

Full Length Research Paper

Biological assessment of *Berberis vulgaris* and its active constituent, berberine: Antibacterial, antifungal and anti-hepatitis C virus (HCV) effect

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Berberis vulgaris is a shrub in the family Berberidaceae. *B. vulgaris* as well as other berberine (BER) containing plants are used medicinally in virtually all-traditional medical systems. In the present study, the efficacy of ethanolic extract of dried root powder of *B. vulgaris* as anti bacterial, anti fungal and anti hepatitis C was evaluated. Also, its efficacy for induction of phagocytosis and lymphoproliferation was determined. The data showed that *B. vulgaris* ethanolic extract have strong anti bacterial effect (gram positive and gram negative) at concentrations range from 2.5 to 20 mg/ml. Anti fungal activity was measured according to inhibition of the production of aflatoxine B1 and B2, where ethanolic extract of *B. vulgaris* was able to inhibit the production of 44 and 98.3% of aflatoxine B1 and 67.2 and 89% of aflatoxine B2 at concentration of 0.01 to 0.1%, respectively. On the other hand, the ethanolic extract inhibits hepatitis C virus replication at concentration of 100 µg/ml, at single dose, while inducing lymphoproliferative effect and phagocytic activity in a dose dependant manner. In conclusion, *B. vulgaris* extract can be considered an efficient antimicrobial especially hepatitis C virus (HCV) and *Aspergillus flavus*. This effect could be returned to its immunostimulant capacity as it enhances the activity of peripheral blood mono nuclear cells (PMNC), especially phagocytic cells.

Key words: *Berberis vulgaris*, ethanolic extract, gram positive bacteria, gram negative bacteria, anti-bacteria, aflatoxines, hepatitis C virus, immune stimulation, phagocytosis.

INTRODUCTION

Berberis vulgaris is a shrub in the family Berberidaceae, native to central and Southern Europe, North-west Africa and Western Asia. The fruit is an oblong red berry 7 to 10 mm long and 3 to 5 mm broad, ripening in late summer or autumn; they are edible but very sour, and rich in vitamin C. Barberry is extensively used as food additive and its juice is recommended to cure cholecystitis (Zargari, 1983). *B. vulgaris* as well as other berberine (BER)

containing plants (Lisy, 1989) are used medicinally in virtually all-traditional medical systems, and have a history of usage in Ayurvedic, Iranian and Chinese medicine, dating back at least 3,000 years. Phytochemical analysis of root or stem bark extract of *B. vulgaris* demonstrated the presence of protoberberines and bisbenzyl-isoquinoline alkaloids (berbamine, tetrandrine and chondocurine) for which anti-inflammatory and

immuno-suppressive activities have also been well established (Akhter et al., 1979). Medicinal properties for all parts of the plant have been reported, including tonic, antimicrobial, antiemetic, antipyretic, antipruritic, antioxidant, anti-inflammatory, hypotensive, antiarrhythmic, sedative, antinociceptive, anticholinergic and cholagogue actions, and it has been used in some cases like cholecystitis, cholelithiasis, jaundice, dysentery, leishmaniasis, malaria and gall stones (Khosrokhavar and Ahmadiani, 2010).

The antimicrobials of plant origin are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Parekh and Chanda, 2005). The mechanism of polyphenol toxicity against microbes may be related to the inhibition of hydrolytic enzymes (proteases) or other interactions to inactivate microbial adhesins, cell envelope transport proteins and non specific interactions with carbohydrates (Cowan, 1999). *Aspergillus flavus* is widely distributed in tropical and subtropical zones around the globe. *A. flavus* can infect several agricultural crops such as peanuts, cotton, tree nuts, maize, rice, peppers, spices and figs, resulting in the production of one of the most toxic and potent carcinogenic metabolites known as aflatoxins (Binder and Chin, 2007). *A. flavus* causes a broad spectrum of disease in humans, ranging from hypersensitivity reactions to invasive infections associated with angioinvasion. After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of invasive and noninvasive aspergillosis (Denning, 1998; Morgan et al., 2005). Furthermore, *A. flavus* produces aflatoxins, naturally occurring mycotoxins, which are toxic and among the most carcinogenic substances known (Morrow, 1980). After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide intermediate or be hydroxylated and become the less harmful aflatoxin M1. High-level aflatoxin exposure produces an acute hepatic necrosis, resulting later in cirrhosis, and/or carcinoma of the liver.

Acute hepatic failure is made manifest by hemorrhage, edema, alteration in digestion, and absorption and/or metabolism of nutrients and mental changes and/or coma (Hedayati et al., 2007).

Hepatitis C virus (HCV) infection is a global blood-borne disease that affects almost 3% of the world's population with a morbidity and mortality that are second only to human immune deficiency virus (HIV) among the emerging infections. The highest estimated HCV prevalence in the world has been described in Egypt, with 11 to 14% of the population chronically infected. Prevalence rises steeply with age and antibodies against HCV may be detected in more than half the people between the age of 40 and 50 years in rural areas in the Egyptian Nile Delta (Kamal and Nasser, 2008; Alter, 1997). The conventional antiviral regimen, which is a combination of pegylated interferon and ribavirin, is curative in about half of treated patients depending on the viral strain (genotype). Additionally, this regimen requires prolonged

therapy, is toxic and expensive, and only a fraction of those with chronic HCV infections meet the criteria for treatment. Despite recent success after the introduction of combination therapy with IFN- α and ribavirin, about 60% of patients with HCV genotype 4 fail to respond (Kamal and Nasser, 2008).

IFN (Interferon) therapy has serious side effects including depression, fatigue, flu-like symptoms, hemolytic anemia, alopecia, itchiness, insomnia, arthralgia, myalgia and anorexia, neutropenia, nausea and vomiting, fever and chills (Vuillermoz et al., 2004). Ribavirin is a nucleoside analog (also called a nucleoside reverse transcriptase inhibitor, or NRTI) antiviral drug; the exact way ribavirin mechanism of action is not clearly understood. However, it is believed that ribavirin is phosphorylated inside hepatocytes; phosphorylated ribavirin diminishes the inosine 5'-monophosphate dehydrogenase activity (IMPDH), consequently, the intracellular synthesis and storage of guanine is reduced and finally the viral replication is arrested. In despite of ribavirin being the anti-HCV, ribavirin monotherapy treatment strategy has little effect on HCV RNA levels, but is associated with a decline in serum ALT (Amino transferase) (Dixit et al., 2004).

The aim of this study was to evaluate the antimicrobial activity of *B. vulgaris* and berberine and berberine chloride against pathogenic *Escherichia coli*, *Candida*, *Aspergillus flavus* and HCV.

MATERIALS AND METHODS

Barberry, *Berberis vulgaris*

Barberry were collected from the fields and authenticated by Prof. Salma El-dareir, Botany Department, Faculty of Science, Alexandria University, Egypt. Firstly, the intact plant was removed from the soil, roots were washed more than once and the plant was firmly pressed between paper towels. Then the plant roots were separated, packed within sealable plastic bags and kept out of direct sunlight. Plant roots were separated then dried at room temperature, powdered, sieved, and stored prior to further use.

Extraction of barberry active ingredients

The dried powdery roots of barberry was exhaustively defatted with petroleum ether and subjected to steam distillation method for ethanolic gradient extraction with Soxhlet apparatus. The ethanolic extract was concentrated to minimum volume using rotary evaporator (Büchi, Switzerland) then lyophilized (DISHI, DS-FD-SH10, Xi'an Heb Biotechnology Co, China) to obtain a powder extract of barberry (25%). The barberry extract powder form was kept at -20°C until subjected to further biochemical analysis (El-Sayed et al., 2011).

Preparation of berberine chloride

Different concentration of berberine chloride were prepared by dissolving a certain weight at 1 ml of 10% ethanol.

HPLC chromatography

The ethanolic extract was analyzed using high performance liquid chromatography (HPLC) (Series 500 Bio-Tek Instrument, Milano, Italy) to determine the berberine concentration in the extract. The analytes were separated by a Zorbax Eclipse XDB-C18 column with these features (250 × 4.6 mm i.d., 5 µm particle size) (Agilent, Santa Clara, CA, USA). The mobile phase was gradient, which consisted of water and methanol, the program was started with 40% solvent A (deionized water) and 60% solvent B (methanol) at a solvent flow of 0.8 ml/min and injection volume of 20 µl (Qadir et al., 2009). The operating temperature was maintained at 30°C and the detector was operated at a wavelength of 254 nm. At beginning, the freeze-dried *B. vulgaris* extracts were dissolved in equal volume water: ethanolic solution (1 mg/ml) and were filtered through a 0.22 µm syringe filter prior to HPLC analysis.

Antimicrobial activity

Effect of berberine and berberis crude extracts on fungi growth and aflatoxins production

A. flavus was mixed with 35 ml Sabouraud's dextrose media that contained 351 µl dimethyl sulphuroxide DMSO (+ve control), water (-ve control) or different concentrations of extract or berberine standard (10 and 100 mg %) and left for 10 days until complete growth. The effect of different concentrations on the aflatoxin production (B1 and B2 toxine) was carried out by HPLC chromatographic assay (Huynh, 1984) using these conditions; reverse-phase Hypersil ODS C18 (150 mm × 4.6 mm); mobile phase was isocratic (methanol: water: acetonitrile, 25: 25: 50 (V/V)) with flow rate of 1 ml/min; injection volume was 20 µl. Absorption was detected by fluorescence detection with excitation $\lambda = 365$ nm and emission $\lambda = 464$ nm. The concentration was estimated at wavelength detector 210 nm.

Inhibition zones measurement

All the samples were evaluated by the agar cup diffusion technique (Nermeen et al., 2012) using a 1 mg/ml solution in DMSO. The test organisms were *E. coli* (ATCC 11775) and *C. albicans* (DSM 70014). Each 100 ml of sterile molten agar (at 45°C) received 1 ml of 6 h-broth culture and then the seeded agar was poured into sterile Petri-dishes. Cups (8 mm in diameter) were cut in the agar. Each cup received 0.1 ml of the 10 mg/ml solution of the test compounds. The plates were then incubated at 37°C for 24 or 48 h. A control using DMSO without the test compound was included for each organism. Ampicillin was used as standard antibacterial, while clotrimazole was used as antifungal reference.

Minimal inhibitory concentration (MIC) measurement

The minimal inhibitory concentrations (MIC) of the most active compounds were measured using the two-fold serial broth dilution method (Guillermo Repetto, 2008). The test organisms were grown in their suitable broth for 24 h for bacteria and 48 h for fungi at 37°C. Two-fold serial dilutions of solutions of the test compounds were prepared using 100, 75, 50, 25, 10, 5, 2.5 and 1 mg/ml. The tubes were then inoculated with the test organisms; each 5 ml received 0.1 ml of the above inoculum and were incubated at 37°C for 48 h. Then, the tubes were observed for the presence or absence of microbial growth.

Minimal bactericidal concentration (MBC) measurement

The minimal inhibitory concentrations (MIC) tests were always

extended to measure the minimal bactericidal concentration (MBC) as follows: A loop-full from the tube not showing visible growth (MIC) was spread over a quarter of Miller-Hinton agar plate. After 18 h of incubation, the plates were examined for growth. Again, the tube containing the lowest concentration of the test compound that failed to yield growth on subculture plates was judged to contain the MBC.

Anti-hepatitis C virus activity

Neutral red uptake assay to measure cytotoxicity on mammalian cells

Isolation of lymphocytes from whole human blood using gradient separation by Ficoll-Paque™ Plus (MP Biomedicals, France), 10×10^4 lymphocyte cells were seeded per well in 96 well plates and the plates were incubated in RPMI (Roswell Park Memorial Institute) media containing different concentrations of the berberine and berberis crude extract for 24, 48, 72 and 96 h. The fraction of viable lymphocyte cells was measured by the Neutral red assay (Guillermo Repetto, 2008). The neutral red assay is based on the initial protocol described by Borenfreund and Puerner (1984) and determines the accumulation of the neutral red dye in the lysosomes of viable cells (Fotakis and Timbrell, 2006). Following exposure of different concentrations of berberine and berberis, crude extract to cells were incubated for 3 h with neutral red dye (40 µg/ml) dissolved in culture media RPMI. Cells were then washed with phosphate buffered saline (PBS) and the addition of 1 ml of elution medium (ethanol/glacial acetic acid /water 50/1/ 49%) followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to 96-well plates and absorbance at 490 nm was recorded using microliter plate reader spectrophotometer (Biotek, U.S.A). The simplest estimate of IC_{50} is to plot x-y and fit the data with a straight line (linear regression).

Qualitative in-vitro anti-HCV screening

Peripheral blood mononuclear cells (PBMC) was prepared as discussed in El-Hawash et al. (2006), then infected with 2% HCV-infected serum in RPMI culture medium containing 8% FBS. Each of the tested berberine and berberis crude extract were added at the specified doses. Positive and negative control cultures were included. After 96 h of incubation at 37°C, 5% CO₂, and 95% humidity, a second dose of the test compound was added. The cells were incubated for a further 96 h followed by total RNA extraction. The positive strand and its replicating form (negative strand) were detected by reverse transcription-polymerase chain reaction (RT-PCR) using HCV specific primers to the 5'-untranslated region of the virus according to detail method described by El-Awady et al. (1999).

Lymphoproliferative activity

The ability of *B. vulgaris* extracts to induce lymphoproliferative activity of resting human PBMC was examined. Briefly, peripheral blood was collected from healthy individuals in heparinized tubes (10 U/ml) and PBMC were collected by density gradient centrifugation using sterile lymphocyte separation medium (Ficoll-Paque premium, density 1.077 g/ml, GE Healthcare, USA). Cell count and viability was determined using Trypan blue exclusion test. Finally, the separated PBMC were suspended at 1.0×10^6 cell/ml in RPMI 1640 medium (Lonza, USA) supplemented with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Lonza), 4 mM L-glutamine (Lonza), 100 U of penicillin and 100 µg

Table 1. Berberine concentration in *Berberis vulgaris* crude extract.

Extract	Berberice concentration (mg/mg extract)
Ethanolic extract	0.62

Table 2. Berberine and berberis crude extract on *C. albicans* and *E. coli* 10⁶ to 10⁷ CFU/ml cult on LB broth.

Species	Parameter	MBC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)
<i>C. albicans</i>	Berberine	10	20	10
	Extract	25	17	75
<i>E. coli</i>	Berberine	2.5	25	2.5
	Extract	5	15	10

MIC = Minimal inhibitory concentration, MBC = minimal bactericidal concentration.

streptomycin (Cambrex) and 10% FBS (Lonza). PBMC was incubated with different concentrations (1, 5, 10, 50 and 100 µg/ml) of the *B. vulgaris* extract (B2) and berberine (B1) standard. All samples were assayed in triplicates. Proliferation was assessed using neutral red uptake assay (Guillermo and Jorge, 2008) after incubation for 24, 48, 72 h at 37°C, 5% CO₂, and 95% humidity.

Phagocytosis

Polymorphonuclear cells (PMNC) was isolated according to the method described by Woong et al. (2009). The effect of *B. vulgaris* extract was assessed for induction of phagocytosis and killing of yeast according to the method described by Schmid and Brune (1974) with some modifications, where, 50 µl of 0.4 × 10⁵ PMNC were added to 120 µl of viable *C. albicans* suspension (2 × 10⁶ *C. albicans* cells/ml) plus 20 µl of normal human serum followed by incubation with different concentrations of Echinacea extract at 37°C in 5% CO₂ incubator for 10 and 60 min. Both phagocytosis and yeast killing was measured by using methylene blue according to the method described by Felice et al. (2009) (Dana et al., 2009). With each well gently rinsed with PBS. Cells were stained and fixed by adding 50 µl methylene blue solution (1.25% glutaraldehyde + 0.6% methylene blue in HBSS (Hanks Balance Salt Solution)) to each well. After incubation at 37°C for 60min, the methylene blue solution was removed from the wells, and then plates were rinsed by distilled water eight times at 2000 r.p.m for 5 min.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Primer of Biostatistics (Version 5) software program. Significance of means ± standard deviation (SD) was detected by the multiple comparisons Student-Newman-Keuls test at p < 0.05.

RESULTS

HPLC chromatographic analysis

HPLC analysis showed that 1 mg of berberis ethanolic extract contains 0.6 mg berberine active compound

(Table 1, Figure 1). This data was obtained after the assessment of the limites of detection and quantization which were found to be 19 and 45 ng/ml for berberine and the recovery of berberine standard was found to be 98.2% that gave us good indication about the sensitivity of the developed method.

Antimicrobial activity

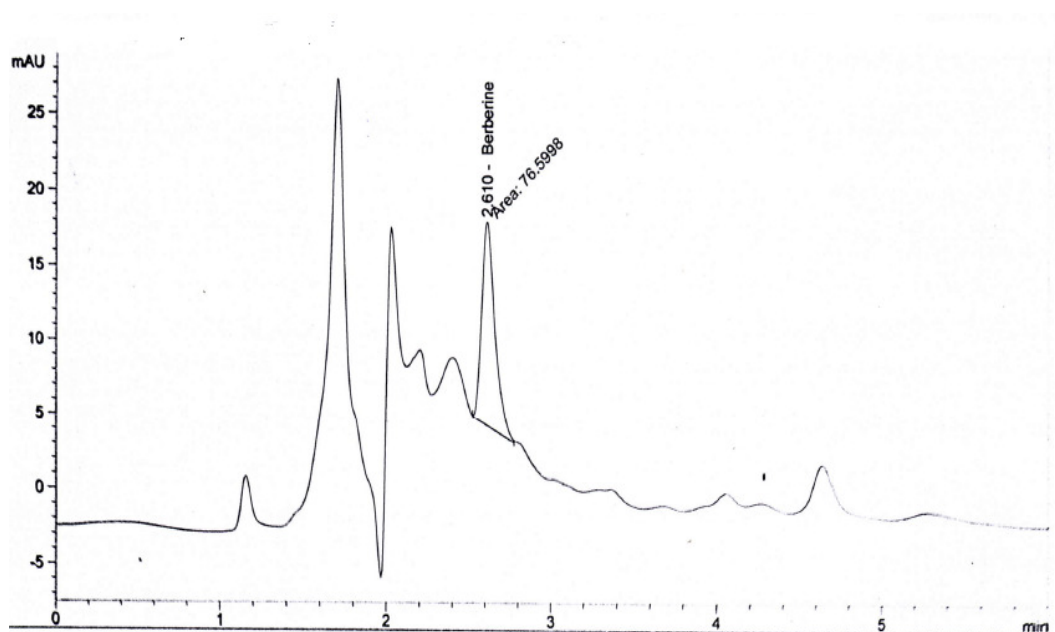
Anti microbial effect of berberis ethanolic extract was tested in comparison with pure berberine stander. As shown in Table 2, both berberis ethanolic extract and berberine stander can inhibit both yeast (*C. albicans*) and bacteria (*E. coli*) growth when used at different concentration. Berberis ethanolic extract showed to have antimicrobial activity at concentration double the concentration of the stander used. This can be attributed to the concentration of berberine compound in the extract (Table 1). On the other hand, berberis ethanolic extract was tested for its effect on the production of aflatoxine produced by *A. flavus*, as shown in Figure 2, berberis ethanolic extract can decrease 44% the production of aflatoxine B1 and 67.2% the production of B2 at concentration of 0.01% while berberis ethanolic extract decreased 98.3% of the production of aflatoxine B1 and 89% of the production of aflatoxine B2 at concentration of 0.1%.

Anti-HCV activity

The effect of both berberis ethanolic extract and berberine stander on HCV replication *in vitro* was examined at concentration of 100 µg/ml. As shown in Figure 3, berberine stander failed to inhibit HCV replication at 100 µg/ml while berberis ethanolic extract was able to inhibit this replication.

Table 3. Phagocytosis activity of berberis ethanolic extract.

Concentration ($\mu\text{g/ml}$)	Phagocytosis index	Percent of phagocytosis	Killing ratio
0	2.731199187	-	0.539534884
1	3.357892954	122.9457364	0.774274905
5	5.271849593	193.0232558	0.872289157
10	11.89447832	435.503876	0.951940192
50	15.84942412	580.3100775	0.985840235
100	17.26371951	632.0930233	0.999264165

**Figure 1.** *Berberis vulgaris* ethanolic extract HPLC chart.

Lymphoproliferative activity

Different concentrations (20, 40, 60, 80 and 100 $\mu\text{g/ml}$) of both berberis ethanolic extract and berberine stander showed to have no growth inhibitory effect on normal blood cells (PBMC). On the contrary, they slightly stimulated the proliferation of PBMC (Figure 4) especially after incubation for 72 h.

Phagocytosis

Berberis ethanolic extract was tested for its ability to induce phagocytosis and killing of living *C. albicans* in resting PMNC. As presented in Table 3, the phagocytosis index as well as percent phagocytosis increases when human polymorphonuclear cells was incubated with different concentration of berberis ethanolic extract (1, 5, 10, 50, 100 $\mu\text{g/ml}$) in a dose dependent manner except at concentration of 50 and 100 $\mu\text{g/ml}$. On the other hand, the killing ration induced by the used concentrations of

berberis ethanolic extract increased gradually.

DISCUSSION

Interest in medicinal plants has burgeoned by the increased efficiency of new plant-derived drugs and the growing interest in natural products. Because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades. The use of plants as medicines dates back to history (Dattner, 2003). Medicinal plants serve as therapeutic alternatives, safer choices, or in some cases, as the only effective treatment. People in separate cultures and places are known to have used the same plants for similar medical problems. A larger number of these plants and their isolated constituents have shown beneficial therapeutic effects, including anti-oxidant, anti-inflammatory, anti cancer, anti-microbial, and immunomodulatory effects (Huffman,

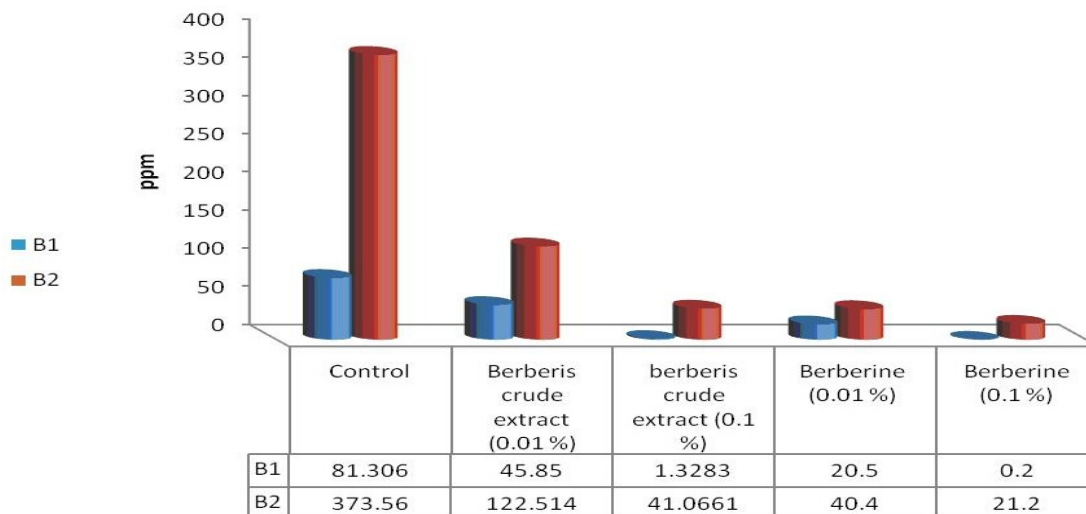


Figure 2. Effect of berberine and berberis crude extract on aflatoxin B1 and B2 production.

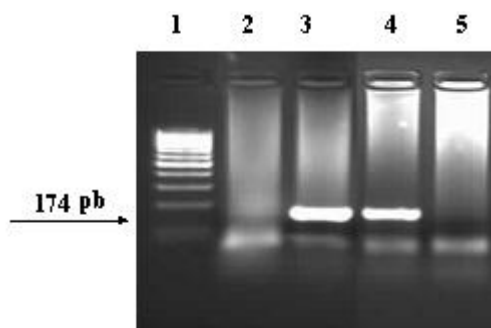


Figure 3. RT-PCR for detection of HCV using nested PCR reaction. Lane one is 100 pb ladder, lane 2 negative control, lane 3 positive control, lane 4 = 100 µg/ml of berberine, lane 5 = 100 µg/ml of berberis ethanolic extract.

2003).

Antimicrobial activity of berberine was preliminarily evaluated for their *in vitro*-antibacterial activity against *E. coli* as Gram-negative bacteria. Berberine also was evaluated for their *in vitro*-antifungal activity against *C. albicans*. Their inhibition zones using the cup diffusion technique (Jain and Kar, 1971) were measured, further evaluation was then carried out on berberine showing reasonable inhibition zones to determine their MIC and MBC using the two-fold serial dilution method (McCartney, 1989).

As revealed from Table 2, both berberis ethanolic extract and berberine stander can inhibit both yeast *C. albicans*) and bacteria (*E. coli*) growth when used at different concentration. Berberis ethanolic extract showed to have antimicrobial activity at concentration double the concentration of the stander used. This can be attributed to the concentration of berberine compound in the extract (Table 1).

According to the MIC and MBC limits derived from the latest National Committee on Clinical Laboratory Standards (NCCLS), we can determine whether the test compound is bactericidal or bacteriostatic to the test organism. If the MBC equals MIC, the test compound is considered a bactericidal but if $MBC > MIC$ the test compound is considered a bacteriostatic. Accordingly, as revealed from Table 2, tested compound were bacteriostatic against *E. coli*. In addition, they were fungicidal against *C. albicans*.

B. vulgaris showed an amazing and unique results as it did not affect the fungus's growth rate but it was completely inhibited toxin production that could be due to the presence of berberine. Several aflatoxin production inhibitors may act at three levels: (1) modulate environmental and physiological factors affecting aflatoxin biosynthesis, (2) inhibit signaling circuits upstream of the biosynthetic pathway, or (3) directly inhibit gene expression or enzyme activity in the aflatoxin production pathway as shown in Figure 5. Till now, the mode of action of most inhibitory compounds is still unknown (Holmes et al., 2008).

Several reports indicate the pharmacological, biochemical and anticancer effects of berberine (5, 6-dihydro-9, 10-dimethoxybenzo[g]-1, 3-benzodioxole-5,6-aquinoizum), which is a natural isoquinoline alkaloid present in roots, rhizome and outer bark of an important medicinal plant species, *Berberis* (*B. aquifolium*, *B. vulgaris*, *B. aristata*, etc.). Mahata et al. (2011) provided extensive data which indicated the pharmacological effect of commercially available berberine on human papilloma viruses and associated cancer. Also, berberine showed to have antiviral effects on the influenza virus both *in vitro* and *in vivo*. The possible therapeutic mechanism of berberine on influenza-induced viral pneumonia could be by inhibiting the virus infection, in addition to improving the pathogenic changes by inhibiting release of

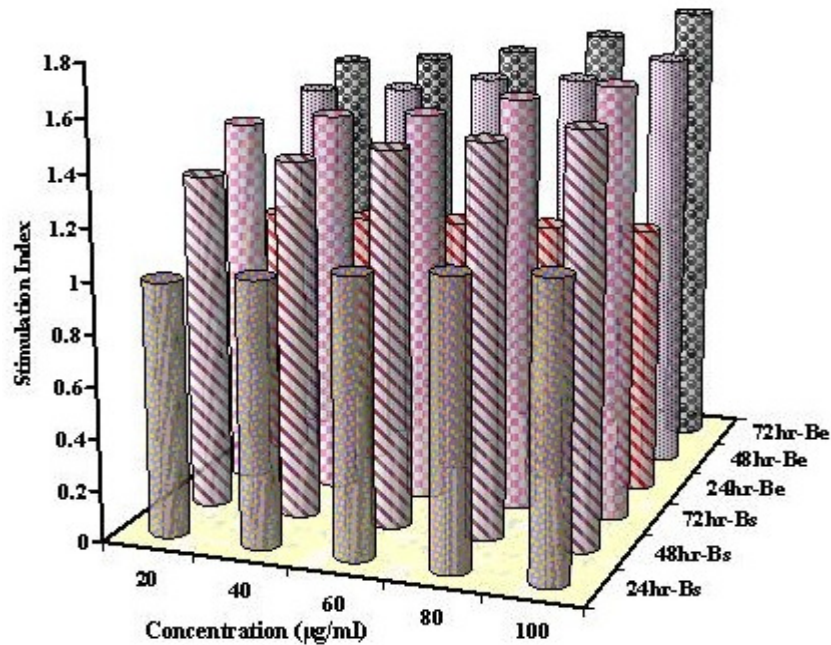


Figure 4. Effect of different concentrations of berberine (Bs) and berberis ethanolic extract (Be) on proliferation of normal peripheral blood mono nuclear cells (PBMC) at 24, 48 and 72 hrs. Data were represented as stimulation index (SI).

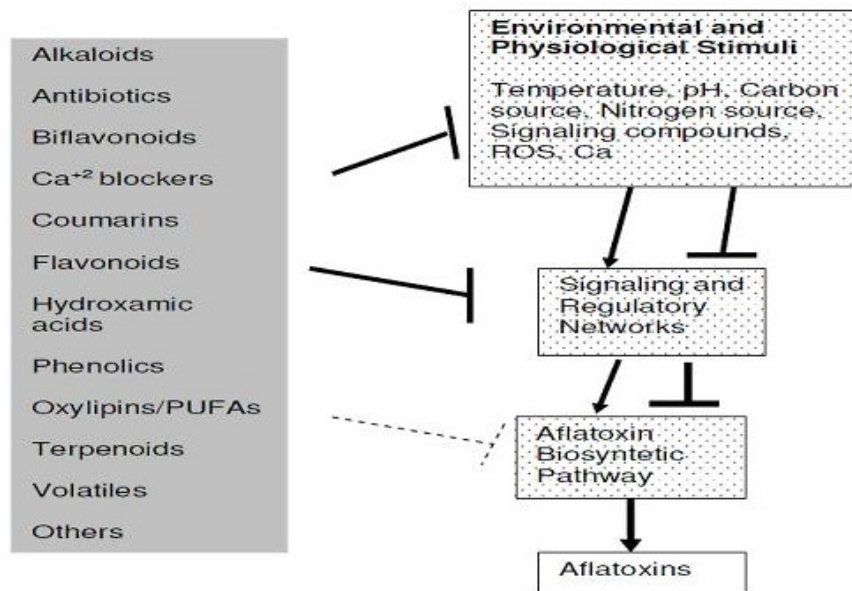


Figure 5. Inhibition of aflatoxin synthesis.

inflammatory molecules by immune cells (Wu et al., 2011).

In the present study, Berberis ethanolic extract and berberine stander was tested for their ability to inhibit replication of HCV in an *in vitro* system, as provided in our results that berberis ethanolic extract can inhibit HCV

replication while berberine did not. Accordingly, the therapeutic mode of action could be through activation of the immune cells and especially phagocytosis (Liu et al., 2010). Several reports indicate the modulatory immune stimulation of berberine (Kim et al., 2003; Marinova et al., 2000). As shown in the present study, both berberine

standard and berberis ethanolic extract can induce healthy PBMC to proliferate. On the other hand, berberis ethanolic extract increase phagocytosis and killing of ingested *Candidia* in the PMNC.

Conclusion

Berberis ethanolic extract from the dried roots of *B. vulgaris* that contain 62% berberine have strong and bacterial anti viral effect as well as immune modulatory function.

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