



Investigating the impacts of various solvents fractions of *Bupleurum lancifolium* on the anti-microbial and antioxidant potentials

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ABSTRACT

Introduction: In the last thirty years, interests in searching for plants with potential antimicrobial and antioxidant activities have been increased due to their probable health benefits. This study aimed to investigate the antimicrobial and antioxidant effects of various solvents fractions of *Bupleurum lancifolium*. **Methods:** The antioxidant activities of four fractions of *Bupleurum lancifolium* were assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, while the antimicrobial activity was assessed by broth microdilution method. The antimicrobial activity of four plant fractions was examined against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella sonnie* and *Enterococcus faecium* American Type Culture Collection reference strains. **Results:** The fractionation extraction method succeeds in the assessment of the antibacterial and antioxidant activity of *B. lancifolium* plant. Hence the methanolic fraction has antioxidant potential with 33.03% of inhibition according to the Trolox antioxidant standard molecule, while *n*-hexane and methanol fractions showed powerful antibacterial activity against *E. faecium* and *S. sonnie* strains with Minimum Inhibitory Concentration (MIC) 1.5625 mg/ml for both fractions. **Conclusion:** All plant fractions have potent antioxidant and antibacterial activities. Further investigations, i.e. isolation, identification, and clinical assessment, of the active compound(s) are needed for the possible formulation of new therapeutic alternatives.

KEY WORDS: *Bupleurum lancifolium*; Antibacterial; Antioxidant; Palestine

INTRODUCTION

Herbal remedies have been used as therapeutic agents for thousands of years and utilized in various forms such as powders, infusions, tinctures, poultices, and decoctions [1, 2]. The discovery of medications from medicinal plants started with the isolation of single active molecules such as salicylic acid, morphine cocaine, atropine, codeine, digoxin, quinine, vincristine and quinine, and most of these drugs are still in use [3, 4].

However, the developing techniques in chemical drugs synthesis led to a major reduction in the usage of herbal products and there was concern that the use of some herbal medications for therapeutic purposes might be a banned. However, phytochemical products are still important for investigating new medications especially for the incurable diseases on which chemical drugs failed in their treatment. For example, some kinds of therapeutic

agents for certain types of diseases, such as antihypertensive, antimalarial, anticancer, and anti-migraine medication, have benefited greatly from natural products [5, 6].

Herbals and other natural products derived drugs continue to provide phytochemists with valuable pharmacologically active compounds and/or chemical models that utilized as starting molecules for developing of new medications. In addition, more than 80% of the used drugs nowadays are produced from natural products and/or inspired by a natural compound [7, 8].

Globally estimated about 100 million species or organisms living on earth and higher plants forming a group of some 250,000 species out of which only 6% has investigated for biological activities and 15% for their chemical constituents. This means that the scientists investigated only little amounts of research from the world huge natural products resources [7].

In nature, there are about 190 plants species of *Bupleurum* plant genus that distributed in the North Temperate regions mainly in North Africa, Mediterranean, and Eurasia. The *Bupleurum* plant genus considered as one of the largest genera of the family Apiaceae. These plants species are easily recognized by their parallel venation of the leaves, conspicuous bracteoles and bracts on the inflorescences. On the other hand, pollen morphology exhibits little variation in this genus. The fruit is ellipsoid or oblong to ovoid-oblong, slightly laterally compressed and their mericarp is subpentagonal and rarely rounded in the cross section [9, 10]. Many of *Bupleurum* plant species have been screened to evaluate their content of flavonoid, essential oils, fatty acids as well as the antioxidant properties [11-14].

The Palestinian flora comprises ten species of *Bupleurum*, which are including *Bupleurum boissieri*, *Bupleurum brevicaulis*, *Bupleurum gerardii*, *Bupleurum libanoticum*, *Bupleurum nodiflorum*, *Bupleurum odontites*, *Bupleurum orientale*, *Bupleurum semicompositum*, *Bupleurum subovatum* and *Bupleurum lancifolium* [15].

Bupleurum lancifolium Hornem. is annual herbaceous plant about 10-60 cm height which belongs to the Apiaceae family. Its stems divaricately branched from base and striate. The leaves are oblong-lanceolate to linear-lanceolate, acuminate, tapering at base, while cauline leaves are perfoliate, ovate to ovate-oblong or lanceolate with rounded base. Bracteoles are connate at base, patulous, ovate to orbicular and mucronate. Flowers petals are ovate with truncate or emarginated inflexed apex. *Bupleurum lancifolium* Fruiting pedicels are thickened and much shorter than their fruits. The mericarps are dark brown, prominently ribbed, and densely tuberculate in furrows.

B. lancifolium is native to Algeria, Austria, Apennine Peninsula, Balkan Peninsula, Central Asia, Cyprus, Egypt, Iraq, Iberian Peninsula, Libya, Palestine, and Syria. Nowadays it is cultivated in Belgium, Luxemburg and British Isles [11, 15].

The leaves of *B. lancifolium* contain quercetin and isorhamnetin flavonoids, while the oil of the seeds contains palmitic, γ -linolenic, linoleic, 1,3-cyclooctadiene and linolenic acids [11].

Bupleurum roots are one of the most frequently used herbs in Chinese herbal medicine [16]. The genus *Bupleurum* contains saponin glycoside (Saikosaponins) and polyphenols which have potential hepatoprotective agents against acute and chronic hepatic injury [14]. In folk medicine, some *Bupleurum* members are used in the treatment of various diseases, such as malaria, fever, and, influenza [17].

To the best of our knowledge, there is little information about the biological and chemical properties of the *Bupleurum* species growing in Palestine. Therefore, more studies on the *Bupleurum* species are necessary. The present paper describes a study undertaken in order to determine antimicrobial and antioxidant

activities of four solvent fractions *B. lancifolium* species collected from the Palestine.

MATERIAL AND METHODS

Instrumentation

Shaker device (Memmert, Germany), rotavap (Heidolph, Germany), grinder (Moulinex, China), balance (Rad wag, AS 220/c/2, Poland). lyophilizer (Mill rock technology, China), micropipettes (Finnpipette, Finland), incubator (Nuve, Turkey), syringe filter 0.45 μ m pore size (Microlab, China), micro-broth plate (Greiner bio-one, Canada), and multi 12-channel micropipette (Eppendorf, Germany).

Chemical Reagents

Ethanol, sodium hydroxide, *n*-hexane, and acetic acid were obtained from Lobachemie, India, and FeCl₃, Millon's reagent, Benedict's reagent Dimethyl sulfoxide (DMSO) were acquired from Riedeldehan, Germany, and Molish's reagent, sulfuric acid, Iodine solution were obtained from Alfa-Aesar, England. Chloroform, DPPH (2,2-Diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and HCl were bought from Sigma-Aldrich, Germany. In addition, Magnesium ribbon and Ninhydrin solution obtained from Alfa Agar, England, Nutrient broth from Himedia, India, and BBL Mueller Hinton II broth, Difco Sabouraud Dextrose Agar were acquired from Dickinson and company sparks, USA. Bacto tryptic soy broth was obtained from Dickinson and company sparks, USA, and macConkey agar was purchased from Himedia Laboratories, India.

Bacterial Isolates

Antibacterial activity of plant extracts was investigated against bacterial isolates obtained from American Type Culture Collection. The isolates included *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella sonnei* (ATCC 25931) and *Enterococcus faecium* (ATCC 700221).

Collection and Preparing of Plant Materials

The aerial parts from *B. lancifolium* plant were collected in June 2016 from Jerusalem region in West Bank of Palestine. The collected plant was botanically classified by the pharmacognosist Dr. Nidal Jaradat, Department of Pharmacy, Faculty of Medicine and Health Sciences, An-Najah National University. The shade dried voucher specimen was archived in the Herbarium of the Pharmacognosy Laboratory under herbarium number Pharm-PCT-447.

After washing under running water to remove intercalating debris and soil particles, the plant material was shade dried at ambient temperature for two weeks. The dried plant material was then finely ground using a mechanical grinder and subsequently stored in air-tight containers.

Fractionation of Plant Extracts

The powdered plant material was sequentially macerated in different solvents that having different polarities to achieve serial fractional extraction process; beginning by the maceration of 50 g of powdered plant material in 500 ml *n*-hexane for 2 days with occasional agitation at room temperature. After filtration, the solvent removed by using rotary-evaporator under pressure at a temperature not exceeding 35°C. After the filtration in the first step, the remaining plant material was macerated in 500 ml acetone and was treated exactly the same as above. The same plant material then macerated in 500 ml methanol and was treated exactly the same as above. Finally, the same material macerated with 500 ml water and this fraction lyophilized by using the freeze dryer apparatus.

The percent recovery of the four fractions extracts calculated according to the following formula:

$$\% \text{ yield extract (\% w/w)} = A/B \times 100$$

Where;

A = weight of the dried extract.

B = weight of powdered plant material.

The yields for *n*-hexane, acetone, methanol and aqueous fractions were 2.68% (w/w), 9.8% (w/w), 14.36% (w/w) and 12.5% (w/w), respectively.

Phytochemical Analysis

Preliminary phytochemical analysis of secondary and primary metabolic compounds such as cardiac glycosides, flavonoids, saponin glycosides, terpenoids proteins, phenols, carbohydrates, starch, steroids, reducing sugar, monosaccharide and tannins was carried out according to the standard pharmacopeia analytical methods.

Determination of MIC

Antimicrobial activities of plant fractions were determined by micro-broth dilution method according to Forbes et al 2016 and Wikler 2007 [18, 19]. A volume of 100 µl of Mueller-Hinton broth was pipetted in each well of micro-titration plate. To the first well, 100 µl of plant fraction (50 mg/ml) was added and 100 µl was transferred from the well to the next well after mixing by the micropipette. This procedure was repeated up to well number 11 from which 100 µl were discharged after mixing. Well number 11 was the negative control of bacterial growth and well number 12 was the positive control of bacterial growth. Each plant extract was examined in duplicate. From a bacterial suspension (5×10^7 CFU/ml), 1 µl was pipetted to all wells except for well number 11. The inoculated plate was incubated at 35°C for 24 hours. The lowest concentration of plant fraction that inhibited visible bacterial growth was considered as the MIC.

Free Radical Scavenging Assay Using Trolox as Standard Equivalent

A stock solution of a concentration of 100 µg/ml in methanol was firstly prepared for each plant fraction and Trolox (standard reference compound). The working solutions at the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 µg/ml) were prepared by diluting from the stock solution with methanol.

Then DPPH (free radical to be inhibited) was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above-prepared working concentration in a ration of 1:1:1, respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only (control solution). The solutions were incubated in dark for 30 min at room temperature. Afterwards, the absorbance readings were recorded at 517 nm. The percentage of antioxidant activity of the plant's fractions and the Trolox standard were calculated using the following formula:

$$\text{Percentage of inhibition of DPPH activity (\%)} = (B - S)/B \times 100\%$$

Where:

B= Absorbance of the control solution,

S = Absorbance of the plant fraction.

The antioxidant half maximal inhibitory concentration (IC₅₀) for the plant fractions and Trolox were determined by using the BioDataFit fitting program as follows:

$$\% \text{ Inhibition} = (\text{Trolox IC}_{50} / \text{fractions IC}_{50}) \times 100\%$$

Statistical Analysis

Free radical scavenging activities of four *B. lancifolium* fractions were determined in triplicates. The results were expressed as means ± standard deviation (SD). The obtained data were compared and analyzed by using unpaired *t*-tests. The statistical significance was considered when the *p*-value was <0.05. Statistical significance is expressed in terms of * when the *p*-value < 0.05, ** when the *p*-value ≤ 0.001, and *** when the *p*-value ≤ 0.0001.

RESULTS

Phytochemical Screening of Four *B. Lancifolium* Fractions

Phytochemical screening tests for the fractions from *B. lancifolium* plant showed the presence of active phytochemical classes including saponin glycosides, carbohydrates, tannins, steroids, cardiac glycosides, alkaloids, reducing sugars, phenols, terpenoids, and flavonoids (Table 1).

Table 1: Phytochemical screening tests of the aqueous, methanolic, acetone and *n*-hexane fractions from *B. lancifolium*.

Class	Hexane	Acetone	Methanol	Aqueous
	Fraction	Fraction	Fraction	Fraction
Saponin glycoside	-	-	-	+
Protein	-	-	-	-
Starch	+	-	+	-
Phenols	-	+	+	+
Carbohydrates	+	-	+	-
Tannin	-	+	+	+
Steroids	+	+	+	+
Reducing sugar	+	-	+	-
Monosaccharide	+	-	+	-
Terpenoids	+	+	+	+
Flavonoid	-	+	+	+
Cardiac glycosides	+	+	-	-

Free Radical Scavenging Activity

The free radical scavenging activity of the *n*-hexane (non-polar solvent), methanolic (polar protic solvent), acetone (polar aprotic solvent), and aqueous (polar protic solvent) fractions of *B. lancifolium* were tested using the DPPH method with Trolox as a reference standard. Concentrations spanned the range of 1-100 µg/ml from each extract as well as from Trolox. Control was determined using DPPH diluted in the corresponding solvents (aqueous, acetone, methanolic and *n*-hexane) without plant fractions. The obtained results are shown in Figure 1.

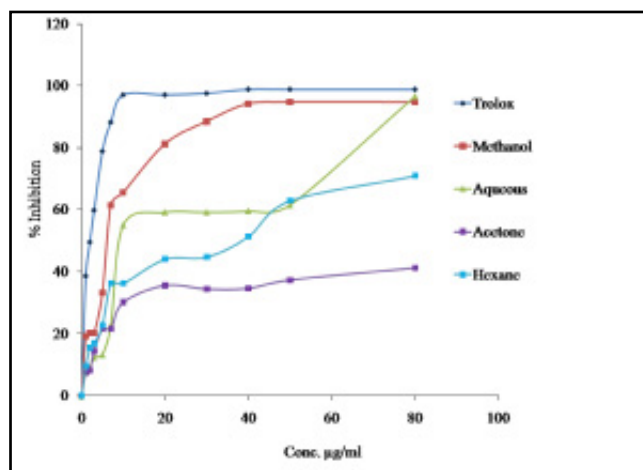


Figure 1: Free radical inhibitory effect of four *B. lancifolium* fractions.

The calculated half maximal inhibitory concentration (IC₅₀) of Trolox was 2.18 ± 1.54 µg/ml. The IC₅₀ for the aqueous, acetone, methanolic and *n*-hexane fractions of aerial parts of *B. lancifolium* are shown in Table 2.

Table 2: IC₅₀ values of the aqueous, acetone, methanolic and *n*-hexane fractions of *B. lancifolium*

Fractions	IC ₅₀ , µg/ml, ± SD	% of inhibition, ± SD
<i>n</i> -hexane	26.9 ± 1.11*	8.1% ± 1.21
Acetone	169.8 ± 1.69**	1.28 ± 1.55
Methanol	6.6 ± 0.99*	33.03 ± 1.09
Aqueous	15.6 ± 1.04***	13.97 ± 1.22

p*-value <0.05, ** when the *p*-value ≤ 0.001, and * when the *p*-value ≤ 0.0001.

Antibacterial Activity

By using microbroth dilution method, the results showed that the four fractions of *B. lancifolium* plant exhibited the growth of bacterial isolates in this study. The highest antibacterial activity (the lowest MIC) was caused by both methanolic and hexane fractions with MIC 1.5625 mg/ml against *S. sonnie* and *E. faecium*, while no inhibition was caused by acetone against *S. aureus* and *n*-hexane fraction against *E. coli* (Table 3).

Table 3. Minimum inhibitory concentrations (MICs) of *B. lancifolium* fractions.

Bacterial isolates	MIC(mg/ml)			
	Acetone fraction	Aqueous fraction	Methanol fraction	Hexane fraction
<i>S. aureus</i>	NI	6.25	12.5	6.25
<i>E. coli</i>	25	6.25	6.25	NI
<i>S. sonnie</i>	6.25	12.5	1.5625	6.25
<i>E. faecium</i>	12.5	25	6.25	1.5625
<i>P. aeruginosa</i>	3.125	3.125	3.125	3.125

NI: No inhibition

DISCUSSION

In the present study, *B. lancifolium* which growing wildy in the West Bank area of Palestine was fractioned by using four solvents with various levels of polarity and screened qualitatively. In addition, their antibacterial and antioxidant activities were assessed. To the best of our knowledge, this is the first investigation on the phytochemical and biological activities of *B. lancifolium* plant.

In the last few years, interest in the investigation for natural antioxidant and antibacterial products has increased dramatically. Moreover, the *in vitro* studies of free radical scavenging potentials of flavonoids have gained importance due to their potential antioxidant activity. Moreover, this class of secondary metabolic compounds has been found in all species of *Bupleurum* genus [20, 21]. Indeed, a study which was conducted by Saleh *et al.* revealed the presence of quercetin and isorhamnetin flavonoids in ethanolic extract of *B. lancifolium* plant [22].

In the last few decades, it has become clear that antibacterial agents such as antibiotics are losing their effectiveness due to the bacterial resistance [23]. Therefore, there is a continuing need to search for new antibacterial compounds. To achieve this, natural products have been considered as one of the fundamental antibacterial sources.

The antioxidant results here showed that the aerial parts from *B. lancifolium* have antioxidant potentials in various levels of inhibition according to the used solvent. Among them, the methanolic fraction extract has the highest antioxidant potential with 33.03% of inhibition activity compared to the reference standard (Trolox), followed by an aqueous fraction with 13.97% of inhibition, *n*-hexane with 8.1% of inhibition while the weakest antioxidant was acetone fraction with 1.28% of inhibition in comparison with Trolox.

Regarding the antibacterial assessment by using the microbroth dilution method, the results showed that the acetone and aqueous fractions of *B. lancifolium* exhibited strongly the *P. aeruginosa* bacterial growth with the same MIC value 3.125 mg/ml, while the highest exhibition of the methanolic fraction was against *S. sonnie* with a MIC value of 1.5625 mg/ml. The best antibacterial activity for the *n*-hexane fraction was against *E. faecium* bacterial isolate.

According to the *E. faecium* bacteria which can be a high resistant to drugs that acquired its drug resistance by plasmids and conjugative transposons, as well as chromosomal genes that encoded the resistance. Some strains have become resistant to gentamicin, penicillin, vancomycin, tetracycline, teicoplanin, and erythromycin. The spread of the disease occurs among patients in hospitals due to transferring the pathogen by hands or medical instruments.

Moreover, the best antibacterial activity against *P. aeruginosa* bacteria were all fractions with same MIC value 3.125 mg/ml, while the best antibacterial activity against *E. faecium* with MIC value 1.5625 mg/ml. Furthermore, the best antibacterial activity against *S. aureus* was for the aqueous and *n*-hexane fractions with the same MIC value 6.25 mg/ml. The best antibacterial activity against *E. coli* were aqueous and methanol fractions with the same MIC value (6.25 mg/ml) and finally the best antibacterial activity against *S. sonnie* was methanol fraction with MIC value 1.5625 mg/ml.

A previous investigation on *B. lancifolium* leaf constituents by Shafaghat, reported the presence of palmitic acid, γ -linolenic acid, linoleic acid, 1,3-cyclooctadiene and linolenic acid also reported that the antioxidant activity of hexane leaves extract was 435 μ g/ml. The same study showed that the *n*-hexane extract of leaves inhibited antimicrobial activity against *Bacillus subtilis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae* by using disc diffusion method [11].

A study conducted by Gevrenova *et al.* found that IC₅₀ values for methanol extract of *B. affine*, *B. baldense* and *B. flavum* were 116.47 μ g/ml, 31.87 μ g/ml, and 22.12 μ g/ml, respectively [21].

In comparison with other studies conducted on the same *Bupleurum* species and other species, the presently studied Palestinian *B. lancifolium* plant showed potential antibacterial and antioxidant activities more than other *Bupleurum* species.

In brief, the fractionation method succeeds in the assessment of the antibacterial and antioxidant activity of *B. lancifolium* plant. Hence, the methanolic fraction extract has antioxidant potential with 33.03% of inhibition according to the Trolox standard antioxidant molecule, while *n*-hexane and methanol fractions showed powerful antibacterial activity against *E. faecium* and *S. sonnie* strains with MIC 1.5625 mg/ml caused by both fractions.

CONCLUSION

The aerial parts of *B. lancifolium* four solvents fractions (acetone, *n*-hexane, methanol and aqueous) exert variable *in-vitro* antioxidant and antibacterial activities. The obtained results showed that the methanolic fraction has potent antioxidant activity. Obviously, the methanol and *n*-hexane fractions affected the growth of *E. faecium* and *S. sonnie* bacterial strains. Further investigations such as isolation and identification of the active compounds are needed for the possible formulation of new therapeutic alternatives.

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Conflict of Interest: None declared