Palestinian Medical and Pharmaceutical Journal

Volume 8 | Issue 1 Article 8

4-1-2023

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Abstract

Natural products with high antioxidant activity are critical for preventing oxidative stress, resulting in various degenerative and metabolic health problems. The study aimed to examine various extracts' qualitative and quantitative phytochemical contents. (aqueous, methanol, hexane, and acetone.) of Bituminaria bituminosa, in addition to evaluating their antioxidant capability. Standard analytical procedures were used to estimate the quantitative and qualitative tests for B. bituminosa. Four solvent extracts and the 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay were also used to assess the in vitro antioxidant activity. The hexane extract has a high flavonoid content (103.95 ± 4.7 mg of RUE/g derived from extract (dry), while the acetone extract has the highest amounts of hydrolyzable tannin and anthocyanin with values of 84.33 ± 1.56 mg of GAE/g of extract (dry) and 17.5 ± 0.7 mg of CAE/g of extract (dry), respectively. It also has the potential to be an antioxidant, with an IC50 value of 17.37 ± 1.97 µg/ml. This investigation reveals that hexane extract of B. bituminosa is a possible natural antioxidant source and can form the basis for therapeutic applications. As a result, the plant has the potential to be used in a future in-vivo study to explore its efficacy and safety in animal models. This research shows that B. bituminosa hexane extract is a possible source of naturally occurring antioxidants and could be used to develop therapeutic applications

Keywords

Bituminaria bituminosa, Tannin, Flavonoids, Anthocyanins, Antioxidant

Palestinian Medical and Pharmaceutical Journal (PMPJ). 2023; 8(1): 71-80

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Received: (24/1/2022), Accepted: (14/7/2022)

ABSTRACT

Natural products with high antioxidant activity are critical for preventing oxidative stress, resulting in various degenerative and metabolic health problems. The study aimed to examine various extracts' qualitative and quantitative phytochemical contents. (aqueous, methanol, hexane, and acetone.) of Bituminaria bituminosa, in addition to evaluating their antioxidant capability. Standard analytical procedures were used to estimate the quantitative and qualitative tests for B. bituminosa. Four solvent extracts and the 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay were also used to assess the in vitro antioxidant activity. The hexane extract has a high flavonoid content (103.95 ± 4.7 mg of RUE/g derived from extract (dry), while the acetone extract has the highest amounts of hydrolyzable tannin and anthocyanin with values of 84.33 ± 1.56 mg of GAE/g of extract (dry) and 17.5 ± 0.7 mg of CAE/g of extract (dry), respectively. It also has the potential to be an antioxidant, with an IC50 value of 17.37 ± 1.97 µg/ml. This investigation reveals that hexane extract of B. bituminosa is a possible natural antioxidant source and can form the basis for therapeutic applications. As a result, the plant has the potential to be used in a future in-vivo study to explore its efficacy and safety in animal models. This research shows that B. bituminosa hexane extract is a possible source of naturally occurring antioxidants and could be used to develop therapeutic applications.

Keywords: *Bituminaria bituminosa*, Tannin, Flavonoids, Anthocyanins, Antioxidant.

INTRODUCTION

Traditional herbal medicine is one of the oldest medicinal sciences in various cultures and nationalities. Even though most of its therapies have been mainly unapproved and inexact, more individuals are resorting to it these days. People choose to utilize herbs since they are inexpensive, widely available, do not require a prescription, have fewer side effects, and are helpful in various situations[1].

An imbalance in oxygen metabolism and an increase in reactive oxygen species in living cells and tissues causes several disorders, including accelerated aging, Alzheimer's disease, Parkinson's disease, and a variety of other neurological and metabolic diseases (2). Due to their ability to scavenge free oxygen radicals, antioxidants are effective medicinal agents against various neurological illnesses and other conditions. The major sources of antioxidant molecules are healthy diets rich in vegetables, fruits, and herbal medications, which are receiving considerable interest from pharmaceutical businesses and the food sector [3].

Bituminaria bituminosa L. is commonly referred to as pitch trefoil or Arabian pea and belongs to the Leguminosae (Fabaceae) family. It is a perennial herbaceous wild plant native to the Mediterranean [4].

The use of B. bituminosa's leaves and legumes as pasture for livestock and goats is one of the most popular uses. [5, 6]. In folk medicine, it is utilized as a vulnerary cicatrizing and disinfectant agent [7]. Furthermore, it treats urinary infections, spasms, fe-

ver, hair loss, and epilepsy. [5, 8, 9]. *B. bituminosa* has considerable pharmaceutical interests and played a vital potential role in the pharmaceutical industry due to the characteristic secondary metabolites found in its aerial parts, like bitucarpin A and erybraedin C furanocoumarins (psoralene and angelicin), and phenylpropanoids [7, 10]. Also, it represents a source of isoflavones (genistein) [7, 11], daidzin [8]), isoflavonoid (8-prenyldaidzein) [11, 12], plicatin B [7, 11], flavone (isoorientin) [8], phenolic acids, and lignans [5]. Furthermore, glycosylated flavonoids (apigenin) and saponins were the most abundant detected compounds [5]. Moreover, *B. bituminosa* contains high concentrations of psoralen and angelicin, which have antibacterial or antifungal activities [13, 14].

B. bituminosa leaves are used as a traditional edible plant in Palestine, where they are eaten as a salad or cooked with onion [15].

Hence, this work intends to quantify and qualitatively examine the phytoconstituents of B. bituminosa leaves and their antioxidant properties.

METHODS

Chemicals

Loba Chemie provided methanol, acetone, and hexane (India). 2,2- diphenyl-1-picrylhydrazyl and Trolox were purchased from Sigma-Aldrich (Germany).

Instrumentation

The antioxidant activity was assayed using a UV-visible spectrophotometer (Jenway,7315), England. A grinder (Uno, Moulinex model) was used for milling the dried plants. The samples were precisely weighed using a balance (Radw ag, AS220/c/2), Poland. Filter papers (Macherey-Nagel, MN617 and Whatman no.1) the USA.

Bituminosa leaf collection and preparation

The *B. bituminosa* leaves were harvested in Nablus during its flowering season in February 2018. (the north region of the West Bank). Dr. Nidal Jaradat, a pharmacognosist, conducted the plant taxonomical classification; specimens were placed in the Pharmacognosy Laboratory, Faculty of medicine and health Sciences, An-Najah National University, with a code Pharm-PCT-2084.

Serial exhaustive extraction

The leaves of *B. bituminosa* were carefully separated and cleaned with distilled water twice. The separated leaves were cleaned and dried in the shade at room temperature for three weeks to avoid cross-contamination and damage. Finally, the dried leaves were pulverized to a fine powder, which was then kept in canvas bags for use at a later time.

The serial exhaustive extraction method was used, which is a standard extraction method that is based on using successive solvents with increasing polarity, beginning with a non-polar solvent (hexane) and progressing to a more polar (water) solvent to ensure that a wide range of polarity of compounds can be extracted [16]. Approximately 25 g of dried plant materials were weighed, chopped into small pieces, and processed into powder with a mechanical grinder. After that, about 25 grams of plant powder were suspended in 50 ml n-hexane, which is a hydrophobic solvent that is relatively safe, largely unreactive, quickly evaporated, and cheap for two days at ambient temperature, with intermittent agitation in the shaker device at 100 rounds per minute, then using filter paper and a Buchner funnel, the plant extract was filtered, and the extracting solvent dried using rotary evaporator under high pressure at a temperature of no more than 35°C. Following the filtration procedure, The remnant plant material was extracted in acetone and processed in the same manner as the prior acetone extraction. The residual material was also extracted in methanol and processed the same way as prior extraction methods. The remaining plant material was extracted with water and dried in a freeze-drier apparatus similarly. Each organic solvent was dried in a rotary evaporator at a temperature of no more than

35°C under high pressure. All extracted materials were kept at 4°C for future use. [16, 17].

Qualitative tests

According to the standard analytical procedures described by Harborne and Trease et al., the *B. bituminosa* four solvents extracts were qualitatively screened to detect the presence of secondary and primary metabolic compounds, including tannins, terpenoids, steroids, saponins, phenols, cardiac glycosides, alkaloids, flavonoids, carbohydrates, protein, monosaccharide, reducing sugar and starch [18, 19].

The quantitative flavonoid, anthocyanin, and hydrolyzable tannin composition

The flavonoid content was determined using the Chang et al. technique. [20]. A spectrophotometer with a wavelength of 510nm was used to measure the absorption. The flavonoid content of each fraction was measured using the Rutin calibration curve and represented as milligrams (mg) of Rutin Equivalent per gram (g) (mg RUE/g extract).

Additionally, the anthocyanin concentration of B. bituminosa methanol, hexane, acetone, and aqueous extracts was measured by acidification of vanillin. A stock solution of 100 µg/ml was initially prepared for each extract and Catechin (as a standard reference compound). Next step, each extract, and Catechin were serially diluted with distilled water to get concentrations of (10, 30, 50, and 70 g/ml). Following that, a series of working solutions were made by adding 3ml vanillin solution (4 % w/v methanol) and 1.5ml HCL in concentrated form to 1ml of each extract's dilution; the working solutions were then incubated for15 mins at room temperature. Finally, the UV-Visible spectrophotometer was calibrated using distilled water as a blank solution, and Each working solution's absorbance at 500nm was measured.

Additionally, the hydrolyzable content of tannin of the four investigated fractions was measured by the Folin-Ciocalteu assay. Briefly, The analysis was performed using a methanolic solution with 1mg/ml for each extract fraction. The mixture was done by mixing 0.5 ml of methanolic plant fraction, 2.5 ml of 10% Folin-Ciocalteu's substance dissolved in water, and 2.5 ml of a 7.5% $NaHCO_3$. Afterward, the samples were maintained at 45 °C for 45 min in a thermostat, and a spectrophotometer was used to determine the absorbance at a wavelength of 765 nm. The same method was followed for the Gallic acid standard solution; then, different successive dilutions were used to blot the calibration line (10, 20, 30, 40, 50, 100 µg/ml). The concentration of Gallic Acid Equivalent was expressed in mg of GAE/g of each extract fraction based on measured absorbance.

Antioxidant potential

The DPPH radical approach was used to evaluate antioxidant activity; this approach is fast, simple, inexpensive, and not specific to any particular antioxidant compound; additionally, it may be applied to liquid or solid samples. [21]. About 10 g of each plant extract and Trolox standard material were weighed to prepare a stock solution of about 1mg/ml dissolved methanol. Each stock solution was serially diluted (2, 5, 10, 20, 50, 100 g/ml) to create the group of working solutions [21]. Freshly DPPH 0.002% w/v was prepared; in a 1:1:1 ratio, methanol was added to the DPPH solution and various concentrations of working solutions; the resultant solutions were then incubated for 30 minutes at room temperature and in the dark. The absorbance readings were taken at 517 nm with methanol as a blank, and the absorbance of the solution containing DPPH was first recorded with methanol only. The preparation and measurement methods were done three times for the plant extracts.

The following formula was used to calculate the inhibitory activity of DPPH by four plant extracts:

DPPH inhibitory activity =((*A0* - *A1*)/*A0*) X 100%)

A0 and A1 are the absorbance of the blank and the sample, respectively. The antioxidant activity is expressed as IC_{50} mg/ml, the extract dose needed to produce a 50% decrease in absorbance at 517 nm; thus, a lower value of IC_{50} means a higher antioxidant activity.

Statistical Analysis

For the four fractions of B. bituminosa leaves, the IC50 values for antioxidants and quantitative tests were determined in triplicate. The data were analyzed using multiple comparisons ANOVA and presented as means ± standard deviation.

RESULTS

Qualitative assessment

The tests identified the presence of different phytochemical classes in *B. bituminosa* leaves in four extracts from secondary and primary metabolites, as shown in Table (1).

Table (1): Phytochemical screening of four preparations of B. bituminosa leaves.

Tests	Hexane	Acetone	Methanol	Aqueous
Biuret test	-	-	+	+
Fehling test for reducing sugars.	-	-	-	+
Molisch test for complex polysaccharides.	-	-	+	+
Iodine test for starch	-	-	-	-
Ferric chloride test for Phenols	-	+	+	+
Gelatin test for Tannins	-	+	+	+
Shinoda test for Flavonoids	+	-	-	-
Foam test 'Saponin'	-	-	-	-
Legal's test 'Glycosides'	-	-	-	-
Salkowaski test 'Terpenoids'	+	-	-	-

Quantitative phytochemical tests

The quantitative hydrolyzable tannin concentration in four extracts of B. bituminosa leaves was determined using the Gallic acid calibration curve, as shown in **Figure (1)**. The following equation calculated the hydrolyzable tannin content in each extract:

$$Y = 0.0098x + 0.0215, R^2 = 0.9929$$

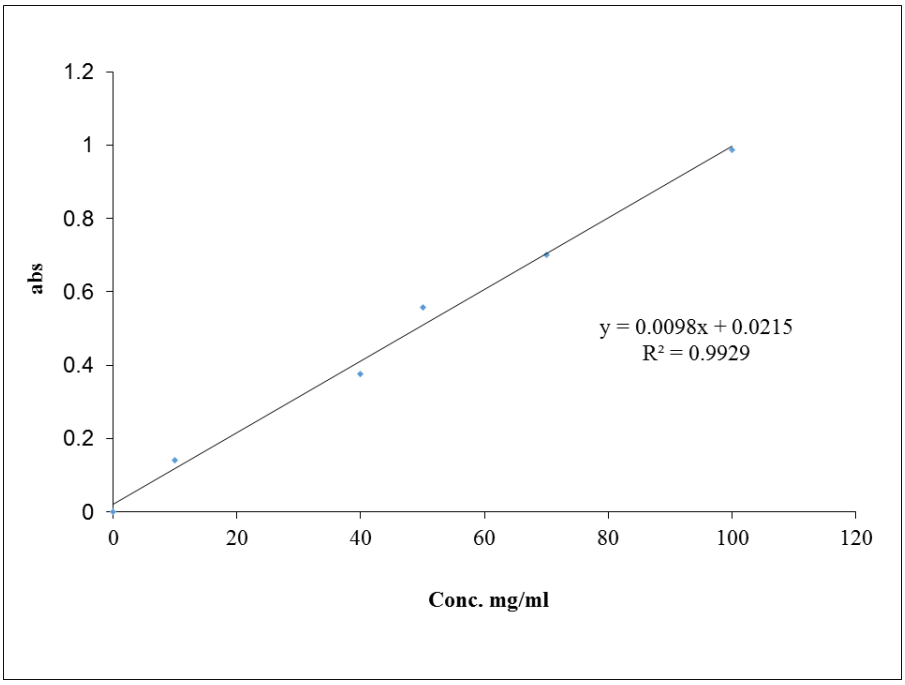

Figure (1). Gallic acid standard calibration curve.

The formula was used to calculate the total flavonoid using the standard Rutin calibration curve presented in **Figure (2)**: y = 0.0032x + 0.0086, R^2 = 0.994.

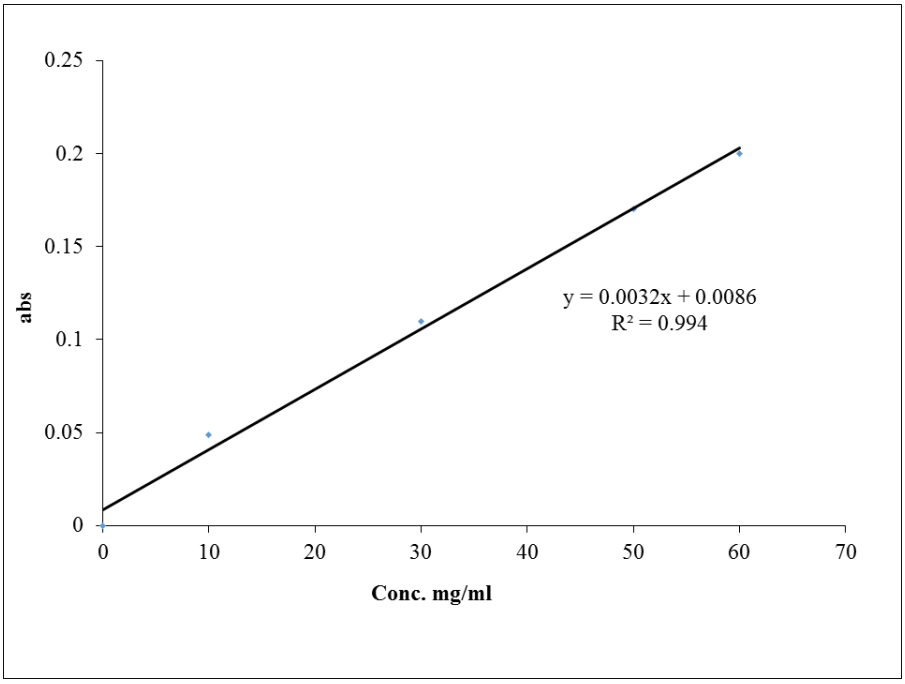

Figure (2): Standard calibration curve of Rutin.

Furthermore, the following equation was used to calculate the total anthocyanin content of each extract based on the Catechin standard calibration curve shown in **Figure (3)**: y = 0.0011x + 0.0023, R^2=0.991. Y- is the absorbance at 500nm and X represents the total anthocyanin contents.

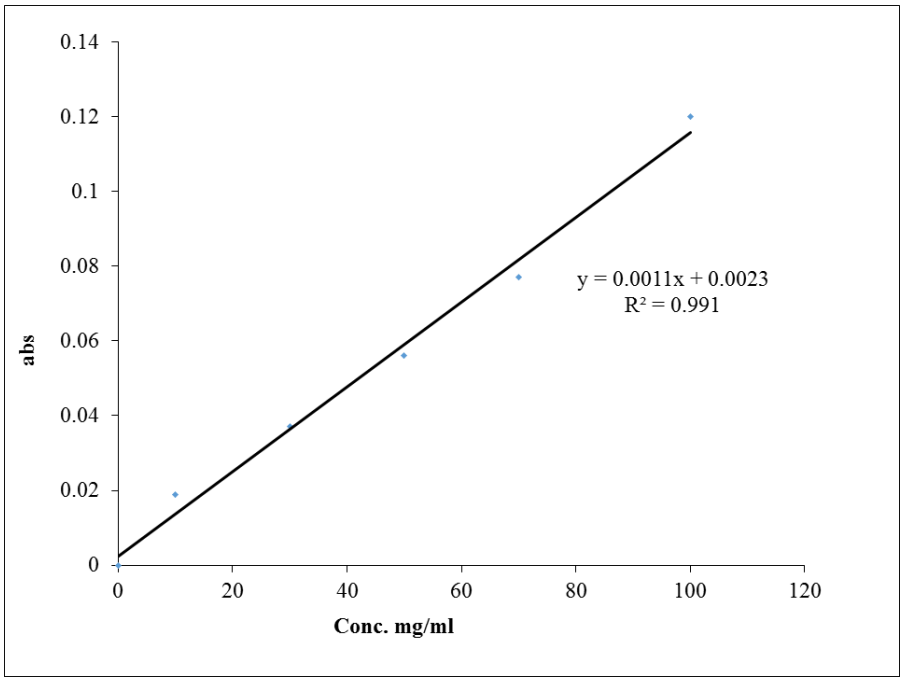

Figure (3): Calibration curve of Catechin.

Table (2) summarizes the quantitative hydrolyzable flavonoids, tannins, and anthocyanin contents of four *B. bituminosa* leaf extracts.

Table (2): Quantitative hydrolyzable tannins, anthocyanin, and flavonoid contents of *B. bituminosa* leaf four extracts.

Plant extracts	Total flavonoids content, RUE mg /g of extract (dry), ±SD	Total hydrolyzable tannin content, GAE mg /g of extract (dry) t ±SD	Total Tannin contents, CAE mg /g of extract (dry), ±SD
Hexane extract	103.95±4.7	-	-
Acetone extract	-	84.33±1.56	17.5±0.7
Methanol extract	-	33.22±1.56	2.21±0.014
Aqueous extract	-	64.32±0.78	2.21±0.014

Antioxidant potential

The antioxidant activity was determined using the DPPH test with Trolox. Trolox is a vitamin E analog having antioxidant activity, serving as a positive control. The DPPH inhibitory activity by *B. bituminosa* leaf aqueous, acetone, hexane, and methanol extracts are shown in **Figure (4),** and the calculated IC_{50} values are shown in table 3.

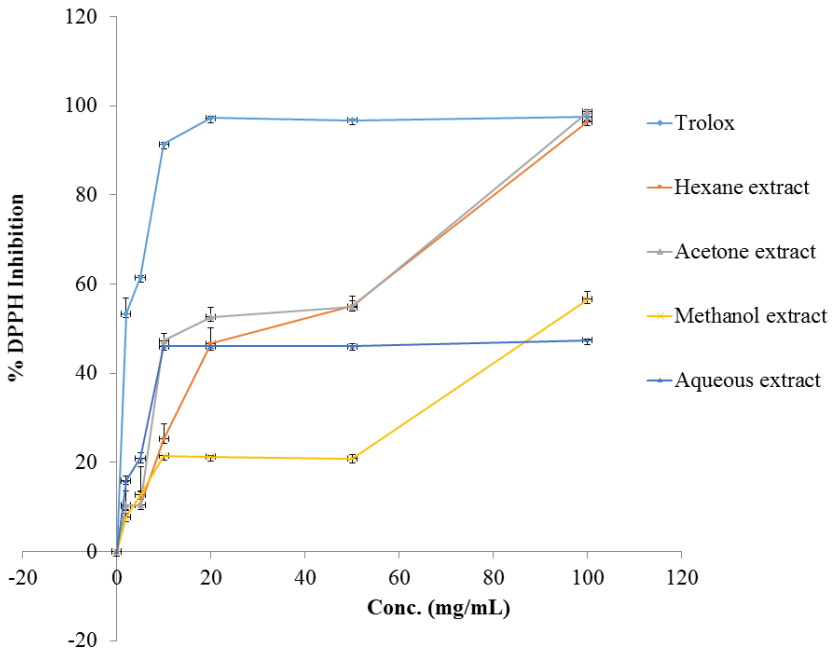

Figure (4): *B. bituminosa* leaf four extracts and Trolox *in-vitro* DPPH inhibitory activities.

Table (3): *In-vitro* antioxidant activity, IC_{50} values, and DPPH inhibitory effects of *B. bituminosa* leaf four extracts.

Conc.	Trolox, ±SD	Hexane, ±SD	Acetone, ±SD	Methanol, ±SD	Aqueous, ±SD
0	0±0	0±0	0±0	0±0	0±0
2	53.43±3.46	10.28±5.3	10.4±3.1	7.79±0.72	15.99±0.86
5	61.51±0.34	10.36±8.7	10.4±3.1	12.73±0.67	20.94±1.3
10	91.41±0.34	25.24±3.4	47.31±1.6	21.48±0.04	46.09±0.45
20	97.29±0.34	46.68±3.5	52.63±2.1	21.29±0.31	46.09±0.45
50	96.8±0.34	55.05±2.2	54.87±1.4	20.9±0.85	46.09±0.45
100	97.54±0.69	96.51±0.32	98.62±0.53	56.66±1.68	47.43±0.17
Antioxidant activity IC_{50} value, (µg/ml)	3.31±0.92	21.87±3.9	17.37±1.97	234.4±0.71	56.23±0.61

DISCUSSION

Herbal treatments have been used for the treatment of numerous illnesses since antiquity. Polyphenols are secondary metabolic chemicals in plants that have significant morphological and physiological significance. The secondary metabolites of polyphenols, such as anthocyanins, tannins, phenolic acids, and flavonoids, have various chemical structures and can protect against various diseases, including oxidative stress and cancer. Furthermore, several studies have shown that herbal products have possible antioxidant capabilities, which can help reduce the risk of various chronic diseases.

The *B. bituminosa* phytochemical screening revealed the presence of different bioactive classes, including flavonoids in the hexane extract, phenols and tannins in the methanol, acetone, and aqueous extracts. The quantitative tests revealed that the hexane extract has a high total flavonoids content (103.95 ± 4.7 mg of RU/g of extract (dry)), while the acetone extract has the highest total hydrolyzable tannin and anthocyanin contents with values of 84.33 ± 1.56

mg of GAE/g of extract (dry) and 17.5 ± 0.7 mg of CAE/g of extract (dry), respectively.

The acetone extract has the highest contents of hydrolyzable anthocyanins and tannins contents also have the highest antioxidant activity of IC_{50} value 17.37 ± 1.97 µg/ml, followed by the hexane extract, which has a high content of flavonoids and has the antioxidant potential of 21.87 ± 3.9 µg/ml. Moreover, the aqueous extract did not contain flavonoids; while it contains a considerable amount of hydrolyzable tannin and a low amount of anthocyanins, this extract has moderate antioxidant potential with an IC_{50} value of 56.23 ± 0.61 µg/ml. In addition, the methanol extract did not contain flavonoids and had low contents of hydrolyzable tannins and anthocyanins. This extract has a weak antioxidant activity of IC_{50} values of 234.4 ± 0.71 µg/ml.

More research is needed to see whether B. bituminosa hexane extract is toxic to living cells, as well as to see what effect it has in vivo using a mouse model and whether it can affect or influence physiological processes. It will also be interesting to see how this extract affects gene expression and the activity of proteins and enzymes in cell oxidation processes. Furthermore, more research is required to determine the exact mechanism by which this extract functions as an antioxidant and the downstream targets engaged in this pathway.

CONCLUSION

The study's findings could develop a new class of free radical inhibitors that selectively decrease oxidative stress. According to the quantitative tests, the hexane extract has the highest total flavonoid content, while the acetone extract has the highest total hydrolyzable tannin and anthocyanin content. Moreover, the acetone extract has the highest contents of hydrolyzable tannins, and anthocyanins have the highest antioxidant activity. B. bituminosa could serve as a beginning point for developing more effective/ideal therapeutic candidates, or it could open opportunities for developing a potential scaffold for a new class of natural antioxidant medications.

Ethics approval and consent to participate

Not applicable

Consent for publication

The authors give the Publisher the Author's permission to publish the work.

Availability of data and materials

The corresponding author's data supporting this study's findings are available upon reasonable request.

Author's contribution

Malik Alqub: conceptualization, writing-original draft, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, validation, visualization, and writing review & editing. **Nidal Jaradat**: formal analysis, investigation, methodology, resources, software, validation, visualization, and writing review & editing.

Competing interest

The authors declare that they have no competing interests

FUNDING

Not applicable

ACKNOWLEDGMENTS

The authors would like to knowledge An-Najah National University

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