	--
13	12.4625	0.7044	2,5-Cyclohexadiene-1,4-dione, 2-(2-hydroxyphenyl)-	$C_{12}H_8O_3$	--
14	12.5463	0.3664	Valine, N-methyl-N-neopentyloxycarbonyl-, octadecyl ester	$C_{30}H_{59}NO_4$	--
15	12.6906	2.6803	4,6(1H,5H)-Pyrimidinedione, 1,3-diethyldihydro-2-thioxo-	$C_{14}H_{16}N_2O_2S$	--
16	12.7512	2.1766	Tetracarbonyl-(diisopropylamino-ethoxycarben)-iron	$C_{13}H_{19}FeNO_5$	--
17	12.807	0.9128	6-Ethyl-3-methylthio-1,2,4-triazolo(3,4-b)-1,3,4-thiadiazole	$C_6H_8N_4S_2$	--
18	12.8768	0.5506	2H-Benz[e]inden-3-ol, 3,3a,4,5-tetrahydro-3a-methyl-, (3S-cis)-	$C_{14}H_{16}O$	--
19	13.0631	1.2275	Valine, N-methyl-N-neopentyloxycarbonyl-, octadecyl ester	$C_{30}H_{59}NO_4$	--

TABLE 3. Cont.

Peak N.	R.T	Peak area (%)	Compound name	Formula	Biological activity
20	13.091	1.9638	4-Carbamoyl-2,2,5,5-tetramethyl-3-imidazoline-3-oxide-1-oxyl	$C_8H_{14}N_3O_3$	--
21	13.1794	1.1137	L-Norvaline, N-hexyloxycarbonyl-, tetradecyl ester	$C_{26}H_{51}NO_4$	--
22	13.4076	5.6787	4,6(1H,5H)-Pyrimidinedione, 1,3-diethyldihydro-2-thioxo-	$C_{14}H_{16}N_2O_2S$	--
23	13.5938	8.5569	l-Leucine, N-isobutoxycarbonyl-N-methyl-, hexadecyl ester	$C_{28}H_{55}NO_4$	--
24	13.6636	6.3816	4,6(1H,5H)-Pyrimidinedione, 1,3-diethyldihydro-2-thioxo-	$C_{14}H_{16}N_2O_2S$	--
25	13.9895	2.4805	Tetracarbonyl-(diisopropylamino-ethoxycarben)-iron	$C_{13}H_{19}FeNO_5$	--
26	14.05	2.093	1,4-Benzenedicarbonitrile, 2,3,5,6-tetrafluoro-	$C_8F_4N_2$	--
27	14.171	2.6145	L-Norvaline, N-hexyloxycarbonyl-, decyl ester	$C_{22}H_{43}NO_4$	--
28	14.3619	0.9701	Tetracarbonyl-(diisopropylamino-ethoxycarben)-iron	$C_{13}H_{19}FeNO_5$	--
29	14.4131	0.8531	4-Penten-1-ol, 5-phenyl-3-piperidino	$C_5H_{10}O$	--
30	14.4597	1.6289	Thiazolo[5,4-f]quinoline, 2-methyl-		--
31	14.5854	1.4541	4-Penten-1-ol, 5-phenyl-3-piperidino	$C_5H_{10}O$	--
32	14.6738	1.016	Tetrafluorophthalonitrile	$C_8F_4N_2$	Fungicidal (Robert et al., 1967)
33	14.739	1.2294	Tetracarbonyl-(diisopropylamino-ethoxycarben)-iron	$C_{13}H_{19}FeNO_5$	--
34	14.8321	1.723	l-Norleucine, N-neopentyloxycarbonyl-, pentadecyl ester	$C_{27}H_{53}NO_4$	--
35	14.8833	1.554	l-Norleucine, N-neopentyloxycarbonyl-, dodecyl ester	$C_{24}H_{47}NO_4$	--
36	14.9345	2.9097	Gamma-Sitosterol	$C_{29}H_{50}O$	Anticancer and antioxidant, cause decreases in serum cholesterol (Misawa et al., 2008)
37	15.1021	1.2829	trans-4-Aminocyclohexanol, N,N',O-tris(trimethylsilyl)	$C_{15}H_{37}NOSi_3$	--
38	15.6468	0.3374	4-Bromo-2-cyano-5-phenyl-penta-2,4-dienethioic acid amide	$C_{12}H_9BrN_2S$	--
39	15.8283	0.6638	1,3,5-Triazine-2,4-diamine, 6-chloro-N'-ethyl-N,N-dihexyl-	$C_{14}H_{18}CIN_5$	--
40	15.9447	0.1088	4-Bromo-2-cyano-5-phenyl-penta-2,4-dienethioic acid amide	$C_{12}H_9BrN_2S$	--
41	16.1588	1.0089	1,3,5-Triazine-2,4-diamine, 6-chloro-N'-ethyl-N,N-dihexyl-	$C_{14}H_{18}CIN_5$	--
42	16.6058	2.5982	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	$C_{30}H_{48}O$	Antioxidant (Durairaj et al., 2014)
43	17.2203	3.1137	Nonacosane	$C_{29}H_6O$	Medicinal importance as an anti-inflammatory, antibacterial, antiulcergenic (Bhakuni et al., 2001)

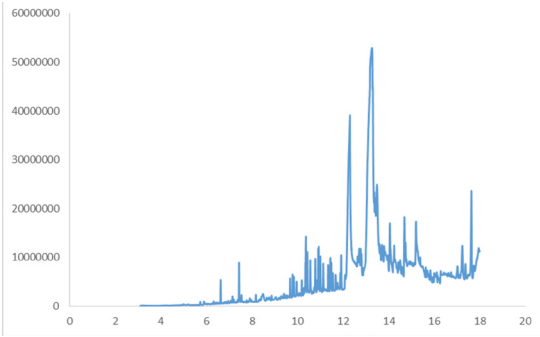

Fig. 4. GC-MS spectrum of phytoconstituents of *Pulicaria undulata* petroleum ether containing oil.

TABLE 4. Phytoconstituents of *P. undulate* essential oil extracted by petroleum ether extract.

Peak N.	R.T	Peak area (%)	Compound name	Formula	Biological activity
1	6.6248	0.2642	Undecane (alkane hydrocarbon)	$C_{11}H_{24}$	--
2	7.4255	0.4695	Dodecane (alkane hydrocarbon)	$C_{12}H_{26}$	--
3	9.6694	0.2672	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	Antibacterial, antiviral and antifungal Ertürk et al. (2016)
4	9.7811	0.2682	Decane, 5-phenyl	$C_{16}H_{26}$	--
5	9.8369	0.2574	Benzene, (1-propylheptyl)-	$C_{16}H_{26}$	--
6	9.958	0.1679	Benzene, (1-ethyldecyl)-		--
7	10.1815	0.2115	Benzene, (1-methylnonyl)	$C_{16}H_{26}$	--
8	10.3676	1.222	Benzene, (1-butylheptyl)-	$C_{17}H_{28}$	--
9	10.4328	0.4759	Benzene, (1-propyloctyl)-		--
10	10.5585	0.4752	Benzene, (1-ethylnonyl)-	$C_{17}H_{28}$	--
11	10.7727	0.4748	Benzene, (1-methyldecyl)-	$C_{17}H_{28}$	--
12	10.8471	0.2077	Methyl tetradecanoate	$C_{15}H_{30}O_2$	--
13	10.903	0.571	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	--
14	10.9309	0.5846	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	--
15	11.0008	0.5089	Benzene, (1-propylnonyl)-	$C_{18}H_{30}$	--
16	11.1265	0.3884	Benzene, (1-ethyldecyl)-	$C_{18}H_{30}$	--
17	11.3406	0.362	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$	--
18	11.4337	0.4812	Benzene, (1-hexylheptyl)-	$C_{19}H_{32}$	--
19	11.4756	0.3969	Benzene, (1-butylnonyl)-		--
20	11.5455	0.2276	Benzene, (1-propyldecyl)-	$C_{19}H_{32}$	--
21	11.6758	0.3247	Benzene, (1-ethylundecyl)-	$C_{19}H_{32}$	--
22	11.8806	0.2395	Benzene, (1-methyldodecyl)-	$C_{19}H_{32}$	--
23	11.9132	0.5237	Hexadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	Antioxidant, decrease blood cholesterol, anti-inflammatory (Das et al., 2014)
24	12.0808	0.2692	9-Hexadecen-1-ol, (Z)-	$C_{16}H_{32}O$	--
25	12.2996	16.5123	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Anti-inflammatory, antioxidant, hypocholesterolemic nematicide, pesticide, anti androgenic flavor, hemolytic, 5-alpha reductase inhibitor, potent mosquito laryicide (Aparna et al., 2012)
26	12.6348	0.9523	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	Antihypertensive, Increases HDL & decrease LDL (Schneider et al., 2011)
27	12.7046	1.5845	Oleanitrile	$C_{18}H_{33}N$	--
28	12.7651	0.735	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{18}H_{32}O_2$	Anti-inflammatory, Hypocholesterolemic Cancer preventive (Arora & Kumar, 2018)

TABLE 4. Cont.

Peak N.	R.T	Peak area (%)	Compound name	Formula	Biological activity
29	12.8024	0.8428	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	--
30	13.2679	37.4343	9,12-Octadecadienoic acid (Z,Z)- Octadecenoic acid (Z) methyl ester	$C_{19}H_{34}O_2$	--
31	13.3889	2.4527	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	--
32	13.4774	5.9337	Linoleic acid	$C_{18}H_{32}O_2$	--
33	14.05	1.3571	9-Octadecenamide, (Z)-	$C_{15}H_{35}NO$	Anti-inflammatory activity and antibacterial activity (Mary & Giri, 2017)
34	14.2409	1.2583	9,12-Octadecadien-1-ol, (Z,Z)-	$C_{18}H_{34}O$	--
35	14.4317	0.3615	Cyclopentadecanone, 2-hydroxy-	$C_{15}H_{28}O_2$	--
36	14.5062	0.3583	Cyclopropaneoctanal, 2-octyl-	$C_{19}H_{36}O$	--
37	14.6924	1.3181	1,9-Tetradecadiene	$C_{14}H_{26}$	--
38	14.7343	0.588	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	--
39	14.7716	0.3628	Linoleic acid	$C_{18}H_{32}O_2$	--
40	15.2744	0.3525	E-11-Hexadecenal	$C_{16}H_{30}O$	--
41	15.6282	0.2345	4,4-Dimethyloxazoline, 2-nonadec-12-enyl-	$C_{24}H_{45}NO$	--
42	15.833	0.3489	Squalene	$C_{30}H_{50}$	Anti cancer, Anti microbial, Anti oxidant, Anti tumor chemo preventive (Mary & Giri, 2017)
43	15.9959	0.1715	11,13-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	--
44	16.2892	0.2602	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Antimicrobial (Orishadipe et al., 2012)
45	17.0247	0.3803	Cholest-5-en-24-one, 3-(acetyloxy)-, (3.beta.)-	$C_{29}H_{46}O_3$	--
46	17.2342	1.2459	Hexacosane	C26H54	Antimicrobial (Singh & Singh, 2003)
47	17.3692	0.3651	Ergosta-4,6,22-trien-3.beta.-ol	$C_{28}H_{44}O$	--
48	17.6346	2.6804	Stigmasta-3,5-diene	$C_{29}H_{48}$	--
49	17.737	0.2221	Ethinyl Estradiol	$C_{20}H_{24}O_2$	--
50	17.9511	0.2603	Phenoxathiin	$C_{12}H_8OS$	--

TABLE 5. Screening of antifungal effect of the methanolic extract and essential oil of *Pulicaria undulata* against the tested fungal pathogens

Test fungus	* Diameter of inhibition zone (mm)		
	Methanolic extract (200mg/ml)	Essential oil (200mg/ml)	Itraconazole (0.2mg/ml)
C. albicans AUMC 9142	20.0±0.00	18.7±0.58	28.0
A. flavus AUMC 10311	24.0±1.00	17.3±0.58	27.0
A. fumigatus AUMC 51	20.3±0.58	16.7±1.50	27.0
T. mentagrophytes AUM C11661	29.3±0.58	24.0±0.00	29.0
M. canis AUMC 11663	29.7±1.50	**ND	28.0
M. boulardii IFM 56403	31.7±0.58	19.0±0.00	26.0

*The experiments were replicated three times and each value is the mean ±SD.

**ND: Not detected.

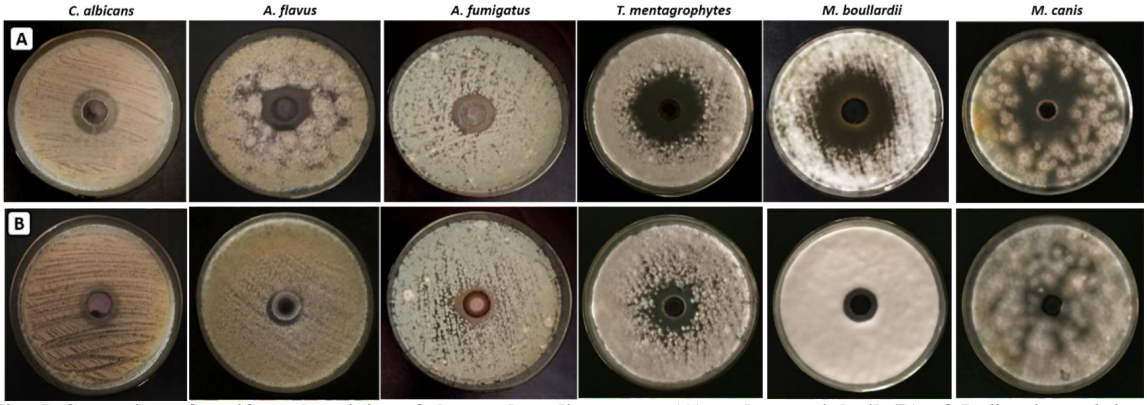

Fig. 5. Screening of antifungal activity of the methanolic extract (A) and essential oil (B) of *Pulicaria undulata* against the tested fungal pathogens.

TABLE 6 Minimum Inhibitory and Fungicidal concentrations (MIC & MFC) of methanolic extract and essential oil of *Pulicaria undulata* against the tested fungi.

Test fungus	Methanolic extract		Essential oil	
	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
C. albicans AUMC 9142	4.25	17	34	> 68
A. flavus AUMC 10311	8.5	> 68	68	> 68
A. fumigatus AUMC 51	8.5	> 68	68	> 68
T. mentagrophytes AUMC11661	4.25	8.5	34	> 68
M. canis AUMC 11663	4.25	8.5	*NT	NT
M. boulardii IFM 56403	2.12	4.25	34	> 68

- MFC/MIC ratio > 4 indicates fungistatic activity, whereas MFC/MIC≤4 indicates fungicidal activity.
- The experiments were replicated three times and the values of MIC and MFC are the means of the three replications.
- *NT: Not tested.

Evaluation of cytotoxicity against VERO cell line

The evaluation of cytotoxicity is an important part of pharmacological validations, and hence the MTT colorimetric assay was conducted to investigate cytotoxicity of the methanolic extract of *P. undulata* against VERO cells. Figure 6 showed an inhibitory effect against the tested cell line as IC50≥ 5.87mg/ml suggesting concentrations below this value as safe ones for further steps as a pharmaceutical product.

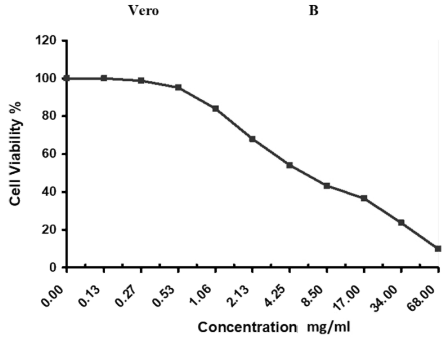

Fig. 6. Survival curve plotted between viable cells (%) and concentration of methanolic extract (mg/ml) obtained from *Pulicaria undulata*.

Discussion

Evidence indicates that the local traditional medicines comprise a potential source of primary healthcare in developing countries (Cordell, 2011; Mahomoodally, 2013). In this connection, several works report that many plants serve as a good source of antioxidants and other bioactive compounds containing phenolics, amino acids, ascorbic acids and alkaloids (Dejussi et al., 2013; Chhetri et al., 2015; Ajaib et al., 2015; Boumaraf et al., 2016). These natural compounds serve as prototypes for the development of more active antimicrobial compounds with less toxicity and with prospects of a large variety of drugs for human consumption (Lewis & Ausubel, 2006; Saravanan et al., 2011). Our results revealed that the aerial portions of *P. undulate* could be utilized as a promising traditional medicine and as a source of some nutraceuticals and antioxidants such as phenols, flavonoids, cardiac glycosides, glucosinolates, alkaloids, terpenes, steroids and coumarins, which is in agreement with the work of Algabr et al. (2010) and Al-Naqeb (2015). As

these phytochemicals are characterized by ROS scavenging abilities (Fecka et al., 2007), they have been recommended as anti-inflammatory, antilukemic (Al-Yahya et al., 1984) and antibacterial activity (Ali et al., 2012; Fawzy et al., 2013; Al-Naqeb, 2015). Furthermore, flavonoids have used as potential therapeutic agents against a wide variety of diseases comprising radical damage (Costa et al., 2015) and terpenes are the most potent drugs against serious diseases such as cancer (Ebada et al., 2010), malaria and heart disease (Parshikov et al., 2012), and bioinsecticides (Liebgott et al., 2000).

As the methanolic *P. undulata* extract exhibited greater DPPH radical scavenging potential and total antioxidant capacity than those of the petroleum ether extract containing oil, it is recommended that the methanolic extracts of *P. undulate* is a potent source of natural antioxidants. Our recommendation is supported by the findings of Kubola & Siriamornpun (2008) and Farahat et al. (2013) that there is a positive relation between the phenolic content and antioxidant activities. Similarly, presence of ascorbic acid, GSH, saponins, cardiac glycosides and alkaloids in the methanolic extract of *P. undulata* could be also involved in the antioxidant biological activity and thereby medicinal uses as reported by Cook & Samman (1996) and Shi et al. (2007). The phytochemical composition of *P. undulate* is also confirmed by the results of GC-MS analysis. A total of 43 compounds and 50 major phytocomponents was identified in the methanolic and petroleum ether extracts, respectively. These compounds are characterized by their antimicrobial, antibacterial, antifungal, antioxidant and anti-inflammatory properties which points to possible new antifungals from various natural sources including higher plants (Graybill, 1996; Maertens & Boogaerts, 2000). Further, the evaluation of MIC and MFC indicated that methanolic and petroleum ether extract of *P. undulata* had an antifungal effect on the tested pathogens whereas petroleum ether extract containing essential oil was less efficient. Since no previous work recorded the antifungal activity of *P. undulata* against *Aspergillus flavus, A. fumigatus*, and dermatophytes, we believe that this is the first report on the antifungal effect of *P. undulata* on these medically important fungi. The antifungal activity of *P. undulata* demonstrated in this study is supported by several previous scientific reports (Hashem, 2011; Jamalian et al., 2011; Massiha & Muradov, 2015) who indicated that a wide variety of plants extracts had fungistatic and fungicidal activities toward different fungal isolates at various concentrations.

The great efficiency of methanol extract compared with other extracts might be due to the extracting capacity of this alcohol to provide a better extraction of phytochemical constituents (Ali-Shtayeh & Abu Ghdeib, 1999). Therefore, the superior antifungal activity of *P. undulata* methanolic extract may be attributed to their major antimicrobial phytochemical constituents (Table 3) (Keskın et al., 2012; Jasim et al., 2015). It has been also reported that the thiazole moiety is the fundamental part of the structure of therapeutic agents which is widely used as antimicrobial agent, antifungal agent, and nonsteroidal anti-inflammatory drug (Karthikeyan, 2009; Bharti et al., 2010; Mishra et al., 2017). In the current investigation, the methanolic extract clearly had measurable amounts of different pyrimidinedione (34.67%) and thiazole (10.55%) derivatives, which are known to have antifungal and antibacterial activities (Farghaly & Hassaneen, 2013; Liu et al., 2014). Moreover, the great performance (as antifungal bioactivity) of the crude methanolic extract relative to the crude essential oil is most likely ascribed to the synergistic effects of its phytochemical constituents.

Owning to the findings that there are adverse toxicity experiences from several medicinal plants (Elgorashi et al., 2003; Unnikrishnan, 2010), evaluation of cytotoxicity using a standard cell-based toxicity assay against Vero monkey kidney cells is important to compare hazards against efficacy and to ascertain the safety of the pharmacologically active extract of *P. undulata* for human use. The result of the MTT assay obtained herein (IC50= 5.87mg/ml) suggest that concentrations below this value could be safe ones. In addition, although the methanol extract of *P. undulata* effectively inhibited all the tested fungal strains, the most sensitive fungi were *M. boulardii* followed by *C. albicans*, T. *mentagrophytes* and *M. canis*. Consequently, the cytotoxicity evaluation of the tested crude extract showed safe levels at least in case of these four fungal strains. However, it was reported earlier that *P. undulata* essential oil displayed moderate cytotoxicity against MCF-7 breast cancer cells (Ali et al., 2011). It is obvious that further mutagenic and toxicological assays with different modes of

action are necessary to establish the complete safety of these plant extracts for pharmaceutical applications.

Conclusion

The results provide a convincing evidence of the antifungal activity of the methanolic extract of *Pulicaria undulata* against the tested fungal species. In addition, it showed fungicidal activities against four of the frequently occurred agents of superficial mycoses. Also, this extract exhibited a good antioxidant activity. Therefore, it is recommended as an alternative medicine and/or a combination therapy for treating these fungal infections. However, further research is needed to isolate the active constituent(s) and to elucidate the exact mode of action of the antifungal and antioxidant activities. Clinical trials are also essential to assess the practical relevance of these *in vitro* results.

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التحليل الكيميائي النباتي و النشاط الحيوي المضاد للفطريات فى مستخلص المثيانول والزيت الأساسى لنبات الربل

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الربل هي واحدة من الأعشاب العطرية الحولية المستخدمة في الطب الشعبي في مصر. تم جمع الأجزاء الهوائية لنبات الربل من الصحراء الساحلية للبحر الأحمر خلال مرحلة الإزهار واستخدمت في التحليل الكيميائي النباتي. وقد تبين أنه مصدر واعد لكلٍ من الفينولات، الفلافونويدات، التيربينويدات، السابونينات والقلويدات، الغلوكوزينولات، والجليكوسيدات القلبية والتى يمكن أن تشارك بشكل فعال في تطوير الأدوية واستكشاف استراتيجيات بديلة لمبيدات الفطريات الأصطناعية الضارة. وأظهرت النتائج أن أكبر تراكم للمركبات النشطة بيولوجيًا كان في المستخلص الميثانولي و كان ذلك مصاحبًا لنسب أعلى في القدرة المضادة للأكسدة و كذلك في قيم IC50 مقارنةً بالقيم المقابلة للزيوت الطيارة المستخلصة بالإيثر النفطي. وكشف تحليل الكروماتوجرافيا الغازية المقترنة بمطياف الكتلة (GC-MS) للمستخلصات النباتية عن وجود حوالي 10 مكونات نباتية ذات نشاط حيوي و تتضمن: Cyclotrisiloxane) و hexamethyl-, وCyclotetrasiloxan و octamethyl- و Cyclopentasiloxane و decamethyl و Dodecanoic acid و methyl ester و Tetrafluorophthalonitrile و gamma-Sitosterol و Nonacosane, 4,4,6a,6b,8a,11,11,14b- ,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-Octamethyl picen-3-one و -Thiazolo[4,5-f]quinoline, 7-methyl). وأظهر المستخلص الميثانولي نشاطاً مضادًا للفطريات ضد السلالات الفطرية الستة الممرضة محل الدراسة و التي تضمنت *Aspergillus flavus* و *A. fumigatus* و *Candida albicans* و *Microsporum canis* و *M. boulardii* و *Trichophyton mentagrophytes*. وكذلك ، فقد أشارت القيم الخاصة باختبار الحد الأدنى من التركيزات المثبطة للنمو(MIC) واختبار الحد الأدنى من التركيزات المبيدة للفطريات (MFC) إلى أن المستخلص الميثانولي كان له تأثير مبيد للفطريات (fungicidal) على كل من *M. boulardii* IFM 56403 و *T. mentagrophytes* AUMC 11661 و*M. canis* AUMC 11663 و*C. albicans* AUMC 9142، هذا و قد أظهر اختبار السمية الخلوية لهذا المستخلص مستويات آمنة في حالة التركيزات المبيدة لهذه السلالات الفطرية الأربعة. في حين كان مستخلص الأثير النفطي المحتوي على الزيوت الطيارة له قدرة تثبيطية ضد الفطريات المختبرة لكن بكفاءة أقل. وتعد نتائج هذه الدراسة بمثابة توسيع لنطاق البحث وكذلك تأكيد لنتائج التقارير السابقة التي قد أشارت إلى التأثير المضاد للفطريات لنبات الربل على بعض الفطريات المهمة طبياً.