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Phytochemical Analysis and Antibacterial and Cytotoxic Properties of *Barleria lupulina Lindl.* Extracts

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Abstract

The ethanolic and aqueous extracts of *Barleria lupulina* leaves displayed antibacterial activity against five human bacterial pathogens viz., *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhi Klebsiella pneumoniae*. The ethanolic extract was more inhibitory than the aqueous extract against all the test pathogens, which caused the maximum growth inhibition of *P. aeruginosa* at 100% concentration. In contrast, aqueous extract did not inhibit the growth of any bacterial pathogens. MIC of ethanolic extract was 2.5 mg/mL against *E. coli, S. aureus* and *P. aeruginosa*, and 10.0 mg/mL against *S. typhi* and *K. pneumonia*. GC-MS analysis displayed the presence of twelve phytochemical compounds among which benzofuranon, hexadecanoic acid, ethyl 9,12,15-octadecatrienoate, and 3,7,11,15-tetramethyl-2-hexadecanoic acid were the most prominent ones. These extracts also displayed cytopathic effects against HepG2 cell line performed by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and neutral red uptake (NRU) assay which demonstrated the varying levels of cell death of HepG2 cells by ethanolic extract. The ethanolic extract of *B. lupulina* bears a significant amount of phytochemical compounds that pose antibacterial as well as anti-cancerous properties.

Keywords: Antibacterial activity; *Barleria lupulina*; Cytopathic; GC-MS; Phytochemical analysis

Introduction

Plant-derived natural products, such as flavonoids, terpenoids, alkaloids and steroids have received considerable attention in recent years due to their diverse and effective pharmacological properties including antibacterial, antioxidant and antitumor [1]. Issasbadi et al. reported that the use of plant is a cost effective and environment friendly. Several plants of Acanthaceae family are used as medicinal plant among which *Barleria lupulina* Lindl. is an important one which comprises of several medicinal properties. It is a herb which is widely distributed throughout tropical Asia. Leaves, stems and roots of *B. lupulina* and flower of *B. prionitis* possess potential antibacterial and anti-inflammatory activities [2-4]. However, cytotoxicity of *B. strigosa* extract has been described by Nuttaporn et al. [5]. Several phytochemicals including barlerin alkaloid is derivative from *B. lupulina* which possess antimicrobial and anticancerous properties. Iridoid glucosides, bataine and alkaloids have also been reported from *B. lupulina* plant [6-8]. Traditionally, leaves of this plant are used to treat snake bites, dog bites, swelling, bleeding wounds and rheumatism. The extracts of the plant also possess anti-HSV-2 activities [9], ameliorate secondary complications of diabetes including cataract [10,11], antiarthritic [12], anti-inflammatory [13], antimicrobial [14], anti-clastogenic, anti-tumor, and anti-cancer activities besides having radiation protection [15].

The cases of cancer are increasing day-by-day due to several reasons. The emergence of multi-drug resistance among bacterial pathogens throughout the world is kindled because of over prescription of antibiotics. Therefore, use of traditional medicinal plant is required as an alternative drug to cure various types of diseases. The present investigation was designed to assess the antibacterial properties of ethanolic and aqueous extracts of *B. lupulina* against five bacterial pathogens and cytotoxic effects on HepG2 cells, and also to identify the bioactive compounds present in its leaves.

Materials and Methods

Collection of plant material

The leaves of *B. lupulina* were collected from the Botanical Garden, Department of Botany and Microbiology, Gurukula Kangri University, Haridwar (India) during the month of June 2014, and identified by the experts of the department (specimen identification No Bot. and micro/199). The plant leaves were washed with running tap water to remove the adhered dust, dirt and unwanted particles from their surfaces. The leaves were shade dried at room temperature for 15 days and separately homogenized in domestic blender to get fine powders. The powders were stored in airtight container at room temperature for further studies.

Bacterial cultures

Five standard human enteric pathogenic bacteria viz. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 27853, *Pseudomonas aeruginosa* ATCC 25922, *Klebsiella pneumoniae* MTCC 432 and *Salmonella typhi* MTCC 733 were procured from the Laboratory of the Departmental of Botany & Microbiology, Gurukula Kangri University. All the pathogens were sub cultured on nutrient agar slants and preserved at 4°C for further study.

Preparation of crude extracts

The dried leaf powder (100 g) was subjected to hot extraction in Soxhlet continuous extraction apparatus with 300 mL of ethanol and water (1:3 ratio) for 48-72 h. The solvent was gently evaporated at room temperature to get the final volume of 100 mL. The extract was filtered and considered as 100% concentrated extract. Further, the extract was diluted with respective solvent to get 75%, 50% and 25% concentrations for antibacterial activity assay. Aqueous extract was prepared by boiling 30 g leaves powder in 150 mL distilled water till the volume was reduced

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to one fourth of its original volume. 100% concentrated ethanolic and aqueous extracts were separately evaporated under vacuum distillation unit at 60°C. Both the extracts were stored in refrigerator for further use.

Evaluation of antibacterial activity of extracts

Antibacterial assay was carried out by agar well diffusion method [16]. A loopful colony of each bacterium was impregnated into 5 mL nutrient broth and incubated at 37°C for 4-6 h with vigorous shaking. Each bacterial culture was separately swabbed uniformly on the surface of solidified Mueller Hinton agar (MHA) medium with sterile cotton swab. Agar well was made with the help of a sterile cork borer (6 mm) in the MHA agar plate. *B. lupulina* extract (100 µL from 25%, 50%, 75% and 100% concentration) was separately poured into the wells using pre-sterile micropipette tips and incubated at 37°C for 24 h. Pure solvents were separately used instead of extract as a negative control for each bacterial strain, whereas streptomycin and ciprofloxacin were used as positive control. After incubation at 37°C for 24 h the diameter of zone of inhibition of treatment and control sets were measured. Inhibition (I) of the growth of bacterial colony was calculated by using the formula: I = T – C, where T = diameter of total inhibition zone in treatment, C = diameter of inhibition zone in control.

Determination of minimum inhibitory concentration (MIC) and survival of bacteria

MIC of extract was carried out to find out the lowest concentration of extract that inhibits the visible growth of test bacteria by standard tube dilution method [17]. A loopful culture from the slants was separately inoculated in nutrient broth and incubated at 37°C for 24 h. Fresh medium (20 mL) was seeded with 0.25 mL of 24 h old broth culture. The extract was dissolved in dimethyl sulphoxide (DMSO) to obtain 200 mg/mL stock solution. 0.2 mL solution of test material was added to 1.8 mL of the seeded broth which was further serially diluted up to 8 tubes with respect to positive control (streptomycin and ciprofloxacin). The concentration of extract ranged from 1.25 to 20 mg/mL. Thereafter, the tubes were incubated at 37°C for 24 h and results were recorded after completion of incubation period.

Phytochemical analysis

High Pressure Thin Layer Chromatography (HPTLC) of the extract: The ethanolic extract was dissolved in ethanol (2 mg/mL) to adjust the final volume. The extract was applied with the help of Linomat syringe (100 µL) using the Linomat applicator 5 on the HPTLC plates (20.0 × 10.0 cm). 5 and 7 µL of extract samples were separately applied as a band (6 mm). Distance between two bands was 12.0 nm. Dimension of slit was 6.00 × 0.30 mm. Silica gel plate acts as stationary phase and ethyl acetate (100): acetic acid (11): formic acid (11): water (28) was used as a mobile phase. The band of plate was developed through capillary action of mobile phase in CAMAG twin trough chamber (20 × 10 cm). Thereafter, the plate was taken out from the chamber and dried in air. CAMAG HPTLC Densitometer (Scanner 201377) was used to scan plate in absorbance mode at 366 nm and the scanning data was subjected for integration through the CAMAG Visualizer (201673). The plate was heated at 120°C for 20 min. The spots of the TLC plate were detected by using the spray reagent (anisaldehyde reagent). Development of different spots revealed different R_f values of phyto-constituent, which were recorded for further study.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis: The ethanolic extract was analyzed through GC-MS by using a Varian-Bruker Scion SQ mass spectrometer system equipped with DB5 capillary column (0.25 mm thickness and 30 m in length). Extract was diluted (2 mg/mL) in ethanol and 1 µL was taken as the injection volume. An aliquot of 20:1 ratio was injected as split mode into the GC-MS. The temperature was initially hold at 40°C for 4 min, rate of 20°C/5 min to 280°C, and the running time was 45 minutes using with maintaining the temperature (280°C). Helium was used as a carrier gas with a constant flow at 1 mL/min.

Cytotoxicity assay

Preparation of test material for MTT and NRU assay: Stock solution was prepared one day in advance with 40% ethanol and MQ water for ethanolic extracts and aqueous extracts, respectively. Multiple aliquots of each sample of extracts were made.

Cell line and culture condition: Cytotoxicity assay was performed using Hep G2 cells (a perpetual cell line consisting of human liver carcinoma cells) derived from the liver tissue (National Centre for Cell Science, Pune) which had a well-differentiated hepatocellular carcinoma. Hep G2 cells were maintained and cultured in EMEM (Eagle's minimum essential media) with 10% fetal bovine serum. Desired cell growth was maintained in humidified atmosphere with 5% CO_2 saturation at 37°C throughout the experiment. Cells were plated in 96-well microtitre plate to get a cell density of 2×10^4 cells per well.

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazollium bromide (MTT) assay: Cytotoxicity of ethanolic and aqueous extracts of *B. lupulina* on Hep G2 cell line was determined by MTT assay as described by Mosmann [17] with some modification. After 24 h incubation of seeded Hep G2 cells, medium of 96 well plates was replaced with fresh medium and treated with different concentrations of extract (0-1000 µg/mL) along with negative control of untreated cells. The plate was incubated at 37°C in 5% CO_2 incubator for 24 hours. MTT (0.5 mg/mL) solution (0.5 µL) was added to each treated well. It was incubated again as earlier 2-3 hours prior to the termination of experiment. At the end, the culture supernatant containing MTT was removed and 100 µL of DMSO was added to each plate containing Hep G2 cells. The plate was gently rotated to solubilize the purple crystal formazan. Absorbance was read after 10 minutes using a plate reader at 550 nm and 660 nm (Bio-Tek Instruments Inc. Vermont, USA).

Neutral red uptake (NRU) assay: Cytotoxicity of ethanolic and aqueous extracts of *B. lupulina* on Hep G2 cell line was also determined by NRU assay. This assay was performed similar to MTT assay. Only 0.5 mg/mL of NRU and 100 µL of 1% glacial acetic acid in 40% alcohol were used in place of MTT and di-methyl sulfoxide (DMSO), respectively.

Statistical analysis

The experiments were executed in triplicates. Statistical analysis was done using GraphPad Prism5 and Microsoft Excel 2008. Data have been expressed as mean significant at $p<0.05$ level.

Results

Evaluation of antibacterial activity of extracts

The ethanolic extract of *B. lupulina* significantly ($p<0.05$) inhibited the growth of all the bacterial pathogens except *K. pneumonia,* whereas the aqueous extract did not inhibit the growth of any bacteria. However, ethanolic extract at 100% concentration posed more lethal effect followed 75%, 50%, and 25% concentration. *P. aeruginosa* was highly sensitive to all the concentrations of the extract exhibiting the maximum zone of inhibition. The antibiotic ciprofloxacin caused the maximum growth inhibition of all bacteria at all concentrations as compared to streptomycin (Table 1 and Figure 1).

Concentrations of extract	Zone of inhibition (mm) of different bacterial species				
	E. coli	*S. typhi*	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*
100%	9.33 ± 1.52	7 ± 2.64	7.33 ± 0.57	13.33 ± 4.72	R
75%	9 ± 0.0	5.33± 2.30	6.66 ± 0.57	11 ± 3.46	R
50%	6.66 ± 0.57	3.33 ± 2.30	5.66 ± 0.57	8.66 ± 3.51	R
25%	2 ± 0.0	6 ± 0.0	4.66 ± 0.57	7.33 ± 3.05	R
Streptomycin	23 ± 1.0	12.67 ± 0.57	24.67 ± 0.57	25.67 ± 1.52	23 ± 1.0
Ciprofloxacin	31 ± 1.0	18.67 ± 0.57	31 ± 1.0	33.67 ± 0.57	13.67 ± 0.57
Mean values ± Standard deviation; R: Resistance					

Table 1: Antibacterial activity of ethanolic extract of *B. lupulina* at different concentrations.

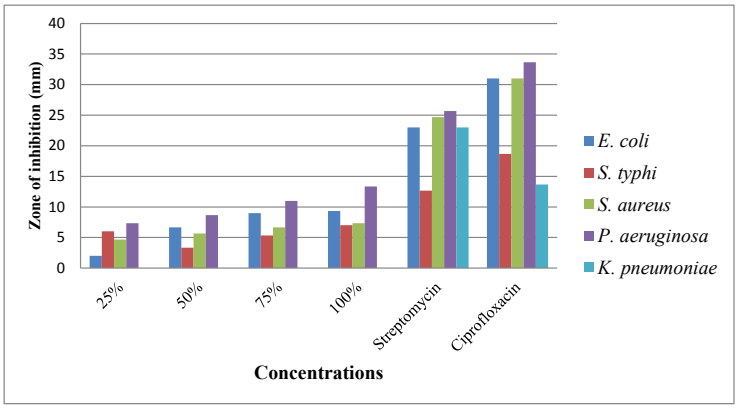

Figure 1: Mean values of diameters of zone of inhibition of bacterial pathogens caused by ethanolic extract of *B.lupulina*.

Name of extracts	MIC (mg/ml) of different bacterial species				
	E. coli	*S. aureus*	*P. aeruginosa*	*S. typhi*	*K. pneumoniae*
Ethanol	2.5	2.5	2.5	5.0	5.0
Aqueous	10.0	5.0	10.0	10.0	10.0

Table 2: Minimum inhibitory concentration (MIC) of ethanolic and aqueous leaf extracts of *B. lupulina*.

Figure 2: Different phyto-constituents separated from ethanolic extract of *B. lupulina* on different tracks at 366 nm on HPTLC plates.

Determination of minimum inhibitory concentration (MIC) of extract

The MIC of ethanolic extract against *E. coli, S. aureus* and *P. aeruginosa* was 2.5 mg/mL, whereas MIC against *S. typhi* and *K. pneumonia* was 5.0 mg/mL. In contrast, MIC of aqueous extract against all the bacterial pathogens was 10 mg/mL (Table 2).

High Pressure Thin Layer Chromatography (HPTLC) of the extract

Different phyto-constituents showing different R_f values viz., 0.17, 0.25, 0.32, 0.43, 0.60, 0.70, and 0.80 were recorded on HPTLC plates from 5 µL sample of ethanolic extract of *B. lupulina*. Similarly chemical constituents having 0.16, 0.25, 0.32, 0.35, 0.44, 0.60, 0.73 and 0.81 R_f values were noted from 7 µL sample of extract. No extra spot was obtained under UV (254 nm) fluorescence mode. More or less similar R_f values were observed in both concentrations of extract. Spots of light pink colour appeared after spraying anisaldehyde (Figure 2) and may show presence of alkaloids and terpenoids group of compounds [18].

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Twelve compounds were identified through GC-MS analysis of ethanolic extract. Then the compounds were matched with NIST library. Some phytoconstituents viz., benzofuranon, hexadecanoic acid, ethyl 9,12,15-octadecatrienoate, and 3,7,11,15-tetramethyl-2-hexadecanoic acid etc. were identified (Table 3).

Cytotoxicity assays

MTT assay: The ethanolic extract of *B. lupulina* had the highest cytotoxicity effect on Hep G2 cells at its all concentrations as compared to that of aqueous extract. The ethanolic extract was effective at 50 µg/mL concentration, while aqueous leaf extract inhibited cell growth at 1000 µL/mL. Only 30% cell death was observed in both cases after 24 h of treatment by MTT assay (Figure 3). In MTT assay, relative percentage of live Hep G2 cells gradually declined with increase in concentration of extracts. But the significant inhibition was observed after 100 µg/mL concentration of the extract. Figure 4 showed cytopathic effect including cytoplasm vacuolation, cell shrinkage, lysis and death.

NRU assay: Cytotoxicity of both extracts were analysed through

RT*	Name of the compound	Molecular formula	Peak area	Total % of peak area
14.76	Tetradecane	$C_{14}H_{30}$	112073872	5.255
15.23	1,H-3a-7-methanoazulene	$C_{15}H_{24}$	126665448	5.939
15.79	Cis-thiopsine	$C_{15}H_{24}$	46636904	2.187
18.42	Benzofuranon	C_8H_6O	50920784	2.388
21.028	Hexadecane	$C_{16}H_{34}$	105627568	4.953
26.341	Phytol acetate	$C_{22}H_{42}O_2$	213710384	10.021
26.750	3,7,11,15, tetramethyl-2-hexadecanoic acid	$C_{21}H_{42}O_2$	36470580	1.710
27.043	3-Eicosyne	$C_{20}H_{38}$	87560240	4.106
28.732	Hexadecanoic acid	$C_{17}H_{34}O_2$	153626656	7.203
30.247	Oxiranehexadecyl (phytol)	$C_{20}H_{40}O$	891487168	41.801
30.938	Ethyl 9,12,15 Octadecatrienoate	$C_{20}H_{34}$	155076272	7.271
40.718	Squalene	$C_{30}H_{50}$	54701708	2.565

*RT: Retention time

Table 3: Main bioactive compounds identified through GC-MS analysis of ethanolic extract of *B. lupulina* leaves.

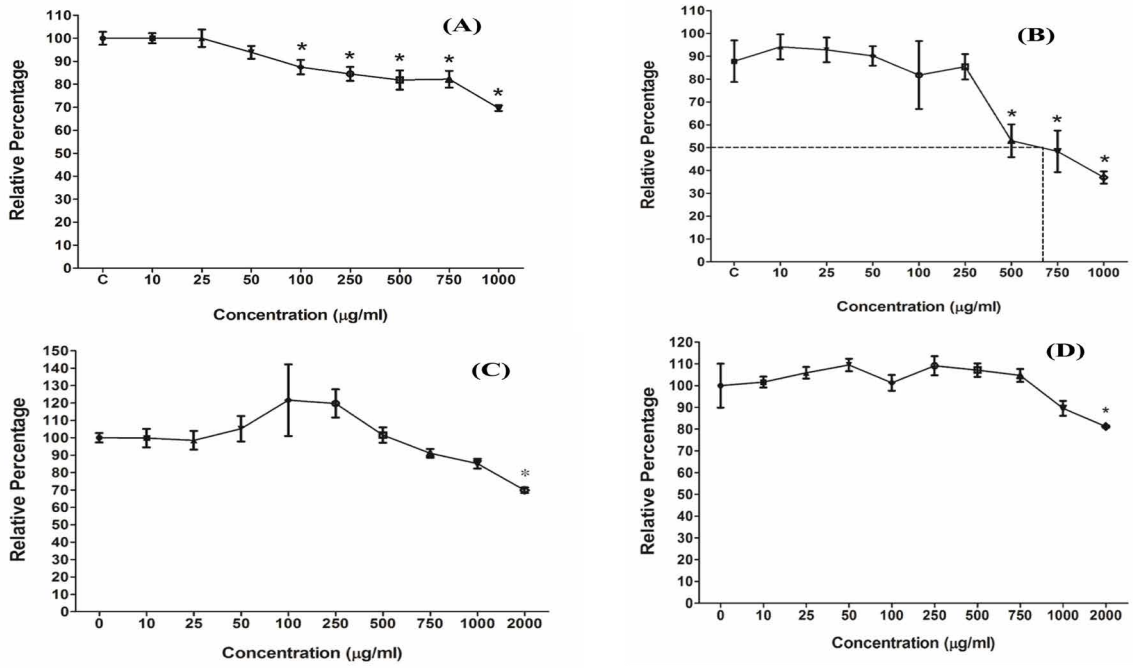

Figure 3: Effect of *B. lupulina* extracts on relative percentage of live Hep G2 cells; (A) MTT assay of ethanolic extract, (B) NRU assay of ethanolic extract, (C) MTT assay of aqueous extract, (D) NRU assay of aqueous extract. p<0.05 between treatments at different concentrations of the both extracts.

NRU assay on Hep G2 cell line; more effect of ethanolic extract was recorded than aqueous extract. The highest non-cytotoxicity dose of ethanolic extract was observed at 250 µg/mL concentration. Ethanolic extract caused 52% cell death at 750 µg/mL concentration. Hence, IC_{50} value was found 650 µg/mL. Furthermore, approximately 20% cell inhibition was recorded at 1000 µg/mL of aqueous extract with different cytopathic effects (Figures 3 and 4).

Discussion

In the present investigation ethanolic extract of *B. lupulina* was found more inhibitory to growth of enteric bacterial pathogens than that of aqueous extract. However, ethanolic extract was highly effective on *P. aeruginosa* but least effective on *K. pneumoniae*. It is the first report on effect of ethanolic extract of *B. lupulina* on enteric bacteria. We found better inhibitory effect of ethanolic and aqueous extracts by agar well diffusion methods than that reported by Doss et al. [19]. This difference may be explained to be due to adoption of different assay methods. Bioactive phytochemicals would have been present in high amount in agar wells than the filter paper discs. In contrast, ethanolic extract at 2.5 mg/mL MIC inhibited the growth of *E. coli, S. aureous* and *P. aeruginosa* as compared to that of aqueous extract all four pathogens were inhibited at 10 mg/mL except *S. aureus* (5.0 mg/mL). These differences may be due to more solubility of antimicrobial phyto-constituents in ethanol than water as well as extraction methods and execution of experiments.

In primary screening different phyto-constituents of ethanolic extract of *B. lupulina* were separated on different tracks on HPTLC plates that were identified as terpenoid and alkaloid groups of compounds. Sur [20] also reported six spots of ethanolic extract of *B. lupulina* under UV (254 nm) in preparative TLC. This result corresponds to the presence of steroid, terpenoid, glycoside, flavonoid, tannin and carbohydrate [20] which supports our findings. The presence of acetylbarlerin, barlerin and shanzhiside methyl ester compounds has earlier been identified from methanol extract of *B. lupulina* [21]; the R_f values of our findings are similar to that of earlier workers [20,21].

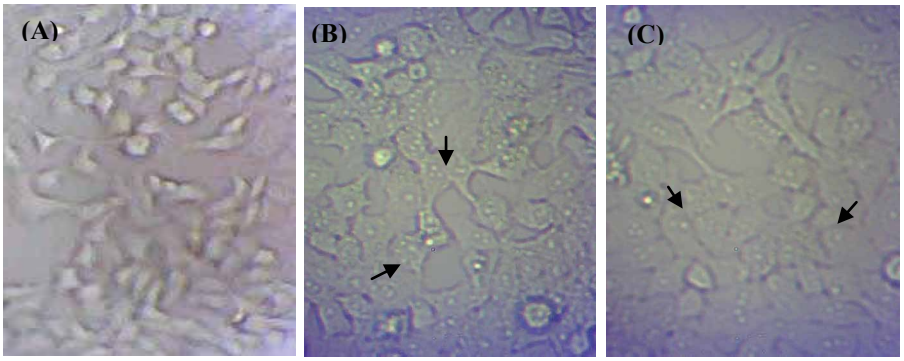

Figure 4: Cytopathic effects on Hep G2 cells treated by ethanol- and water-soluble extracts of *B. lupulina* (*see* arrows); (A) normal cells, (B) vacuolation and shrinkage of cells treated with ethanolic extract, (C) vacuolation and death of cells treated with aqueous extract.

Several chemical constituents have been identified through GC-MS analysis of the ethanolic extract (Table 3). Some compounds viz., phenol,2,4,bis (1,1-dimethylethyl)-tetradecanoic acid, 12- methyl-, methyl ester, hexadecanoic acid, methyl ester, phytol and octadecanoic acid have also been reported by Omulokoli et al. [22] and Namuli et al. [23]. 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 essential oil of *B. lupulina* by Sarmad et al. [24]. But, in this communication we identified slightly different constituents. Some other phytochemical compounds viz., benzene (1-methyl decyle), benzoic acid 4-methoxy-methyl ester, propenoic acid, benzyl benzoate and 2 (4H)-benzofuranone were identified from acetone- and methanol-soluble extracts of *B. lupulina*. Presence of similar or slightly different phyto-constituents has recently been reported by Kumari and Dubey [25].

MTT and NRU dyes were used to visualize and count the viable Hep G2 cells treated with ethanolic and aqueous extracts of *B. lupulina*. The cytotoxic dose was higher but effective on Hep G2 cell line possibly due to adoption of different extraction method. IC_{50} dose could not be measured in both extract by using MTT. It is well known that methods, temperature and time of extraction, solvent type, concentration of solvent, etc. can affect the extraction of phytochemical constituents [26]. Ethanolic extract of *B. lupulina* exhibited the highest selective index (SI) (781.5) with lowest 50% inhibitory concentration (IC_{50}) dose (0.02 µg/mL) against HSV-2 cells as reported by Wirotesangthong and Rattanakiat [26]. Yoosook et al. [9] found higher IC_{50} values of *B. lupulina* than that of Wirotesangthong and Rattanakiat [27]. Therefore, Wirotesangthong and Rattanakiat accepted these differences because of use of different methods of extraction and anti-viral assay.

Multifarious cytotoxicity of *B. lupulina* extracts alone or in combination with other plant extracts has been reported by other workers [28,29]. But it is the first report on the cytotoxic effect of ethanolic extracts of *B. lupulina* on Hep G2 cell line. In our findings the Hep G2 cells treated with both ethanolic and aqueous extracts of *B. lupulina* leaves separately displayed of growth inhibition, vacuolation, cell shrinkage and cell lysis (Figure 4). Higher level cell death was caused by ethanolic extract than aqueous extracts as detected by NRU assay (Figure 4). Therefore, these differences gave different results.

Conclusion

It may be concluded that the *in vitro* data cannot be directly extrapolated to the *in vivo* conditions as several other factors are taken into account. Cytotoxicity on Hep G2 cells and antibacterial activities of ethanolic extract of *B. lupulina* leaves are being reported for the first time. Presence of several phytochemical constituents in the extracts of *B. lupulina* leaves might be responsible for antibacterial effects. This study supports the ethano-botanical use of leaves of *B. lupulina* due to the presence of antibacterial and anti-cancerous phyto-constituents.

Conflicts of Interest

The authors declare no conflicts of interest.

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