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EVALUATION OF ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF DIFFERENT EXTRACTS OF LAVANDULA PUBESCENS GROWING IN ALBAHA REGION, KSA

SALEH B. ALGHAMDI[1,A] AND KHALED A. ABDELSHAFEEK[2,3]

[1]Bioloy department, faculty of science, Albaha university, Albaha, KSA

[2]Chemistry department, faculty of science, Albaha university, Albaha, KSA

[3]Medicinal plants department, pharmaceutical industries division, national research Centre, Dokki, Giza, Egypt.

***Corresponding author: Saleh B. Alghamdi: E Mail: sb.alghamdi@bu.edu.sa; Telephone: 00966580483984**

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ABSTRACT

Lavandula pubescens belongs to the family *Lamiaceae*. This plant commonly grows in Albaha region of Saudi Arabia. It contains many classes of phytoconstituents such as volatile oils, terpenes, flavonoids, phenolics; therefore possesses different biological activities such as antibacterial, antifungal and antidepressant etc. Different extracts (Petroleum ether, chloroform, ethyl acetate, butanol, hydro-methanol and water) were prepared by maceration of dried above ground fragments of *Lavandula* in different solvents. The volatile components of the pet ether extract were identified by GC/MS which showed the occurrence of α-*iso*methyl ionone (45.38%), caryophyllene oxide (29.08%) and Carvacrol (18.08%) as main components. The antimicrobial action of various extracts was assessed using Gram positive bacteria (*Staphylococcus aureus and Bacillus subtilis)* and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeuroginosa.* The antifungal activity was also tested using fungi *Aspergillus flavus* and *Candida albicans*. The results showed that, pet. ether extract exhibited highest activity

against *E. coli* and *S. aureus* with MIC=32mg/ml. While the cytotoxicity results depicted that, the Inhibitory activity of the hydoalcoholic extract (70%) is maximum and was established as most active with $IC_{50} = 21.4 \pm 1.3$ µg/ml against colon carcinoma (HCT-116) cell lines.

Key words: *Lavandula pubescens*, *Lamiaceae*,Extracts, Antimicrobial, Cytotoxicity

INTRODUCTION

Infectious ailments are amid the factors that cause deaths and are associated with approximately one-half of the mortality rate in tropical countries. Poor communities especially belonging to remote towns and locals in developing countries utilize folk medicine for cure of infectious diseases. Medicinal plants are consumed as decoctions, infusions, teas and juice preparations to treat the respiratory infections and directly used on wounds or burns. A lot of work has been done and still is being going on for the search of bioactive compounds to treat microbial infections as more effective and safer therapeutic agents (Muhammad et al 2013).Cancer is serious threat in both developed and developing countries. It has been ranked as 2nd major disease all over the world. Traditional formulations may serve as strong new drugs. Therefore the researches are interested to isolate the active constituent of the plants. Phytomedicines are being utilized for cure of different diseases in Egypt, China, India and Greece (Mohammad Shoeb, 2006).

Hanamanthagouda et al in 2010 stated that, *Lavandula* Genus (family Lamiaceaae) is very common in the Mediterranean region and includes annuals aromatic foliage and flowers. It was the essential oil isolated from *Lavandula* species which has been used in aromatherapy, sedative. antibacterial, antifungal, antidepressant, epilepsy, migraine, reduce spasms in colic pain and carminative purposes. (Barocelli et al 2004, and Hajhashemi et al 2003). Also, the main constituents of *Lavandula* genus have extensively been studied. It contains the volatile compounds, triterpenes, phenolics cinnamic acid derivatives and flavonoids (Torras-Claveria et al., 2007; Papanov et al., 1992).

Ibrahim El-Garf, et al in 2015 isolated many flavonoids (luteolin, apigenin, luteolin 7-*O*-β-Dglucoside, apigenin 7-*O*-β-D-glucoside, hypolaetin 8-*O*-glucuronide, hypolaetin 4-methyl ether 8-

O-glucuronide, isoscutellarein 8-*O*-glucuronide and luteolin 7-*O*-β-D-glucuronide) from *L. coronopifolia* and *L. pubescenes*.

Considering the diverse medicinal uses of the *Lavandula* plant, this study was focused to investigate the components of pet. ether extract of *Lavandula pubescens,* collected from Albaha region of Saudi Arabia. Additionally, our study was also designed to study antimicrobial and cytotoxic activity of different extracts of *Lavandula pubescens*.

MATERIALS AND METHODS

Plant material

The *Lavandula pubescens* plant was collected from Albaha region , KSA, in June 2017. The plant was identified by Dr. Hedir Abdelkader, a taxonomist at Biology Department, faculty of Science, Albaha University, KSA. A voucher specimen was deposited at Biology Department, Albaha University. The above ground portion of plant have been dried in shade for 10 days with reversing daily. After completely drying, the plants were finely powdered for extraction.

Preparation of the plant extracts

The 100g of dried plant parts were extracted with pet. ether (b.r. 40-60°C) in a Soxhlet for two days till exhaustion to afford a greenish color extract. A portion of this extract was passed over fuller's earth to remove the colored pigments to afford a clear yellowish extract and the solvent was evaporated under reduced pressure using rotatory evaporator to give the pet. ether extract which was analyzed using GC/MS. The solvent of other portion of the pet. ether extract was evaporated to give a semisolid oily residue (6L) which was used for biological tests.

GC-MS analysis:

The GC-MS study of volatile compounds in pet ether was carried out using gas chromatography-mass spectrometry instrument present at the Department of Medicinal and Aromatic Plants Research, National Research Center, Dokki, Giza, Egypt with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system has been outfitted to TG-5MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness). The investigation was done using helium as carrier gas at a flow

rate of 1.0 ml/min and a split ratio of 1:10, using the following temperature program: 60 ^{o}C for 1 min; rising at 3.0 ^{o}C /min to 240 ^{o}C and kept for 1 min. The injector and detector were kept at 240 ^{o}C. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were injected. The Mass spectra were obtained by electron ionization (EI) at 70 eV, utilizing spectral range of m/z 40-450. Most of the compounds were identified by comparing their retention indices relative to C8-C26 (n- alkane series) and by comparing their mass spectra fragmentation and retention times with those already reported in the Wiley spectral library collection and NSIT library, addition to the previous literature (Adams, 1995, Wafaa et al 2014 and Mclafferty et al 1989). The individual components have been acknowledged by comparing their mass spectra.

Preparation of different plant extracts:

Five samples; 50g each were put into five conical flasks and macerated individually, in 100ml of chloroform, ethyl acetate, n-butanol, methanol and water respectively for two days with shaking from time to time. The mixture was filtered to remove saturated solvents and new solvents were added. The process was repeated three times till complete extraction and the solvents were evaporated as described above to afford the chloroform extract(1L), ethyl acetate extract(2L), n-butanol extract(3L), methanol extract(4L) and water extract(5L) respectively which were investigated for biological activities.

Antimicrobial Activity test:

The antimicrobial activity was evaluated using different organisms as shown in the table -1, using a modified Kirby-Bauer disc diffusion method (Pfaller, *et al*., 1988). Six microorganisms (bacterial and fungal strains acquired from Micro Analytical Center, Faculty of Science, Cairo University), have been investigated. *Staphylococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051) were used as Gram-positive bacteria; *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145) were used as Gram-negative; *Candida albicans* (ATCC 7102) and *Aspegillus flavus* (ATCC 19433) as were used as fugal strains. Ampicillin and Amphotericin B (Bristol-Myers Squibb, Switzerland) have been used as standard antibacterial and antifungal drugs; respectively however, the filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were

used as a negative control. The region with no growth in the region of the disc is being designated as zone of inhibition or clear zone. 100 µl of microbial suspension have been spread onto agar plates. Approved standard (M38-A) Disc diffusion technique for filamentous fungi (NCCLS, 2002) for investigating susceptibilities of filamentous fungi to antifungal agents was used. Approved standard method (M44-P) for yeasts (NCCLS, 2003) was used. Plates inoculated with *A. flavus* were incubated at 25 ^{o}C for 48 hours; *S. aureus, B. subtilis*, *E. coli* and *P. aeuroginosa* were incubated at 35-37 ^{o}C for 24-48 hours. The *C. albicans* was incubated at 30^{o}C for 24-48 hours and finally the diameters of the inhibition zones were measured (Bauer *et al.,* 1966).

Determination of minimum inhibitory concentration (MIC)

MIC values were determined as described by the NCCLS (1999).

Evaluation of cytotoxicity on HCT-116 cell lines:

This work was done at Al-Azhar University, The Regional Center for Mycology & Biotechnology. The Mammalian cell lines HCT-116 (colon carcinoma) were obtained from VACSERA Tissue Culture Unit.

Chemicals Used

Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza. Crystal violet stain (1%) was prepared by mixing 0.5% (w/v) crystal violet and 50% methanol and then final volume was made up with with ddH_2O and filtered through a Whatmann No.1 filter paper.

Cytotoxicity assay

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well and 100µl of growth medium was kept in each well. Fresh medium containing different test samples were added after 24 h. Two-fold dilutions of samples were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA). The microtiter plates were incubated at 37 ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells have been dedicated for each concentration of the test sample.

Control cells were incubated without test samples and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. The viability of cells was determined by a colorimetric method as reported earlier (Mosmann, T., 1983). Media were aspirated and the crystal violet solution (1%) was added to each well and kept for 30 minutes and the procedure was completed as described by Gomha et al 2015.

RESULTS AND DISCUSSION

The GC /MS data of volatile components of the pet. ether extract (Fig.1and Table 1), showed the presence of twenty one volatile components which were identified as α-*iso*methyl ionone as the major compound (21.40%). Other major compounds were carvacrol (14.35%) and caryophyllene oxide (27.16%). The volatile constituents belonging to many chemical classes that characterize the volatile components like: oxygenated monoterpenes(19.16%), oxygenated sesquiterpenes (28.74%), oxygenated nor-sesquiterpenes(24.97%), sesquiterpenes hydrocarbons (6.45%), oxygenated diterpenes (5.66%) in addition to some acids, aromatics and steroids which constituted approximately 11%. The data obtained isn't in agreement with that reported by Wafaa et al in 2014 who identified forty six compounds from the volatile oil of *L. coronopifolia* of Saudi Arabia, with the main components as phenol-2-amino-4,6-bis (1,1dimethylethyl) (51.18%), carvacrol (4.35%), n-hexadecanoic acid (3.60%), trans-2-caren-4-ol (3.57%), 17-pentartiacontene (2.59%) and caryophyllene oxide (2.16%), whereas our results discovered the existence of caryophyllene oxide as a main component(27.78%) with carvacrol and α-*iso*methyl ionone (14.87% and 21.4% respectively. While, Ait Saidab et al in 2010 found that, the main compounds of the oil of *L. coronopifolia* growing in Morocco were carvacrol (48.9%), caryophyllene (10.8%) and caryophyllene oxide (7.7%). The obtained data by Gouda et al in 2017 where they studied the volatile oil of *L. pubescens* growing in Egypt, identified twenty eight components showing 87.39% of the total oil with Carvacrol (22.39 %), cis-β-Farnesene (13.25 %) and β-Bisabolene (12.9 %) as main constituents, while the same plant oil in Yaman has been found rich in carvacrol (60.9-77-5%), with lesser concentration of carvacrol methyl

ether (4.0-11.4%), caryophyllene oxide (2.1-6.9%), and terpinolene (0.6-9.2%)(Al-Badani et al 2017). Also the essential oil of Tunisian *L. coronopifolia* was characterized by elevated proportion of Trans-β- ocimene (26.9%), carvacrol (18.5%), β-bisabolene (13.1%) and myrecene (7.5%), while the essential oil of *L. coronopifolia* collected from Jordon has been autheticated by the incidence of linalool with percentage of 41.2% and 40.7% for flower and leaves respectively, while 1,8-cineole (25.4% , 7.7% and 7.3%) have been recognized in aerial portion such as leaves and flowers respectively. It was clear that, the chemical composition of the volatile constituents isolated from different *Lavandula* species obtained from various places are quite dissimilar both in quality and quantity regarding to their components.These differences in essential oil compositions could be owing to differences in the ecological conditions, collection time of the plant materials, the environmental conditions such as water, nutrient stress or stage of growth of the plantand temperature (Messaoud et all 2011).

The antimicrobial action of the obtained extracts were studied against six microorganisms using the agar disc diffusion assay as shown in Table (2). The activity can be classified into four levels: weak activity (IZ zone =9-12mm), moderate activity (IZ zone =13-15mm) strong activity (IZ zone =16-21mm), and highly active (IZ more than 21mm). The data (inhibition zone in mm) of various microorganisms showed that, the pet. ether extract (6L) exhibited the maximum antimicrobial activity against all the selected strains of microorganisms, especially in case of *E. coli* (IZ=17mm, MIC=32mg/ml), and moderate activity against *C. albicans* (IZ=13mm, MIC=50mg/ml). All extracts demonstrated no activity against the fungus *A. flavus.* The chloroform extract (1L) exhibited weak activity against all tested bacterial strains (IZ=11-12mm), while, the activity of ethyl acetate and butanol extracts ranged from weak to moderate (IZ-11-15mm).So, our findings are in contrast to that of Wafaa et al in 2014 where they stated that the antimicrobial activity of *L. coronopifolia* possess no activity against *B. subtilis, S. aureus, E. coli, P. aeruginosa, M. smegmatis* and *C. albicans* at a concentration of 180 µg/ml.

The data in table (3) and figures(2-7) of cytotoxicity of different extracts against HCT-116 (colon carcinoma) celllines exhibited varied inhibitory effects, the alcoholic extract showed 93.0% and 71.64% inhibition of the colon carcinoma cells with IC_{50} = 21.4 ± 1.3 µg/ml as shown in fig. 5. Also the present results proved that, the butanol, water and pet. ether extracts displayed feeble inhibitory action against colon carcinoma cells with IC_{50} for all of them = > 500 µg/ml. The strong activity of the alcoholic extract might be owing to the incidence of polyphenols and some of the flavonoids which found to be present during the phytochemical screening and these compounds have potent antioxidant and anticancer actions as reported by (Czaplinska et al., 2012). This data is in agreement with that of Ali et al in 2014; they found that the ethanolic extract of *L. dentata* exhibit promising cytotoxic activity and also the activity could be owing to presence of some lipodal substances which were accounted to have antitumor activity, whereas, André et al in 2013 proved the cytotoxic effects of the essential oil of *L. angustifolia* against human fibroblasts (GM07492-A).

Table (1): GC/MS data of pet. ether extract of *L. pubescenes*

Peak no.	Compound	RT (min.)	Area (%)	M. wt.	Chemical formula
1	p-Cymen-8-Ol	13.52	1.99	150	$C_{10}H_{14}O$
2	Carvacrol methyl ether	15.24	2.30	164	$C_{11}H_{16}O$
3	Carvacrol	18.08	14.87	150	$C_{10}H_{14}O$
4	β- bisabolene	26.21	3.51	204	$C_{15}H24$
5	(-)-Caryophyllene oxide	29.08	27.78	220	$C_{15}H_{24}O$
6	Falcarinol	39.70	2.94	244	$C_{17}H_{24}O$
7	4-t-Octyl-*O*-cresol	42.03	0.57	220	$C_{15}H_{24}O$
8	6-Methyl-α-Ionone	44.35	3.57	206	$C_{14}H_{22}O$
9	5,6-epoxy-5,6-dihydro- Retinoic acid	45.04	2.92	316	$C_{20}H_{28}O_3$
10	α -*iso*methyl ionone	45.38	21.40	206	$C_{14}H_{22}O$
11	epicedrol	46.61	0.95	222	$C_{15}H_{26}O$
12	Combetastatin A-4	47.09	1.05	316	$C_{18}H_{20}O_5$
13	Retinoic acid	48.79	1.84	300	$C_{20}H_{28}O_2$
14	4-Benzyloxy-6-methoxy-*O*-toluic acid	50.88	2.14	272	$C_{16}H_{16}O_4$
15	Oxymetholone	52.26	0.70	332	$C_{21}H_{32}O_3$
16	17-hydroxy-1,17-dimethyl-Androstan-3-one	52.59	2.93	318	$C_{21}H_{34}O_2$
17	Totara-7a,13-diol	52.69	2.79	302	$C_{20}H_{30}O_2$
18	Ferruginol	54.04	2.03	286	$C_{20}H_{30}O$
19	Epibolin	54.64	0.84	320	$C_{20}H_{32}OS$
20	1,4,8-trimethoxy-9-Anthracenol	57.89	2.32	284	$C_{17}H_{16}O_4$
21	Diisooctyl phthalate	58.91	0.68	390	$C_{24}H_{38}O_4$
Classes of the volatile chemical constituents of *L. pubescens*					
1	Compounds	%			
2	Oxygenated Monoterpenes (OM)	19.16			
3	Sesquiterpene Hydrocarbons (SH)	6.45			
4	Oxygenated Sesquiterpenes (OS)	28.74			
5	Oxygenated nor sesquiterpenes (ONS)	24.97			
6	Oxygenated Diterpenes (OD)	5.66			
7	Steroids	3,63			
8	Acids	4.46			

Table (2) : Antimicrobial activity of different extracts with MIC(mg/ml) of *L. pubescens*

Sample		Inhibition zone diameter (mm)					
		Bacterial species				Fungi	
		G^+		G^-			
		B. Subtilis	*St.aureus*	*E. coli*	*Ps.aeruginosa*	*A. Flavus*	*C. albicans*
Control: DMSO		0.0	0.0	0.0	0.0	0.0	0.0
Standard	Ampicillin	31	24	30	28	--	--
	Amphotericin B	--	--	--	--	16	21
1L		11	12	11	12	0.0	0.0
2L		11	13	13	12	0.0	0.0
3L		11	12	15	12	0.0	0.0
4L		14	12	13	11	0.0	0.0
5L		9	10	13	11	0.0	0.0
6L		16	16	17	15	0.0	13
Determination of MIC (mg/ml)							
3L		--	--	40	--	--	--
6L		26	32	32	38	--	50

Table(3): Cytotoxicity activity of different extracts of *L. pubescens*

Sample conc. (µg/ml)	1L		2L		3L		4L		5L		6L	
	Viab. %	Inhib. %± S.D	Viab.%	Inhib. %± S.D	Viab. %	Inhib. %± S.D	Viab.%	Inhib. %± S.D	Viab. %	Inhib. %± S.D	Viab. %	Inhib. %± S.D
500	39.76	60.24±0.81	31.25	68.75±0.89	70.94	29.06±2.32	6.81	93.19±0.12	87.23	12.77±0.51	54.12	45.88±0.69
250	51.34	48.66±0.92	43.89	56.11±1.37	87.38	12.62±1.06	13.92	86.08±0.27	98.14	1.86±0.28	71.39	28.61±0.43
125	64.29	35.71±0.37	56.73	43.27±1.41	94.42	5.58±0.34	21.84	78.16±0.32	100	0	86.40	13.6±0.31
62.5	78.92	21.08±0.84	71.48	28.52±0.64	99.71	0.29±0.07	33.28	66.72±0.56	100	0	95.34	4.66±0.25
31.25	90.63	9.37±0.25	85.17	14.83±0.35	100	0	42.79	57.21±0.93	100	0	99.72	0.28±0.14
15.6	98.41	1.59±0.17	93.88	6.12±0.24	100	0	54.25	45.75±0.71	100	0	100	0
7.8	100	0	98.62	1.38±0.13	100	0	63.16	36.84±0.42	100	0	100	0
3.9	100	0	100	0	100	0	78.57	21.43±0.31	100	0	100	0
0	100	0	100		100	0	100	0	100	0	100	0

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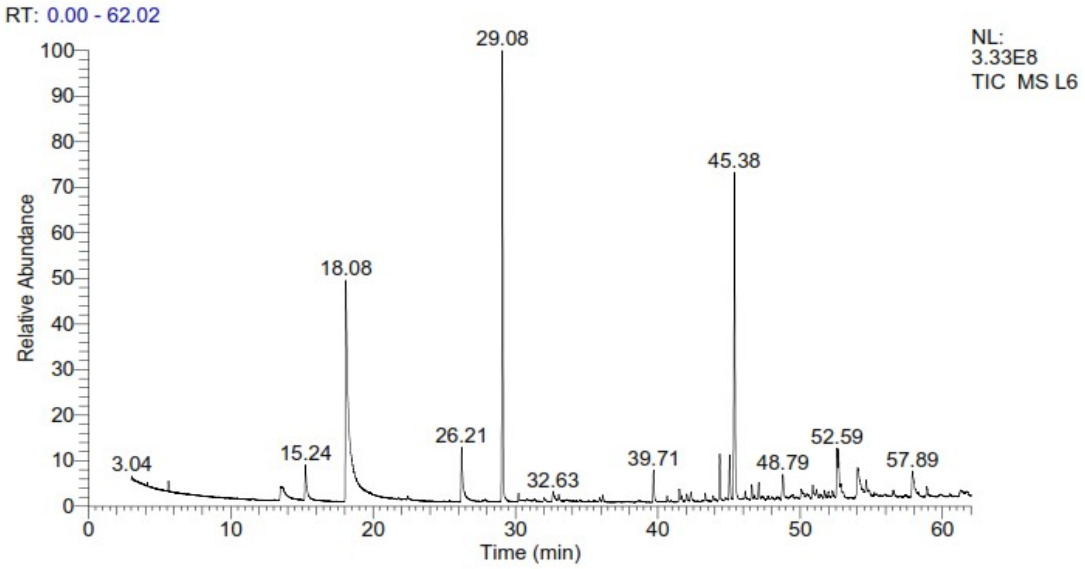

Figure 1. GC/MS Chromatogram of volatile constituents in pet. ether extract of *L. pubescenes*

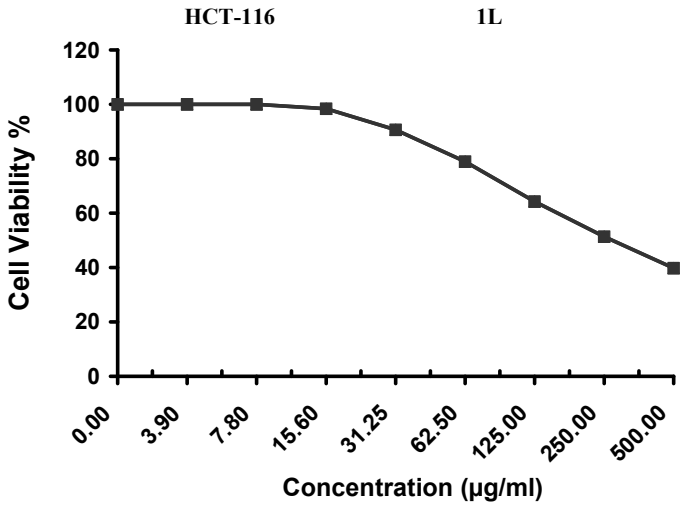

Figure 2. Inhibition of HCT-116 cells by chloroform extract (1L) of *L. pubescens*

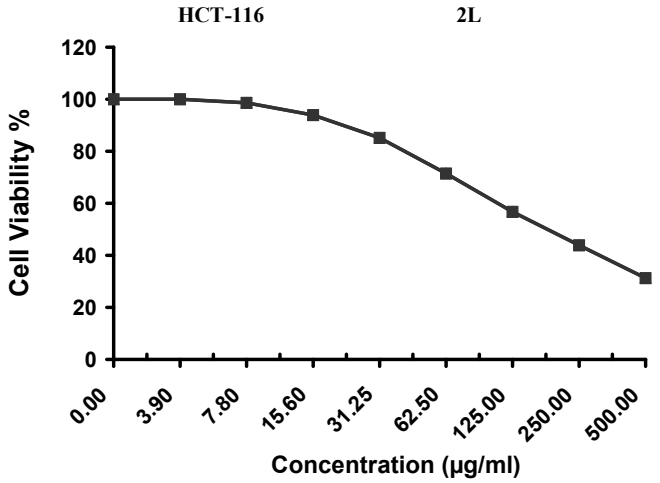

Figure 3. Inhibition of HCT-116 cells by ethyl acetate extract (2L) of *L. pubescens*

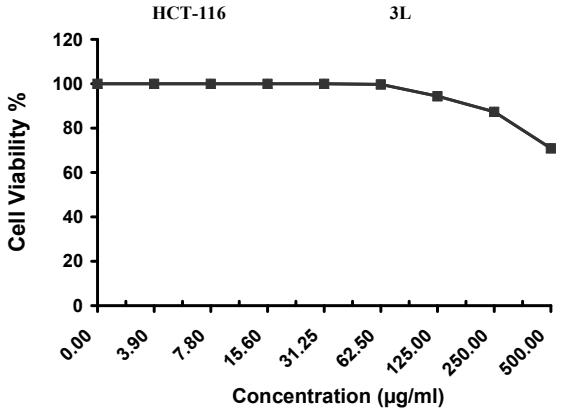

Figure 4. Inhibition of HCT-116 cells by butanol extract of *L. pubescens*

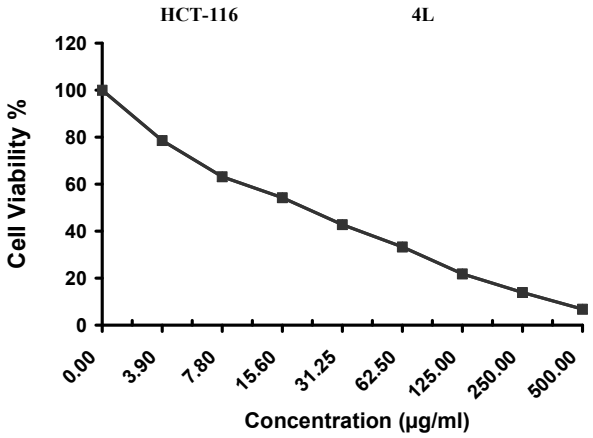

Figure 5. Inhibition of HCT-116 cells by methanol extract of *L. pubescens*

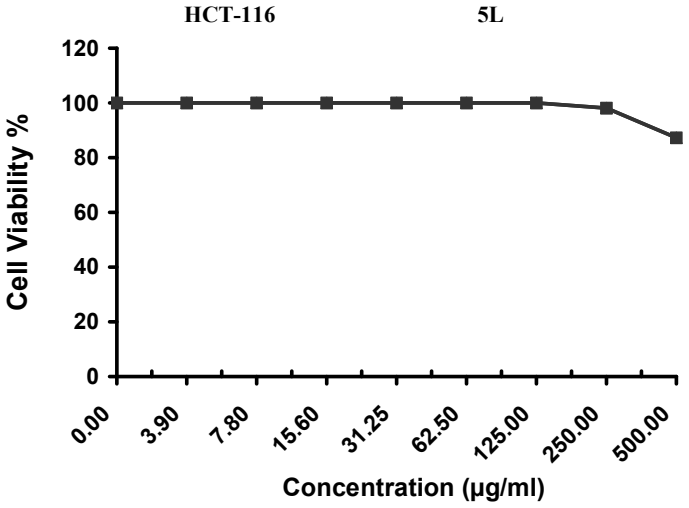

Figure 6. Inhibition of HCT-116 cells by water extract (5L) of *L. pubescens*

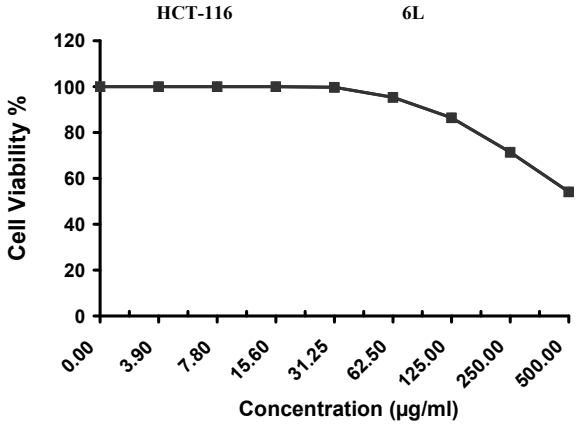

Figure 7. Inhibition of HCT-116 cells by pet. ether extract (6L) of *L. pubescens*

CONCLUSION

The volatile constituents in pet. ether extract of the *Lavandula pubescens* aerial parts were analyzed and identified by GC/MS which showed the occurrence of α-*iso*methyl ionone (45.38%), Caryophyllene oxide(29.08%) and Carvacrol(18.08%) as main components. The results of antimicrobial activity of extracts (Petroleum ether, chloroform, ethyl acetate, butanol, hydro-methanol and water) was evaluated against Gram positive bacteria such as *St. aureus* and *B. subtilis*; Gram negative bacteria such as *E. coli* and *Ps. aeuroginosa* , also using fungi such as *A. flavus* and *C. albicans*. showed that, pet. ether extract exhibited the highest activity against both *E. coli* and *S. aureus* with MIC=32mg/ml. While the cytotoxicity results against colon

carcinoma (HCT-116) cell lines proved that, the Inhibitory activity of the methanolic extract (70%) is maximum and was found the most active with IC_{50} = 21.4 ± 1.3 µg/ml. The study need to further investigation to determine which fraction in the methanolic extract have this activity to be able to isolate and formulate it in a suitable dosage form.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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