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Corresponding author:
Ramesh Chandra Dubey
Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar-249404 India. E.: profrcdubey@gmail.com; Tel.: +919412157616

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Original Research Article

HPTLC AND GC-MS PROFILE OF *BARLERIA LUPULINA* LINDL. EXTRACTS AND THEIR EFFECT ON ENTERIC BACTERIAL PATHOGENS

Reshma Kumari, Ramesh Chandra Dubey
Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar-249404 India.

ABSTRACT

The antibacterial activity of acetone, methanol, and water-soluble extracts of both leaf and stem of *Barleria lupulina* were evaluated against some human pathogens viz., *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aureginosa*, *Klebsiella pneumonia* and *Staphylococcus aureus*. The highest antibacterial activities were exhibited at 100% concentrationof all the extracts against the bacterial pathogens. Acetone-soluble leaf (AL) and acetone-soluble stem (AS) extracts caused the maximum zone of inhibition against *P. aureginosa,* and methanol-soluble leaf (ML) and methanol-soluble stem (MS) extracts against of *Salmonella typhi*. However, *Klebsiella pneumonia* was inhibited by all the extracts. The result of HPTLC analysis revealed the presence of alkaloid having different phyto-constituents having their R_f values. Twenty phyto-constituents were identified from all the extracts in which the major compounds were methyl ester, phytol, benzoic acid and propenoic acid as per GC-MS analysis.

Keywords: *Barleria lupulina,* enteric bacteria, extracts, HPTLC and GC-MS profile

Introduction

Medicinal plants play a key role in management of several diseases including malaria, diarrhoea, dysentery, respiratory infection and skin diseases caused by fungal and bacterial agents [17]. The emergence of multi-drug resistant bacterial strains throughout the world has limited the effectiveness of current synthetic and microbial medicine in recent years [8]. The increasing drug-resistance in bacterial pathogens has further complicated the treatment of infectious diseases. A number of modern drugs have been developed through experience of indigenous people by using medicinal plants [1].

Barleria lupulina Lindl. belonging to the family Acanthaceae has been used as drug plant since the ancient time. It is a large genus comprising of over 300 species of shrubs, and many of which are known for their ornamental and or medicinal values. The plant is commonly known as Sornomukhi, Hophead philipine violet, Visalyakarni, etc. It is native to India and widely distributed in Southern and Western India. The plant is a small shrub, externally used as an anti-inflammatory against insect bites, snake bites, and herpes simplex [19]. It has been reported to possess potent anti-inflammatory, analgesic, anti-leukemic, antitumor, anti-hyperglycemic, anti-amoebic, virucidal, diuretic, bactericidal and antibiotic properties. It has been traditionally used for diabetes, rheumatoid arthritis, eczema, itches, scabies and snake bite; its leaf paste is applied to the affected area [3]. The extract of *B. lupulina* plant is known to have high antiviral activity against HSV-2 [23] and anti-ulcer activity [22]. Its leaf juice is also medicinally important and being used as the coagulating agent on cut wound and leaf paste is used as poultice to relief pain. It has strong inhibitory effect against acne-inducing bacteria [2]. Chemical compounds present in the leaves of *B. lupulina* include barlerin, acetylbarlerin, shanzhiside methyl ester, acetyl shanzhiside methyl ester, ipolamiidoside and iridoid glucosides [11]. Nine iridoid glucosides [21] have been identified in addition to phenyl propanoid glycosides lignin glucoside, aliphatic glycoside and benzyl alcohol glycoside from the aerial part of this plant [9].

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Herbal drugs are believed to enhance the natural resistance of the body against infection. The research work on antimicrobial effect of extracts of *B. lupulina* is meager and scanty. Therefore, in the present investigation the antimicrobial potential and identification of phytochemical compounds in *B. lupulina* through HPTLC and GC-MS of *Barleria lupulina* has been evaluated against some enteric bacterial pathogen.

MATERIAL AND METHODS

Collection of plant sample

The leaves and stem of *B. lupulina* were collected from the Botanical Garden, Department of Botany and Microbiology, Gurukula Kangri University, Haridwar (India) in the month of September, 2014. The plant samples were washed with running tap water to remove the adhered dust and other foreign material and dried in shade at room temperature. The dried samples were separately homogenized to get fine powders that were stored in air tight container at room temperature for further studies.

Preparation of crude extracts

The powdered plant material was subjected to hot extraction in Soxhlet continuous extraction apparatus with acetone, methanol solvents and water, separately for 48 - 72 h. The extracts were filtered separately and considered as the stock that considered 100% concentrated extract. Further all the stock extracts were diluted with respective solvent to get 75, 50 and 25% concentrations of each extract.

Bacterial Strains

Five human pathogenic bacteria such as *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 27853, *Pseudomonas aeruginosa* ATCC 25922, *Klebsiella pneumonia* MTCC 432 and *Salmonella typhi* MTCC 733 were procured from the Laboratory of the Departmental of Botany & Microbiology, Gurukula Kangri Vishvidyalaya. All the pathogens were stored on nutrient agar slants at 4ºC for further study.

Antibacterial activity of *B. lupulina* extracts

Antibacterial assay was performed by agar well diffusion method [16]. A young colony of each bacterium was inoculated into 5 ml nutrient broth and incubated at 37ºC for 4 - 6 h. The test culture of each bacterium was separately swabbed evenly on the surface of solidified Mueller Hinton Agar (MHA) medium. Agar well was made in the MHA agar plate with the help of a sterile cork borer (7 mm). Each *B. lupulina* extract (100 µl) was poured into the wells using pre-sterile micropipette tips. The plates were incubated at 37ºC overnight. For each bacterial strain, pure solvents were used instead of extract as a negative control, whereas streptomycin and ciprofloxacin were used as positive control. The diameter of zone of inhibition was measured after incubation at 37ºC for 24 h, and average values were recorded. The experiment was performed in triplicates and the activity of acetone and methanol were subtracted from the total zone of inhibition.

High Pressure Thin Layer Chromatography (HPTLC) Chemo Profiling of Extracts

The extract samples were dissolved in the respective solvents (2 mg/ml). Ethyl acetate (100): acetic acid (11): formic acid (11): water (28) were used as a mobile phase. Each extract solution was separately applied with the help of Linomat syringe (100 µl) using the Linomat applicator 5 on the HPTLC-plates (20.0 × 10.0 cm). 10 µl of sample was applied as a band of 6 mm and distance between tracks or band 12.0 nm.

Silica gel was applied to act as stationary phaseon the percoated plates. The plates were developed in CAMAG twin trough chamber (20 × 10 cm) with the help of mobile phase. Therefore, the plates were then taken out of the chamber and dried in air. CAMAG HPTLC Densitometer (Scanner 201377) was used as a scanner in absorbance mode at 366 nm in fluorescence and normal mode. The slit dimention was 6.00 × 0.30 mm. The scanner data was subjected for integration through the CAMAG Visualizer (201673) and plates were heated at 120ºC for 20 min. The spots on TLC plates were detected by using the spray reagents as Dragendroff's reagents. The R_f values of phyto-constituent in spots were noted.

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

GC-MS analysis of the extracts was carried out using a Varian-Bruker Scion SQ mass spectrometer system equipped with DB5 capillary column (0.25 mm thickness and 30 m in length). Each extract was separately diluted (2 mg/ml) according to respective solvents of the extracts and 1 µl was taken as the injection volume. It was injected in the split mode with 20:1 ratio. The temperature was initially maintained at 40ºC for 4 min which was gradually increased to 280ºC at a rate of 20ºC/5 min. The transfer line was maintained at a temperature of 280ºC and total run time was 45 min. Helium was used as a carrier gas with a constant flow at 1 ml/min. The electron impact ionization was 70eV. The compounds were evaluated using total ion count (TIC) for constituent identification after comparison with database of spectrum of known component available in the computer library (NIST) which was attached to the GC-MS instrument.

RESULT AND DISCUSSION

Antibacterial properties of *B. lupulina* extracts

Leaf and stem extracts at 100% concentration were most effective followed by 75, 50 and 25% of extracts against five selected human enteric bacterial pathogens (Table 1). The leaf and stem extracts significantly inhibited the growth of the test pathogens. Acetone soluble leaf extract (AL-extract) and stem extract (AS-extract) caused the maximum zone of inhibition against *P. aureginosa* (11 mm, 18 mm) at 100% concentration. Methanol soluble leaf extract (ML-extract) and stem extract (MS-extract) at 100% concentration resulted in maximum growth inhibition of *S. typhi* (21.33 mm and 12 mm, respectively). However, *K. pneumonia* showed complete resistance to ML and AS extracts and least inhibited by the other extracts.AL-extract, ML-extract, AS-extract and MS-extract exhibited inhibition of zone against *S. aureus* of 7, 8, 8.66 and 4 mm, whereas all the extracts caused moderate and more-or-less equal antibacterial activity against *E. coli* (Table 1). The order of sensitivity of all the pathogens to AL-extract and AS-extract was *P. aureginosa* > *S. typhi* > *E. coli* > *S. aureus* > *K. pneumonia*. Similarly the order of sensitivity to ML-extract was *S. typhi* > *P. aureginosa* > *S. aureus* > *E. coli* > *K. pneumonia.* MS-extract resulted in inhibitory effect in the order: *S. typhi* > *P. aureginosa* > *E. coli* > *S. aureus* > *K. pneumonia.* The zone of inhibition caused by ML-extract (21.33 mm) was higher than that of ciprofloxacin (18.66 mm), while streptomycin and MS-extract resulted in zone size of 12 mm. *P. aureginosa* was highly sensitive to both the antibiotics. Streptomycin caused the lowest effect on *S. typhi* and ciprofloxacin did the same on *K. pneumonia*. Aqueous extracts did not cause the zone of inhibition against any pathogens.

AS-extract was more inhibitory to bacterial growth that AL-extract. Similarly, ML-extract was more inhibitory that the MS-extract. Differential inhibitory properties of plant extracts would have been possible due to accumulation of bioactive compounds more in leaves than the stem. Further the presence of more chemical in methanol-soluble extract than the acetone-soluble extracts may be explained to be due to their solubility more in methanol than acetone. The important phyto-chemicals including alkaloids, glucosides, saponins, tannins, flavonoids, steroids, etc. are required by the plants for their growth and protection [10].

Table 1 Antibacterial activity of leaf and stem extracts of *Barleria lupulina.*

Extracts	Zone of inhibition (in mm)*				
AL-extract (%)	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumoniae*
100	8.6 ± 0.57	7.0 ± 0.0	11.0 ± 1.0	8.6 ± 1.15	6.6 ± 0.57
75	7.3 ± 0.57	6.3 ± 0.57	9.6 ± 1.14	7.6 ± 2.08	5.0 ± 1.0
50	6.0 ± 0.0	5.0 ± 0.0	7.0 ± 1.73	4.6 ± 1.15	5.0 ± 0.0
25	4.0 ± 0.0	4.6 ± 0.57	5.6 ± 1.52	2.3 ± 0.57	0.0
AS-extract (%)	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumonia*
100	7.3 ± 0.57	8.6 ± 0.57	18.0 ± 1.0	11.3 ± 2.30	R
75	6.3 ± 0.57	7.0 ± 0.0	17.3 ± 1.15	8.6 ± 1.15	R
50	6.0 ± 0.0	5.6 ± 0.57	13.6 ± 1.52	7.0 ± 1.0	R
25	4.0 ± 0.0	5.0 ± 0.0	8.0 ± 1.0	5.3 ± 1.15	R
ML-extract (%)	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumonia*
100	7.3 ± 0.57	8.0 ± 1.0	14.3 ± 3.21	21.3 ± 4.16	R
75	6.3 ± 0.57	6.6 ± 0.57	10.0 ± 4.35	18.0 ± 2.0	R
50	6.0 ± 0.0	6.0 ± 0.0	8.3 ± 2.08	11.0 ± 2.64	R
25	4.0 ± 0.0	5.3 ± 0.57	6.0 ± 1.0	10.6 ± 3.05	R
MS-extract (%)	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumonia*
100	7.6 ± 0.57	4.0 ± 1.0	11.0 ± 1.0	12.0 ± 2.0	4.0 ± 0.0
75	6.3 ± 0.57	3.3 ± 0.57	9.3 ± 0.57	10.6 ± 1.15	3.0 ± 1.0
50	6.0 ± 0.0	2.0 ± 0.0	7.6 ± 0.57	6.0 ± 0.0	2.6 ± 0.57
25	4.0 ± 0.0	0.0	5.6 ± 1.52	4.0 ± 0.0	1.6 ± 0.57
Streptomycin	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumonia*
	21± 1.0	24.6±0.57	25.6±1.52	12.6±0.57	23 ±1.0
Ciprofloxacin	*E. coli*	*S. aureus*	*P. aureginosa*	*S. typhi*	*K. pneumonia*
	31.0 ±1.0	31.0 ±1.0	33.6±0.57	18.6±0.57	13.6 ±0.57

*, Values are mean of triplicate ± standard deviation; R- resistance to extracts; AL, acetone soluble leaf; AS, acetone-soluble stem; ML, methanol-soluble leaf; MS, methanol-soluble stem.

We have found the maximum number of chemical compounds in ML-extract. Besides, ML-extract was inhibitorier to enteric bacteria than the others. Similarly antibacterial activity by disc diffusion method of methanol-soluble extract of *B. lupulina* with MIC of 0.125 mg/ml^{-1} against *E. coli* and *S. aureus* has been reported by Doss et al. (2011) [6]. Even methanol-soluble extract from the calli of *B. lupulina* has also been reported to have antibacterial activity against *S. aureus* [13]. The presence of alcaloids, starch, tannins, reducing sugar, proteins, flavonoids, amino acid and lignin in different concentration in different tissues of *B. lupulina* has been reported by Mandal and Mandal (2014) [12]. We found better antibacterial result than that reported by Doss et al., 2011 [6], possibly due to adoption of different approaches for chemical extraction and antibacterial testing against enteric pathogens.

Phyto-constituents analysis of extract by HPTLC

HPTLC analysis of extracts revealed the presence of alkaloid as revealed by different spots observed at 366 nm in fluorescence and normal mode (Figure 1 A and B). These spots show different phyto-constituents along with their R_f values (Table 2). The R_f values of AL-extract were 0.20, 0.78 and that of AS-extract were 0.13, 0.40, 0.51, 0.79 (Table 2). Similarly the R_f values of ML-extract were 0.08, 0.12, 0.36, 0.48, 0.69 and that of MS-extract were 0.11, 0.44, 0.71(Table 2). The highest R_f value was found in AS-extract (0.79) and the lowest in MS-extract (0.11).

Table 2. HPTLC analysis of leaves and stemextracts of *B. lupulina* and their R_f values.

Name of extract	Total number of peaks	R_f value	Area of percentage (%)
AL-extract	2	0.20, 0.78	23.66, 76.34
AS-extract	4	0.13, 0.40, 0.51, 0.79	2.41, 30.44, 26.46, 40.69
ML-extract	5	0.08, 0.12, 0.36, 0.48, 0.69	20.19, 29.28, 12.39, 20.77, 17.37
MS-extract	3	0.11, 0.44, 0.71	20.63, 42.46, 36.90

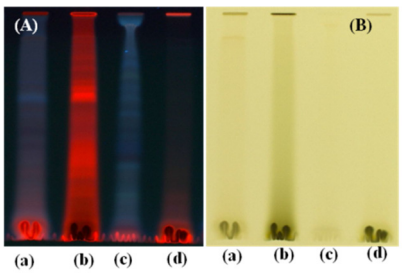
Abbreviations: AL, acetone soluble leaf; AS, acetone-soluble stem; ML, methanol-soluble leaf; MS, methanol-soluble stem.

Table 3. Bioactive compounds identified by GC-MS analysis in leaves and stem of *B. lupulina.*

Name of extract	RT	Name of the compound	Molecular formula	Peak area	Total % of peak area
AL-extract	23.682	Benzene (1-methyl decyle)	$C_{17}H_{28}$	158856816	8.813
	25.77	Benzene (1-methyl decyle)	$C_{17}H_{28}$	150736256	8.362
	26.337	3-Eicosyne	$C_{20}H_{38}$	135089680	7.494
	30.237	Phytol	$C_{20}H_{40}O$	220575504	12.236
	40.717	Squalene	$C_{30}H_{50}$	281571744	15.620
AS-extract	36.490	Bis (2 ethyl hexyle phthalate)	$C_{24}H_{38}O_4$	2.292e+10	95.802
ML-extract	13.86	Benzoic acid 4-methoxy-methyl ester	$C_{10}H_{12}O_3$	124277096	2.803
	16.199	Methyl paraben	$C_8H_8O_3$	54947100	1.239
	18.423	2(4H)-Benzofuranone	$C_{11}H_{16}O_2$	107754000	2.430
	20.269	Cyclopenta (c) pyran-4-carboxylic acid (Dimethoxy)	$C_7H_6O_4$	73017408	1.647
	22.936	2 Propenoic acid	$C_3H_4O_2$	185992736	4.195
	26.341	3-Eicosyne	$C_{20}H_{38}$	89836776	2.026
	26.747	Hexadeconoic acid	$C_{16}H_{32}O_2$	14434957	0.326
	27.755	Phytol	$C_{20}H_{40}O$	613218112	13.830
MS-extract	18.421	2(4H)-Benzofuranone	$C_{11}H_{16}O_2$	65188140	5.654
	20.436	Di-ethyl phthalate	$C_{12}H_{14}O_4$	78071080	6.771
	24.922	Benzyl Benzoate	$C_6H_5CH_2O_2CC_6H_5$	52723008	4.573
	27.76	Hexadecanoic acid	$C_{16}H_{32}O_2$	326817120	28.346
	30.5	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	296655936	25.730
	30.232	Phytol	$C_{20}H_{40}O$	215083760	18.655

Abbreviations: AL, acetone soluble leaf; AS, acetone-soluble stem; ML, methanol-soluble leaf; MS, methanol-soluble stem; RT, retention time.

In HPTLC plate R_f value of MS-extract was 0.44 as pink color spot which confirms the presence of alkaloids. Similar R_f value (0.44) of methanolic extract of a plant of same family, such as other *B. prionitis* has also been reported earlier [7]. Chemical constituents from natural sources have become relatively elementary and have played considerable role in the development of a new drug from medicinal plants [4-5]. Several group of phyto-constituents have antimicrobial potential, such as saponin, tannin, flavonoid, alkaloid and terpenoid, etc. in which several deterpene alkaloids have been found to exhibit antimicrobial and microbicidal properties.



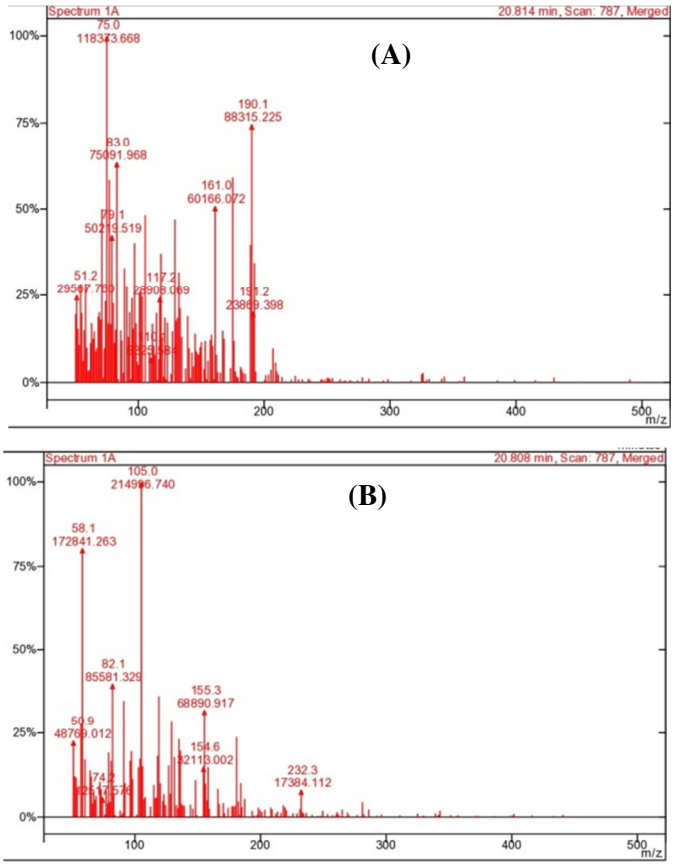


Fig. 1. Different phyto-constituents of *B. lupulina* separated on different tracks at 366 nm on HPTLC plates (A) in fluorescent mode (B) in normal mode (a) AL-extract (b) ML-extract (c) AS-extract (d) MS-extract.

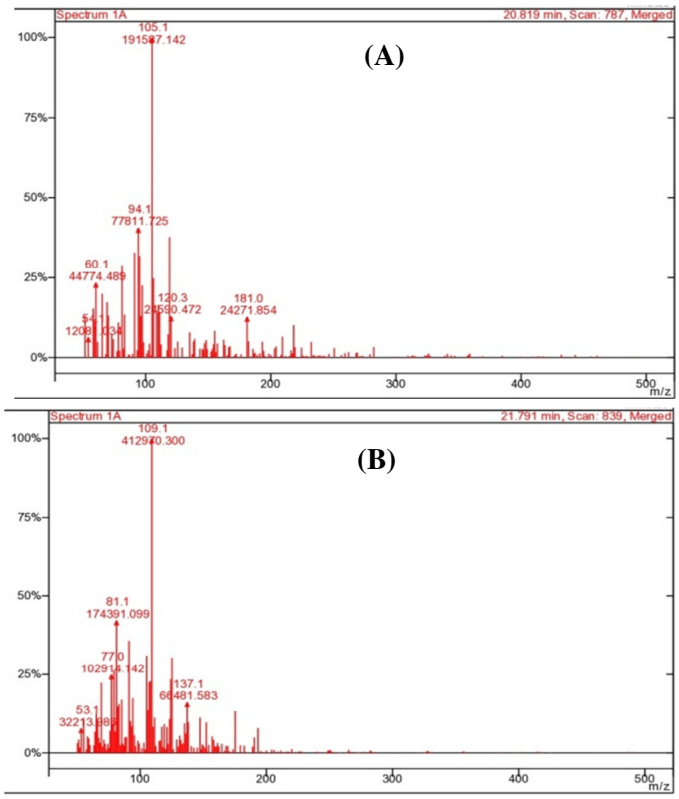


Fig. 2. Chromatograph representing the peak areas of phyto-constituent of *B. lupulina* matched with NIST library (A) acetone-soluble leaf extract (B) acetone-soluble stem extract.

Compounds identified through GC-MS analysis

On the basis of NIST library major five phyto-constituents were identified in the AL-extract, one in AS-extract, eight in ML-extract, and six in MS-extract at different retention time (Table 3) and the total ion graphs are presented in Figure (2 and 3). Benzene, 3-eicosyne, phytol, squalene in AL-extract, bis (2 ethyl hexyle phthalate) in AS-extract, benzoic acid 4-methoxy-methyl ester, cyclopenta pyran-4-carboxylic acid, propenoic acid, phytol, 2(4)-benzofuranone in ML-extract were identified at different retention time followed by and 2(4H)-benzofuranone, benzyl benzoate, hexadecanoic acid, 9,12-octadecadienoic acid, phytol in MS-extract.

Moreover GC-MS analysis reports the similar group of compounds like methyl ester, phytol, benzoic acid and cyclopenta pyran-4-carboxylic acid, hexadecanoic acid, etc. accept benzyle benzoate, ecosyne, propanoic acid, squalene 2(4H)-benzofuranone. The compounds are used as additives for any kind of preservation because they contain microbicidal agents. A new iridoid diglucoside, lupulinoside and eight known iridoid glucosides, acetyl barlerin, ipolamiidoside,6-O-acetylshanzhiside methyl ester, barlerin, shanzhiside methyl ester, mussaenosidic acid, 8-O-acetylshanzhiside, and shanzhiside that have beenisolated from the flowers of *B. lupulina* [20]. Some of the constituents such as phenol,2,4,bis (1,1-dimethylethyl)-tetradecanoic acid, 12- methyl-, methyl ester, hexadecanoic acid, methyl ester, phytol and octadecanoic acid, have proven antimicrobial potential [14-15]. Presence of cyclobutane, 1,1-dimethyl- 2-octyl, 2-hexyl-1-octanol, 1, 2-henzenedicarboxylic acid, mono (2-ethylhexyl) ester and 1-hentetracontanol have been reported in *B. lupulina* [18]. The constituents contain antimicrobial efficacy itself and lend support to the claim that these phyto-constituents identified through HPTLC screening and GC-MS analysis may attribute to the antibacterial property of *B. lupulina*.

On the basis of above results it may be concluded that the leaf and stem extracts in different solvents showed significant antibacterial activity against all selected pathogens. GC-MS analysis of *B. lupulina* extracts identified a total of 20 phyto-constituents which may the responsible for antibacterial activity.

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