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In vitro Antimicrobial Screening of *Hedwigia ciliata* Var. *leucophaea* and Determination of the Ethanol Extract Composition by Gas Chromatography/Mass Spectrometry (GC/MS)

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Many plants contain active substances that are known to be effective in both enhancing the wound healing process and lowering the incidence of wound infections. Previous studies have shown that bryophytes produce a variety of secondary metabolites that present pharmaceutical activities including antimicrobial activity against various pathogenic bacteria and fungi. The aim of this study was to investigate the antimicrobial activity of *Hedwigia ciliata* var. *leucophaea* (*Hc*) against 17 bacterial and 1 fungal strains and analyse the composition by GC/MS. *Hc* samples were collected from Akdag Mountain (Amasya, TURKEY) and subjected to ethanol extraction after air drying. The *in vitro* antimicrobial activities of *Hc* extracts having 9 mg.mL^{-1} concentrations were assessed against a wide range of strains by disk diffusion method and the results were supported by a MIC (minimum inhibitory concentration) test. Our present study has shown that the ethanol extract of *Hc* has antimicrobial activity against several Gram positive and Gram negative microorganisms tested, but its antimicrobial activity is notable especially against *B. subtilis*, *E. faecalis*, *S. carnosus,* and *S. epidermidis*. These results are the very first report of the antimicrobial activity of *Hc* and its composition. The results obtained herein indicate that ethanol extract of *Hc* contains several active metabolites and it is active against several microorganisms. But further researches, especially cytotoxicity and genotoxicity tests are needed to be conducted to conclude whether *Hc* extracts can be used safely in terms of their antimicrobial activity.

Key words: *Hedwigia ciliata* var. *leucophaea*, Bryophyte, Antimicrobial activity, Disk diffusion test, Bacterial strains, Fungal strains.

It has been known for years that mosses were used to treat cardiovascular diseases, tonsillitis, bronchitis, cystitis and skin infections especially in traditional Chinese medicine. In addition, previous studies presented that some of the secondary metabolites produced by mosses are effective in wound healing process and have anti-infective effect on some microorganisms (Altuner *et al*., 2010).

The anti-infective activities of plant-derived products come up focus of interest in recent years. Increasing morbidity and mortality rates of bacterial and fungal infections and accelerating antibacterial and antifungal resistance rates of microorganisms lead to the research on new antimicrobial agents (Altuner *et al*., 2010).

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The secondary metabolites synthesized by plants which are often used as a defence mechanism against microorganisms, insects and herbivores (Samidurai and Saravanakumar, 2009, Altuner *et al*., 2012c). These secondary metabolites have some applications such as being used as antimicrobial agents. The antimicrobial activity of plants has many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997; Reynolds, 1996).

Bryophytes are plants which define about 14,500 species (Veljic *et al*., 2008). In contrast to the extensive utilisation of higher plants as a source of antimicrobial substances, Bryophytes have rarely been considered for this purpose (Basile *et al*., 1998).

It has been known for centuries that mosses are effective in both wound healing process and lowering the risk of infection. In the previous studies it has been presented that some secondary metabolites extracted from mosses have anti-infective effect on some bacteria and fungi (Basile *et al*., 1998; Ilhan *et al*., 2006; Veljic *et al*., 2008; Altuner, 2008; Altuner & Cetin, 2009; Altuner *et al*., 2010a,b,c, 2011a; Savaroglu *et al*., 2011a,b; Onbasili *et al*., 2011,2013; Oztopcu-Vatan *et al*., 2011; Altuner & Canli, 2012).

It has been known for years that mosses were used to treat cardiovascular diseases, tonsillitis, bronchitis, cystitis and skin infections especially in traditional Chinese medicine. North American Indians were used some bryophytes, such as *Bryum*, *Mnium* and *Philonotis*, to heal burns, bruises and wounds (Saroya, 2011).

Since antibacterial and antifungal resistance has been developing in human pathogens against commonly used antibiotics, there is a need for a search about new antimicrobial substances from other sources including plants (Erdogrul, 2002).

Hedwigia ciliata var. *leucophaea* (Family: *Hedwigiaceae* Schimp.) (*Hc*) is a well-known species, easily recognised by its whitish colour, growing over dry acidic rocks. *Hc* is differentiated with a longer hyaline upper part of leaves and strongly papillose leaf cells. *Hc* mainly grows on exposed rocks and prefer base-rich habitats. Although it is reported from Europe, North America and North Africa previously, it's a rare variety (Smith, 2004).

In this study, antimicrobial activity of *Hc* was determined against 17 bacterial and 1 fungal strains. *Hc* samples which were collected from Akdag Mountain (Amasya) were air dried and extracted with ethanol. The *in vitro* antimicrobial effect of *Hc* were investigated against a wide range of strains such as *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 by using the disk diffusion method and the results were supported by a minimum inhibitory concentration (MIC) test.

MATERIALS AND METHODS

Moss Samples

Hedwigia ciliata var. *leucophaea* Bruch & Schimp. samples used in this study were collected from Akdag Mountain (N 40° 442 - E 035° 592), Amasya, which is located between Central Anatolia and the Middle Black Sea region. Voucher specimens were deposited for further reference.

Extraction Procedure

All *Hc* samples were dried out after collection and the samples were ground by a mortar and a pestle. In order to extract active substances, ethanol (Merck, Germany) was chosen as an extraction solvent. Ground samples were shaken in ethanol at 100 rpm for 3 days at room temperature. All the extracts were filtered through Whatman No. 1 filter paper into evaporation flasks (Altuner *et al*., 2011b). The filtrate was evaporated by a rotary evaporator (Heidolph Hei-Vap Value HL/HB-G1) at 30°C. After evaporation the residues were collected and used to prepare 9 $mg.mL^{-1}$ extracts.

Microorganisms

A wide range of Gram positive and Gram negative bacteria and yeast were selected to test

the antimicrobial effect of *Hc*. The strains were chosen from standard strains as much as possible. Other strains which are not standard were all isolated from food and identified in Ankara University, Faculty of Science, Department of Biology.

Bacillus subtilis ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 were used in the study.

Prepartion of Inocula

All bacterial strains were incubated at 37 °C for 24 h. But since the requirements for *C. albicans* is different, *C. albicans* was inoculated at 27 °C for 48 h. Inocula were prepared by transferring morphologically similar colonies of each organism into 0.9% sterile saline solution until the visible turbidity was equal to 0.5 McFarland standard having approximately 10^8 cfu.mL^{-1} for bacteria and 10^7 cfu.mL^{-1} for *C. albicans* (Hammer et. al, 1999; Altuner, 2011).

Disk Diffusion Method

Disk diffusion test was performed as described previously by Andrews (2003). The culture medium was poured into 120 mm sterile Petri dish to give a mean depth of 4.0 mm ± 0.5 mm (Altuner and Çetin, 2009; Altuner and Akata, 2010). 60 µL, 100 µL and 150 µL aliquots of each extract was applied on sterile paper disks of 6 mm diameter end up with 550 µg.µL^{-1}, 917 µg.µL^{-1} and 1375 µg.µL^{-1} sample on each disk (Mahasneh and El-Oqlah, 1999; Silici and Koc, 2006). To get rid of any residual solvent which might interfere with the results, disks were left to dry overnight at 30°C in sterile conditions (Silici and Koc, 2006; Altuner *et al*., 2012a,b). The surface of the plates was inoculated using previously prepared inocula containing saline suspension of microorganisms. Inoculated plates were then left to dry for 5 minutes at room temperature before applying the disks. Disks were firmly applied to the surface of the plate which had an even contact with the agar. Plates were incubated and inhibition zone diameters were expressed in millimetres.

Determination of MIC

Broth dilution method for Minimum Inhibitory Concentration (MIC) determination as described in Basile *et al.* (1998) was performed. Serial 2-fold dilutions were made to obtain a concentration range of 3.9 - 2000 µg.mL^{-1}. The MIC was defined as the lowest concentration of extract inhibiting any visible bacterial growth.

Gas Chromatography-Mass Spectrophotometry Method (GC-MS)

The GC-MS analysis of etanol extract of *Hc* were quantitatively performed by GC-MS (Agilent 19091S-433, 30x0.25x0.25 HPS MS) based on the method defined previously by Hossain and Rahman (2011) with some modifications. Split less injection was performed with a purge time of 1.0 min. The carrier gas was helium at a flow rate of 1 mL.min^{-1}. The column temperature was maintained at 60 °C for 2 minutes, then programmed at 10 °C.min^{-1} to 300 °C and waited for 10 minutes. The inlet temperature was 250 °C, AUX was 290 °C and the solvent delay was 4 min. The identification of the peaks was based on computer matching of the mass spectra with the National Institute of Standards and Technology (NIST Mass Spectrometry DATA CENTER, 2005) library and by direct comparison with published data (Karimi and Jaafar, 2011).

Controls

Empty sterile disks and extraction solvent (ethanol) loaded on sterile disks which were dried at sterile conditions to remove solvent as done in the study were used as negative controls.

Statistics

The statistical analysis was performed using a non-parametric method Kruskal-Wallis one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The diameter of the inhibition zones recorded as the diameter of the zones in millimetres for the samples are given in Table 1 and the MIC

values for the microorganisms in which activity was observed in disk diffusion test are given in Table 2.

No activity was observed for the negative controls; solvents and empty sterile disks.

Table 1 clearly shows that ethanol extracts of *Hc* were presented antimicrobial activity against *B. subtilis* ATCC 6633, *E. aerogenes* ATCC 13048, *E. faecalis* ATCC 29212, *E. faecium*, *S. typhimurium* SL 1344, *S. carnosus* MC1.B, *S. epidermidis* DSMZ 20044 and *S. agalactiae* DSMZ 6784.

According to the data presented in Table 2, the lowest activity was observed in *S. typhimurium* SL 1344 by 1000 µg.mL^{-1} of *Hc* extract, where the highest activity was observed in *B. subtilis* ATCC 6633 by 62.5 µg.mL^{-1} of *Hc* extract.

The GC-MS analysis of ethanol extract of *Hc* is given in Table 3. The ethanol extract of *Hc* is found to be composed of acetic acid, silane, triethoxymethyl, tetraethyl silicate, 1,1-diethoxypentane, hepta-2,4-dienal, d-limonene, 1,2,4,5-tetramethylbenzene, 1-ethyl-2,3-dimethylbenzene, benzoic acid, ethyl ester, 4-methyl benzoic acid, hexose, maltol, orcinaldehide, diethyltoluamide, tetradecanoic acid (myristic acid), 2-phenylmethyl 1,3-cyclohexanedione, dihydrophytol, γ-palmitolactone, farnesol isomer a, n-hexadecanoic acid, hexadecanoic acid, manoyl oxide, doconexent, kaurene, γ-stearolactone, transphytol, linoleic acid, octadecanoic acid, linoleic acid ethyl ester, monopalmitin, monostearin, squalene, α-tocopherol, campesterol, stigmasterol, obtusifoliol, γ-sitosterol, isofucosterol, cycloartenol, vitamin-E acetate, sitostenone, dihydroxyacetone (oxetane), delta-3-carene, phytol and stearic acid.

Table 1. Disk diffusion test results (Inhibition zones in mm)

	60µL	100µL	150µL
B. subtilis ATCC 6633	-	-	16
C. albicans ATCC 10231	-	-	-
E. aerogenes ATCC13048	7	7	7
E. durans	-	-	-
E. faecalis ATCC 29212	-	-	12
E. faecium	-	-	7
E. coli ATCC 25922	-	-	-
E. coli CFAI	-	-	-
K. pneumoniae	-	-	-
L. monocytogenes ATCC 7644	-	-	-
S. enteritidis ATCC 13075	-	-	-
S. infantis	-	-	-
S. kentucky	-	-	-
S. typhimurium SL 1344	-	-	7
S. aureus ATCC 25923	-	-	-
S. carnosus MC1.B	-	8	11
S. epidermidis DSMZ 20044	-	9	15
S. agalactiae DSMZ 6784	7	9	8

"-": No activity observed.

DISCUSSION

Results given in Table 1 and Table 2 clearly show that *Hc* are active against several microorganisms but its antimicrobial activity is notable especially against *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *S. carnosus* MC1.B and *S. epidermidis* DSMZ 20044.

There have been no reports about the antimicrobial activity of *Hc* as far as the current literature is concerned. These results are the very first data about the antimicrobial activity of *Hc* and its composition.

Among the microorganisms which were affected by *Hc* extracts *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *E. faecium*, *S. carnosus* MC1.B and *S. epidermidis* DSMZ 20044 are gram positive where *E. aerogenes* ATCC13048 and *S. typhimurium* SL 1344 are gram negative strains.

It is a well known fact that Gram negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than Gram positive bacteria (Nikaido, 1998). In addition, it was also pointed out previously that Gram negative bacteria are the dominant killers among bacterial pathogens in the Intensive Care Units (*ICU*) (Villegas and Quinn, 2004).

Table 2. MIC values (µg.mL^{-1})

	MIC (µg.mL^{-1})
B. subtilis ATCC 6633	62.5
E. aerogenes ATCC13048	500
E. faecalis ATCC 29212	125
E. faecium	500
S. typhimurium SL 1344	1000
S. carnosus MC1.B	125
S. epidermidis DSMZ 20044	125
S. agalactiae DSMZ 6784	250

Table 3. Chemical composition of *Hc* and their composition percentages.

No	Compounds	Composition (%)
1	acetic acid	0.96
2	silane, triethoxymethyl	0.04
3	tetraethyl silicate	0.22
4	1,1-diethoxypentane	0.04
5	hepta-2,4-dienal	0.08
6	d-limonene	0.04
7	1,2,4,5-tetramethylbenzene	0.06
8	1-ethyl-2,3-dimethylbenzene	0.10
9	benzoic acid, ethyl ester	0.08
10	4-methyl benzoic acid	0.10
11	hexose	2.00
12	maltol	1.02
13	orcinaldehide	0.06
14	diethyltoluamide	0.02
15	tetradecanoic acid (myristic acid)	0.08
16	2-phenylmethyl 1,3 -cyclohexanedione	0.12
17	dihydrophytol	0.20
18	γ-palmitolactone	0.08
19	farnesol isomer a	0.42
20	n-hexadecanoic acid	0.68
21	hexadecanoic acid	7.02
22	manoyl oxide	0.06
23	doconexent	0.48
24	kaurene	0.06
25	γ-stearolactone	0.14
26	transphytol	0.08
27	linoleic acid	1.94
28	octadecanoic acid	0.30
29	linoleic acid ethyl ester	3.58
30	monopalmitin	0.90
31	monostearin	2.48
32	squalene	1.82
33	α-tocopherol	0.74
34	campesterol	4.56
35	stigmasterol	2.60
36	obtusifoliol	0.38
37	γ-sitosterol	1.96
38	isofucosterol	0.22
39	cycloartenol	0.22
40	vitamin-E acetate	0.18
41	sitostenone	0.18
42	dihydroxyacetone (oxetane)	2.06
43	delta-3-carene	1.92
44	phytol	2.70
45	stearic acid	3.02

E. aerogenes was previously identified as one of the "*ICU* bugs" which could cause significant morbidity and mortality. In addition the infection management is complicated due to its resistance to multiple antibiotics (Hidron *et al*., 2008). Although a very low activity was observed against *E. aerogenes*, the results can be accepted as noteworthy. Probably increasing amount of extracts loaded on the empty sterile antibiotic disks may increase the activity

It was reported that although serovar Typhimurium of *Salmonella* has a less alarming public image than serovar Typhi, it is a bigger health problem and it is thought by researchers to be at least 30-fold underreported. There are probably hundreds of millions of cases every year in the world in which serovar Typhimurium kill twice as many people as serovar Typhi which were mostly infants and the elderly people (McClelland *et al*., 2001). According to results, 100 µL of *Hc* extract showed very low antibacterial activity against *S. typhimurium* SL 1344. Since the inhibition zone is quite low, increasing the active substance loaded on the empty sterile antibiotic disks may also increase the activity.

On the other hand *ethanol* extracts of *Hc* are active against several gram positive strains, as stated previously. The results of the disk diffusion tests applied on the gram positive strains are more remarkable than the results of gram negative strains. The activity against especially on *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *S. carnosus* MC1.B and *S. epidermidis* DSMZ 20044 are noteworthy.

The pathogenic potential of *B. subtilis* is generally described as low or absent (De Boer and Diderichsen, 1991). *B. subtilis* is only known to cause disease in severely immunocompromised patients (Galieni and Bigazzi, 1998). Several researchers study antimicrobial activity of some plant extracts on *B. subtilis* ATCC 6633. Khalid *et al*. (2011) compared four different methanolic plant extracts, namely *Pistacia integerrim*, *Polygonum bistorta*, *Swertia chirata* and *Zingiber officinale*. In this study maximum 30 mg of extracts were loaded on sterile antibiotic disks and inhibition zones were found to be 12 mm for *P. integerrim*, 11 mm for *P. bistorta*, 12 mm for *S. chirata* and 17 mm for *Z. officinale*. In our study we observed 16 mm zone for 1.375 mg of *Hc* extract which is about 22 times

lower than the amount used for study conducted by Khalid *et al*. (2011). Comparing these results clearly puts forward how *Hc* is active against *B. subtilis* when compared to some other higher plants.

E. faecalis can cause endocarditis and bacteremia, urinary tract infections, meningitis and other infections in humans (Murray, 1990). It is associated with nosocomial infections including catheter-associated urinary tract infections, central line-associated bloodstream infection and surgical site infections (Hidron *et al*., 2008).

Khalid *et al*. (2011) also studied the activity of some higher plants on *E. faecalis* but used ATCC 14506 strain. Again 30 mg of extracts were loaded on sterile antibiotic disks and inhibition zones were found to be 10 mm for *P. integerrim*, 13 mm for *P. bistorta* and 8 mm for *Z. officinale* and no activity was observed against *S. chirata*. In our study we observed 12 mm zone for 1.375 mg of *Hc* extract. Some other researches about the antimicrobial effect of *Hypericum triquetrifolium*, *Hypericum perforatum* and *Hypericum empetrifolium* against *E. faecalis* ATCC 29212 presented an activity 9 mm, 10 mm and 8 mm of zones respectively.

According to these results it can be concluded that *ethanol* extracts of *Hc* is more active than some higher plants against *E. faecalis* ATCC 29212, which is also true for *B. subtilis*.

S. epidermidis is not usually pathogenic. But they often develop risk for infection for patients with a compromised immune system. These infections can be both nosocomial or community acquired, but they pose a greater threat to hospital patients. *S. epidermidis* is also a major concern for people with catheters or other surgical implants because it is known to cause biofilms that grow on these devices (Queck and Otto, 2008; Salyers *et al*., 2002).

Several studies conducted on the antimicrobial activity of several higher plants against *S. epidermidis*. Mahida and Mohan (2007) tested 10 mg of methanolic extracts of 23 plant extracts, but the highest zone was found to be 20 mm for *Mangifera indica*. In another study *ethanol* extracts of 23 plants were tested against *S. epidermidis* and the highest inhibition zone diameter was given as 18 mm for *Stachys leptoclada* (Sarac and Ugur, 2007).

In our study we observed a 15 mm inhibition zone for 1.375 mg of *Hc* extract against *S. epidermidis* DSMZ 20044 which is relatively high when compared to other previous studies.

The antimicrobial nature of *Hc* extract can be explained by looking at Table 3. The GC/MS analysis clearly puts forward the composition of *Hc* extract, which contains several compounds that have antimicrobial activities.

Dihydroxyacetone (1,3-Dihydroxypropan-2-one) (DHA), which is also known as glycerone in *Hc* is a simple carbohydrate. It is a triose, having formula $C_3H_6O_3$ (Budavari, 1996). DHA was first recognized as a skin colouring agent and also used as an oral drug for assisting children with glycogen storage disease (Wittgenstein and Berry, 1960; Wittgenstein and Guest, 1961; Goldman *et al*., 1961). In addition to these properties DHA has also antimicrobial activity. Manuka honeys containing DHA presented antimicrobial activity especially against multi-resistant strains of *S. aureus*, *S. epidermidis*, *E. coli* and *Pseudomonas aeruginosa* (Lukowski *et al*., 2008; Mavric, 2008; Adams *et al*., 2009; Bäcker *et al*., 2010).

Carene (3,7,7-trimethylbicyclo[4.1.0]hept-3-ene) which is also known as delta-3-carene ($C_{10}H_{16}$), is a bicyclic monoterpene which can be a skin irritant or CNS depressant. Cosentino *et al*. (2003) showed that *Juniperus turbinata* essential oils are active against fungi, particularly against a strain of *Aspergillus flavus*. In addition, they tested antimicrobial activity of single compounds which are present in significant quantities in oils. They proved that delta-3-carene posses the broadest antimicrobial activity and appeared to contribute significantly to the antifungal activity of *J. turbinata* oils (Cosentino *et al*., 2003). Pauli (2006) also found that delta-3-carene is active especially against *Candida utilis*.

Maltol (3-hydroxy-2methyl-4H-pyran-4-one) is neutral, heterocyclic compound having formula as $C_6H_6O_3$. It is also known as larixic acid and widely used as food additive, antioxidant and metal ions chelator (Samejo *et al*., 2009). Some pharmacological activities of maltol have been reported (Aoyagi *et al*., 1974; Soulimani *et al*., 1997), such as showing a central depressing activity (Kimura *et al*., 1980; Aytemir *et al*., 2004). In addition, some maltol derivatives were proved

to have antimicrobial activity especially against *C. albicans* and *S. aureus* (Fassihi *et al*, 2009).

Tetradecanoic acid, which is also called myristic acid, is a saturated fatty acid having molecular formula $CH_3(CH_2)_{12}COOH$. It is previously presented that myristic acid is an antibacterial and antifungal agent (McGaw, 2002; Seidel and Taylor, 2004; Agoramoorthy *et al*., 2007). Skrivanova (2006) showed that myristic acid has antimicrobial activity against *Clostridium perfringens*. Batovska *et al*. (2009) found that myristic acid is active against *L. monocytogenes* with having 125 $\mu g.mL^{-1}$ MIC value. Narasimhan *et al*. (2006) tested the antimicrobial activiy of myristic acid derivatives and presented that myristic acid derivatives are more effective against Gram positive bacteria rather than Gram negative bacteria. It is also concluded that *S. aureus* is the most sensitive organism to myristic acid derivatives (Narasimhan *et al*., 2006). It is also reported that myristic acid has antifungal activity against *Alternaria solani* (Liu *et al*., 2008), *Aspergillus niger* (Altieri *et al*., 2007; Carballeira *et al*., 2005), *Candida albicans* (Kabara *et al*., 1972), *Emericella nidulans* (Altieri *et al*., 2007), *Fusarium oxysporum* (Liu *et al*., 2008), *Penicillium glabrum* (Altieri *et al*., 2007), *Penicillium italicum* (Altieri *et al*., 2007).

Phytol ((2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol) is an acyclic diterpene alcohol having molecular formula $C_{20}H_{40}O$. It is commercially utilised in the fragrance industry and also in cosmetics, shampoos, toilet soaps, household cleaners, and detergents (McGinty *et al*., 2010). Phytol is also reported for its antimicrobial, antiviral, diuretic, anti-inflammatory, strong antioxidant and anti-tumoral activities (Alfred, 2002; Kumar *et al*., 2010; Padmini *et al*., 2010; Uma *et al*., 2011; Revathi *et al*., 2012). Phytol is found to be active especially against *S. aureus* (Inoue *et al*., 2005).

Campesterol ((24*R*)-Ergost-5-en-3â-ol) is a phytosterol having molecular formula $C_{28}H_{48}O$. It has a chemical structure similar to that of cholesterol. It is recommended as a treatment for especially hypercholesterolemia (Párraga *et al*., 2011). Animal studies have presented that campesterol can reduce the size of atherogenic plaques and it can also reduce atherosclerosis, heart disease and cardiac events (Clifton, 2009). In addition, Singh *et al*. (2011) showed that campesterol has a notable antifungal activity especially against *C. albicans*.

Gamma-sitosterol is a phytosterol which is C-24 isomer of betasitosterol. It is present in many plants (Raman *et al*., 2012). Gamma-sitosterol is known to be used as a phytomedicine to treat especially ulcers, bronchitis, diabetes and heart diseases (Balamurugan *et al*., 2011). Gamma-sitosterol is also reported to be used as a folklore medicine because of its very strong antifungal, antibacterial and anti-angiogenic activity (Zhang and Zhou, 2011).

Stigmasterol is one of other phytosterol which is also known as Wulzen anti-stiffness factor. It has a molecular formula $C_{29}H_{48}O$ and it is also chemically similar to animal cholesterol. Stigmasterol is mostly used in producing semi-synthetic progesterone as a precursor (Sundararaman and Djerassi, 1977; PBS, 2007). Recent researches showed that stigmasterol can be used in prevention of certain cancers, inhibiting several pro-inflammatory and matrix degradation mediators which are involved in osteoarthritis-induced cartilage degradation and it also possesses antioxidant, anti-rheumatic, hypoglycemic and thyroid inhibiting properties (Budavari, 1989; Gallina *et al*., 2007; Panda *et al*., 2009). In addition, stigmasterol was previously reported as an antimicrobial agent (Prachayasittikul *et al*, 2009, 2012).

Hexadecanoic acid, which is also known as palmitic acid, is the most common fatty acid found in animals, plants and microorganisms (Gunstone *et al*., 2007). It is mainly used to produce soaps and cosmetics. Hexadecanoic acid is also proposed as an antimicrobial agent in several researches (Glover *et al*., 1997; Risk *et al*, 1997; Bazes *et al*., 2009). Hexadecanoic acid also presented a significant antibacterial activity against *Streptococcus mutans* (Huang *et al*., 2011). In addition, it has antifungal activity against *Alernaria solani* (Liu *et al*., 2008), *Aspergillus niger* (Altieri *et al*., 2007), *Aspergillus terreus* (Altieri *et al*., 2007), *Cucumerinum lagenarium* (Liu *et al*., 2008), *Emericella nidulans* (Liu *et al*., 2008), *Fusarium oxysporum* (Liu *et al*., 2008).

Stearic acid is a saturated fatty acid having molecular formula $C_{18}H_{36}O_2$. Stearic acid is mainly used in the production of detergents, soaps and cosmetics, but an antimicrobial activity potential

against diverse pathogenic microorganism is also proposed in several studies (Zheng *et al*., 2005; Nalina and Rahim; 2007; Chakraborty and Shah, 2011).

Antimicrobial activity of linoleic acid was also determined in several researches. Tsuchida and Morishida (1995) investigated its antimicrobial activity against some Gram positive and Gram negative strains. They found that linoleic acid has high antimicrobial activity mostly against Gram positive strains. It also presented antimicrobial activity against *Bacillus larvae* (Feldlaufer *et al*., 1993). It is also identified that linoleic acid is active against *Alternaria solani* (Liu *et al*., 2008), *Candida albicans* (Kabara *et al*., 1972), *Crinipellis pernicosa* (Walters *et al*., 2004), *Fusarium oxysporum* (Liu *et al*., 2008), *Pyrenophora avanae* (Walters *et al*., 2004), *Pythium ultimum Rhizoctonia solani* (Walters *et al*., 2004).

It is also previously reported that linoleic acid has antibacterial activity (Knapp and Melly, 1986; Kabara *et al*., 1972; Hazell and Graham, 1990; Sun *et al*., 2003; Zheng *et al*., 2005) and antifungal activity against *Crinipellis pernicosa*, *Pyrenophora avanae*, *Pythium ultimum* and *Rhizoctonia solani* (Walters *et al*., 2004).

In our study we observed antimicrobial activity against *B. subtilis*, *E. aerogenes*, *E. faecalis*, *E. faecium*, *S. typhimurium*, *S. carnosus*, *S. epidermidis* and *S. agalactiae*. But we did not observed any activity against *C. albicans*, *E. durans*, *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *S. enteritidis*, *S. infantis*, *S. kentucky*, *S. aureus* in any concentrations tested.

There is no information about the antimicrobial activities of the metabolites found in the composition of *Hc* extract against *B. subtilis*, *E. aerogenes*, *E. faecalis*, *E. faecium*, *S. typhimurium*, *S. carnosus* and *S. agalactiae*. But according to the previous studies, an activity against *S. epidermidis* is not a surprise, because DHA is proposed to be active against *S. epidermidis* (Lukowski *et al*., 2008; Mavric, 2008; Adams *et al*., 2009; Bäcker *et al*., 2010).

The main surprise in our study is not observing any antimicrobial activity against *S. aureus*, *C. albicans*, *E. coli* and *L. monocytogenes*.

Batovska *et al*. (2009) proposed that myristic acid is active against *L. monocytogenes*. In addition DHA is stated to be active against *E. coli* (Lukowski *et al*., 2008; Mavric, 2008; Adams *et al*., 2009; Bäcker *et al*., 2010).

Previous studies stated that DHA (Lukowski *et al*., 2008; Mavric, 2008; Adams *et al*., 2009; Bäcker *et al*., 2010), maltol derivatives (Fassihi *et al*, 2009), myristic acid derivatives (Narasimhan *et al*., 2006) and phytol (Inoue *et al*., 2005) are active against *S. aureus*. Also, maltol derivatives (Fassihi *et al*, 2009), myristic acid (Kabara *et al*., 1972) and campesterol (Singh *et al*., 2011) are found to be active against *C. albicans*.

The reason of not observing any activity against *S. aureus*, *C. albicans*, *E. coli* and *L. monocytogenes* could possibly either due to the concentration of the metabolites in the *Hc* extract or the strains, which could be resistant to the metabolites.

The results obtained herein indicate that ethanol extract of *Hc* contains several active metabolites and it is active against several microorganisms. Its antimicrobial activity is notable especially against *B. subtilis*, *E. faecalis*, *S. carnosus* and *S. epidermidis* when both disk diffusion test and MIC values are taken into account. But further researches especially cytotoxicity and genotoxicity tests are needed to be conducted to conclude whether *Hc* extracts can be used safely in terms of their antimicrobial activity.

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