

**Research Article**

Open Access, Volume 3

**Evaluation of phytochemical constituents and antimicrobial activities of ethyl acetate and butanol fractions of *Periploca aphylla* Decne****Umbreen Rashid<sup>1,2,3\*</sup>; Muhammad Rashid Khan<sup>1</sup>; Jasia Bokhari<sup>1</sup>; Shumaila Jan<sup>1</sup>**<sup>1</sup>Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.<sup>2</sup>Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.<sup>3</sup>Department of Life Sciences, Abasyn University, Islamabad, Pakistan.**\*Corresponding Author: Umbreen Rashid**

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Received: Jun 14, 2022

Accepted: Jul 12, 2022

Published: Jul 19, 2022

Archived: www.jcimcr.org

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DOI: www.doi.org/10.52768/2766-7820/1956

**Abstract**

**Introduction:** Plants have been utilized for the cure of different diseases since ancient times. Medicinal importance of the plants is due to presence of phytochemicals having specific physiological action on the human body. A wide variety of activities have been found in these phytochemicals which might help in preventing chronic disorders. *Periploca aphylla* Decne belongs to the family Asclepiadoideae, is traditionally used for the cure of cerebral fever and as stomachic.

**Objectives:** In this study, phytochemical constituents and antimicrobial potentials of ethyl acetate and butanol fractions of crude methanolic extract of *P. aphylla* were investigated.

**Methodology:** Qualitative determination of Phytochemical constituents (tannins, saponins, flavonoids, cardiac glycosides, terpenoids, coumarins, phlobatanins and anthraquinones) in the extracts was performed using standard procedures. Antibacterial and antifungal activities were determined using agar well diffusion method and tube dilution method respectively.

**Results:** The phytochemical analysis of ethyl acetate and butanol fractions of *P. aphylla* crude methanol extract confirmed the existence of tannins, saponins, alkaloids, flavonoids, cardiac glycosides and terpenoids. Ethyl acetate fraction did not show any activity against the tested bacterial strains except *K. pneumonia*. Whereas butanol fraction was active against all the strains. Ethyl acetate fraction showed inhibition against *A. fumigates* and *F. solani* while did not show any inhibition against *M. pyriformis*. The n-butanol fraction showed inhibition against all the tested fungal strains.

**Conclusion:** It is evident from the above results that *P. aphylla* is a rich reservoir of phytochemical compounds which contributes to its various ethnomedicinal uses.

**Keywords:** *Periploca aphylla*; Phytochemicals; Ethyl acetate; Butanol; Antimicrobial.

**Citation:** Rashid U, Khan MR, Bokhari J, Jan S. Evaluation of phytochemical constituents and antimicrobial activities of ethyl acetate and butanol fractions of *Periploca aphylla* Decne. J Clin Images Med Case Rep. 2022; 3(7): 1956.

## Introduction

Medicinal plants have been the source of medicine to all civilizations since long. Plants are selected for pharmacological analysis using one of the frequent methods i.e. ethnobotany [1]. Traditional medicine is employed as a major health care system in many developing countries [2,3]. The interest in maintaining personal health and safety by using medicinal plants is increasing due to bioprospecting of novel plant-derived drugs along with rising costs of prescribed medicines [4]. The large variety of bioactive molecules produced by plants, are mostly evolved as chemical defense against infection or predation, make them a rich source of medicine. About 74% of 119 pharmaceutical or biotechnology drugs derived from plants are used in modern medicine corresponding to their traditional uses [5,6]. Hence, ethnomedical data provides a better chance to find active plants rather than random approach [7,8].

*Periploca aphylla* Decne. is a medicinally important plant. It belongs to the family Asclepiadoideae having 348 genera, with about 2,900 species. They are primarily located in the tropics to subtropics, particularly in Africa and South America. It is commonly found in the northern parts of Pakistan and has a variety of medicinal uses in the indigenous system of medicine [9].

It is locally named as “Bata”. The milky juice of *P. aphylla* is externally applied to swellings and tumors. It is also used for the treatment of cerebral fever and as stomachic [10]. Latex from its stem is used as chewing gum. The flowers are used as vegetables. Sometimes, they have been used for emetic purposes, expectorant, laxative, diuretic and for wart removal. There are a number of known triterpenes and steroids which have been isolated from this species [11].

There are several chemical substances which produce a definite physiological effect on human body and hence are responsible for the medicinal importance of plants. The most significant of these plant bioactive constituents includes phenolic acids, flavonoids, terpenoids, lignins, tannins, stilbenes, coumarins, alkaloids, quinones, betalains, amines, vitamins and other metabolites having antioxidant potential [12,13].

There has been a universal trend in recent times towards using natural phytochemicals found in teas, berry crops, herbs, vegetables, fruits and beans [14-16]. Moreover, natural antioxidant ingestion reduces the risks of cardiovascular disorders, cancer, diabetes and age related diseases [17,18]. The nature of drugs can be predicted by the preliminary phytochemical screening. It also helps to detect different constituents present in different polarity solvent.

The evaluation of medicinal plants on a large scale for different biological activities is the first essential step to isolate and characterize the active principles which will lead to drug development. Antimicrobial research is required for the discovery and growth of new therapeutic antimicrobial agents. This effort is encouraged due to lack of good antifungal agents and the ongoing trouble of development of resistance to existing antibacterial agents towards novelty [19]. In the past years, numerous reports are presented on the antibacterial activity of plant extracts against human pathogenic bacteria which showed that plants were significant source of potentially valuable structures to develop novel chemotherapeutic agents [20-27].

One of the important alternative approaches to manage diseases and control antibiotic resistance is the isolation of antibacterial active principles from higher plants. The drugs derived from plants are believed to cause less or no side effects in comparison to synthetic antibiotics [28]. In vitro studies have revealed a great deal of phytochemicals which belongs to different chemical classes to have inhibitory effects on all types of microorganisms [29].



**Figure 1:** *Periploca aphylla* in its natural habitat.

## Materials and methods

### Plant material

Plant samples of *Periploca aphylla* were collected in April-May 2010 from Margalla Hills Islamabad, Pakistan. Further identified by Dr. Saleem Ahmad and deposited the voucher specimen at Herbarium of Pakistan Museum of Natural History, Islamabad (Voucher No. 069721). Whole plants were collected and shade dried at temperatures range of 21-30°C. Resulting dried samples were powdered using a blender and kept at room temperature in polythene bags. The extraction was carried out according to the following procedure.

### Preparation of extract

**Methanol extract:** Dried powdered samples of *P. aphylla* (1.5 kg) were soaked in 3 litres of methanol (95%) for one week (3 times) at room temperature with random shaking and stirring. The filtration of extracts was carried out by using Whatman filter paper No. 42 (125 mm). After combining the resulting filtrates, they were concentrated at 40°C in a rotary vacuum evaporator to get a solid, gummy mass (PAME). The extract was stored at -4°C in airtight vials till further use.

**Preparation of fractions:** The methanol extract was partitioned using a liquid-liquid extraction technique and the resultant partitions i.e. ethyl acetate and n-butanol fractions were concentrated by means of a vacuum evaporator and were stored at -4°C in airtight vials till further analysis.

### Determination of phytochemical constituents

The extracts were evaluated for the occurrence of flavonoids, tannins, saponins, phlobatannins, cardiac glycosides, alkaloids, terpenoids and anthraquinone using simple qualitative methods of Harborne [30], Trease and Evans [31] and Sofowora [32].

**Salkowski test for terpenoids:** To a 5 ml of extract/fraction; 2 ml of  $\text{CHCl}_3$  and 3 ml of  $\text{H}_2\text{SO}_4$  (concentrated) were added. Terpenoid constituents were confirmed by the formation of a reddish brown colored interface.

**Test for alkaloids:** An amount of 0.1 g of extract and each fraction was warmed with 4 ml of 1% HCl followed by filtration. Titration of the filtrates (2 ml) was done individually with (a) Dragendroff's reagent and (b) Mayer's reagent. The presence of alkaloids was indicated by yellow or cream precipitate formation.

**Test for saponin:** About 0.1 g of the fraction was mixed with distilled water (10 ml) and then filtered after boiling in a water bath. An aliquot of 2.5 ml of distilled water was added to 5 ml of the filtrate and shake it vigorously. Emulsion was formed after mixing the froth with olive oil (3 drops) and shaken strenuously.

**Test for phlobatannins:** Boiling of 80 mg of crude extract and each fraction was carried out in 1% aqueous hydrochloric acid in a small tube. Formation of red precipitates showed the presence of phlobatannins.

**Keller- Kiliani test (for deoxy sugars in cardiac glycosides):** From the crude extract and each fraction, 5 ml of aqueous extract (10 mg/ml) was taken. Then 2 ml glacial acetic acid, 1 drop of FeCl<sub>3</sub> solution and 1 ml concentrated H<sub>2</sub>SO<sub>4</sub> was added to it. Brown ring was formed at interphase indicating the existence of cardiac glycosides.

**Test for coumarins:** About 0.1 g of extract and each fraction was taken in a test tube. Moistening of Filter paper was done with 1 N NaOH solution and it was used to cover the mouth of the tube. Test tube was placed in boiling water for few minutes. Filter paper was removed to examine under UV light. Yellow fluorescence indicates the presence of coumarins.

**Test for anthraquinones:** Briefly, 0.1 g of extract and each fraction was boiled in 5 ml of 1% HCl followed by filtration. To the filtrate 5 ml of benzene was added and 10 % NH<sub>4</sub>OH was added in the benzene layer after shaking. The colour in the alkaline phase was observed. Violet or red colour formation indicated the existence of anthraquinones.

**Test for tannins:** About 0.1 g of the extract and each fraction was mixed with five ml of H<sub>2</sub>O and filtered. Formation of blue black or brownish green color after treating filtrate with a 0.1% ferric chloride (few drops) indicated the presence of tannins.

**Test for flavonoids:** A portion of the aqueous filtrate of extract and each fraction was mixed with 5 ml of dilute NH<sub>3</sub> solution and add concentrated H<sub>2</sub>SO<sub>4</sub>. A yellow coloration is observed if flavonoid compounds are present.

### Biological assays

#### Anti-bacterial assay

**Preparation of bacterial inoculums:** Four isolated colonies were inoculated in the 30 ml nutrient broth and incubated for 24 h at 37°C so that the growth in the broth was equivalent with McFarland standard (0.5%).

**Antibacterial activity:** Antibacterial potential of crude extract derived fractions was investigated by agar well diffusion method [33] using nutrient agar medium. Exactly two grams of nutrient agar was dissolved in 100 ml of distilled water (pH 7.0) and was autoclaved. It was cooled down to 45°C. Then 100 ml of this media was seeded with 1 ml of inoculum having size of 10<sup>6</sup> CFU/ml as per McFarland standard and after proper homogenization 75 ml was poured into the petri plate of 14 cm diameter. For agar well diffusion method, eleven wells per plate were made by using a sterile cup-borer (8 mm). Extract and

fractions at concentration of 25, 15, 12.5, 10, 7.5, 5, 3, 2 and 1 mg/ml were prepared in Dimethyl Sulfoxide (DMSO). The test compounds (100 µl) were poured into the wells and petri plates were then incubated at 37°C for 24 hours. Simultaneously, roxythromycin and cefixime were used as positive controls each having the concentration of 0.5 mg/ml. DMSO was used as a negative control as well as the dilution medium for the positive controls. The lowest concentration inhibiting growth was taken as the Minimum Inhibitory Concentration (MIC).

#### Antifungal activity

Agar tube dilution method was used to evaluate antifungal activity [34]. The stock solution of extract and positive control; terbinafine were prepared at a concentration of 12 mg/ml by dissolving them in DMSO. As a negative control DMSO was used. 6.5 g of sabouraud dextrose agar was dissolved in 100 ml of distilled water (pH 5.6) to make media for fungus. Then 4 ml of it was added into screw cap tubes. These tubes were autoclaved for 15 min at 120°C and cooled down to 45°C. The stock solution (66.6 µl) was mixed with media to get the final concentration of 200 µg/ml of sabouraud dextrose agar. Then the tubes were solidified in the slanted position at 25°C. An agar surface streak was used by inoculating each tube with a piece of inoculum (4 mm diameter) taken from a seven days old culture of fungi. After seven days of incubation at 28 ± 1°C, visual observation of fungal growth inhibition was made. The % inhibition of growth was calculated with reference to the negative control.

### Results

#### Qualitative studies of phytochemicals

Qualitative phytochemical screening was carried out on all the considered fractions of *P. aphylla* to identify the phytochemical classes, i.e. tannins, saponins, flavonoids, cardiac glycosides, terpenoids, coumarins, phlobatanins and anthraquinones.

**Qualitative screening of *P. aphylla*:** The phytochemical analysis of ethyl acetate and butanol fractions of *P. aphylla* crude methanol extract confirmed the existence of tannins, saponins, alkaloids, flavonoids, cardiac glycosides and terpenoids. However coumarins, phlobatanins and anthraquinones were absent in both fractions (Table 1).

**Table 1:** Phytochemical constituents of fractions of *P. aphylla* methanol extract.

Phytochemicals	Extract/Fractions	
	PAEE	PABE
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Alkaloids	+	+
Cardiac glycosides	+	+
Terpenoids	+	+
Phlobatannins	-	-
Coumarins	-	-
Anthraquinone	-	-

PAEE: *P. aphylla* ethyl acetate fraction;  
PABE: *P. aphylla* n-butanol fraction.

## Anti-bacterial activity

The antibacterial potential of ethyl acetate and butanol extracts of *P. aphylla* were tested against five strains of bacterial test organisms. The antibacterial activity of the extracts and their potency were quantitatively assessed by determining Minimum Inhibitory Concentration (MIC) values. The extracts were tested at various concentrations (1-25 mg/ml), and the evaluated MIC values are reported in Table 2.

**MIC value of *P. aphylla*:** Ethyl acetate fraction did not show any inhibition against *M. luteus*. n-butanol fraction exhibited the activity against *M. luteus* with MIC values of 15 mg/ml. Both the fractions showed activity against *K. pneumoniae* as follows: n-butanol (MIC=5 mg/ml) and ethyl acetate (MIC=12.5 mg/ml). Only the n-butanol fraction was active (MIC=3 mg/ml) against the *B. bronchiseptica* and *E. aerogenes* (MIC=7 mg/ml). Ethyl acetate fraction did not show any activity against *P. aeruginosa* as well while n-butanol fraction showed MIC value of 15 mg/ml (Table 2).

## Anti-fungal activity

Ethyl acetate and butanol fractions of *P. aphylla* were screened in vitro for their antifungal activity against three strains. The growth inhibition was measured and presented in Table 3.

**Anti-fungal activity of *P. Aphylla*:** Inhibition in growth of *A. fumigates* was recorded for n-butanol (21.05%) and ethyl acetate (16.66%).

The n-butanol fraction showed 50% inhibition against *F. solani*, while the ethyl acetate showed 18.75% inhibition. Low activity was shown by n-butanol (6.25%) against *M. pyriformis* whereas ethyl acetate fraction was unable to produce any inhibition in growth (Table 3).

**Table 2:** The MIC values of fractions of *P. aphylla* methanol extract.

Bacterial strains	Minimum inhibitory concentration (mg/ml)			
	PAEE	PABE	Rox	Cef
<i>M. luteus</i>	-	15	0.06	0.05
<i>K. pneumoniae</i>	12.5	5	0.17	0.13
<i>B. bronchiseptica</i>	-	3	0.14	0.16
<i>E. aerogenes</i>	-	7	0.16	0.15
<i>P. aeruginosa</i>	-	15	0.14	0.12

-: not active; PAEE: *P. aphylla* ethyl acetate fraction; PABE: *P. aphylla* n-butanol fraction; Rox: Roxithromycin; Cef: Cefixime.

**Table 3:** Antifungal activity of fractions of *P. aphylla* methanol extract.

Fungal Strains	Percent inhibition (%)			
	Negative	Positive	PAEE	PABE
<i>Aspergillus fumigates</i>	0	100	16.66	21.05
<i>Fusarium solani</i>	0	100	18.75	50
<i>Mucor pyriformis</i>	0	100	0	6.25

PAEE: *P. aphylla* ethyl acetate fraction; PABE: *P. aphylla* n-butanol fraction

## Discussion

Plants have been utilized for the cure of different diseases since ancient times. The medicinal uses of plants growing throughout the world lie in the active constituents having direct action in the body. They are used in conventional as well as herbal medicine. Hence mankind is provided with a valuable gift by nature in the form of herbal drugs [35]. Medicinal importance of the plants is due to presence of phytochemicals having specific physiological action on the human body. A wide variety of activities have been found in these phytochemicals which might help in preventing chronic disorders. Alkaloids have been used for medicinal purposes since long [36]. Besides cytotoxicity, they have been reported to have antispasmodic, analgesic [30,37] and antibacterial properties [38,39]. The phytochemical analysis of *P. aphylla* has also revealed to contain saponins. Saponins are major ingredients in Chinese traditional medicine [40]. They have been acknowledged to inhibit inflammation, have antibiotic potential and defend against hypercholesterolemia [41]. Saponins can coagulate and precipitate red blood cells and are involved in central nervous system activities. Moreover, they can form foams in aqueous solutions and possess hemolytic, bitterness and cholesterol binding properties [39,42]. Thus these characteristics of saponins and their presence in the plant extracts tend to validate the use of *P. aphylla* in traditional medicine. Several triterpenoids isolated from plants displayed good analgesic and anti-inflammatory properties [43,44]. Some of the pentacyclic triterpenoids are used in medicine because of their broad range of biological activities including cytotoxic, anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, analgesic, anti-allergic and anti-cancer [45]. Tannins have been used to treat ulcerated or inflamed tissues and they have significant activity in the prevention of cancer [46,47]. Tannins interfere with protein synthesis and bind to proline rich protein. Thus phytotherapeutically, the plants containing tannins are used to treat throat and mouth inflammations, skin injuries and non-specific diarrhea [48]. Since tannins can precipitate proteins, they protect the underlying layers by acting as waterproof to the external layers of the exposed tissues. Moreover, tannins are also known to possess different physiological effects such as antisecretolytic, anti-irritant, antiphlogistic, antiparasitic and antimicrobial activities [49]. One of the ubiquitous and leading groups of plant metabolites are phenolic compounds [50]. The biological properties of phenolic compounds include antiatherosclerosis, antiinflammation, endothelial function improvement, anticancer, antiapoptosis, cardiovascular protection, cell proliferation and angiogenesis inhibition activities [51]. The medicinal plants rich in phenolic compounds have been reported to have antioxidant properties in many studies [52]. The natural antioxidants derived from the plants are mostly in the form of phenolic compounds for instance phenolic acids, flavonoid, tocopherols [53]. Phenols have been useful in the preparation of cresol and dettol which are antimicrobial compounds. The antioxidative properties and use of *P. aphylla* in different diseases are due to the presence of these phenolic compounds. Flavonoids produced by plants in reaction to microbial infection are hydroxylated phenolic compounds and they have shown antimicrobial activity against broad range of microorganisms in in vitro studies. This activity is might be due to their ability to form complexes with bacterial cell wall and extracellular soluble proteins [54]. Flavonoids show anticancer activities and are effective antioxidants [55]. Flavonoids have been found to act by effecting membrane permeability and inhibiting membrane-bound enzymes like phospholipase A2 and ATPase [56], and the

antioxidant action mechanism of *P.aphylla* may be explained by this property. Moreover, flavonoids as a result of their anion radicals serve as health promoting compounds [57]. The results found in our study imply that these phytochemical compounds might be the bioactive constituents in *P. aphylla* thus making these plants an important reservoir of medicinally important bioactive compounds. The plants provide potentially valuable structures in order to develop novel chemotherapeutic agents. In order to achieve this target, *in vitro* antibacterial assay is a first step [20]. A number of reports are accessible during the past years on the antibacterial potential of plant extracts against human pathogenic bacteria [21-27]. The synthetic antimicrobials are frequently coupled with side effects, whereas the plant derived antimicrobials having vast therapeutic potential can serve the rationale with less adverse effects [58]. Therefore, there is a requirement to constantly explore plant derived antimicrobials. More research is required to identify and resolve the full scale efficiency of antibacterial compounds from these plants. According to our results, the plant extracts were active against Gram negative as well as Gram positive bacteria. This might be an indication to the presence of wide range antibiotic compounds or general metabolic toxins, in addition to the pharmacological active metabolites such as flavonoid glycosides [59], spirostanol and furostanol saponins [60], amides and phytosterols [61]. Antimicrobial potential of the plants is perhaps due to high percentage of phenolics as both are always linked together [62]. It can also be due to the presence of different phytochemical constituents for example flavonoids, terpenoids, alkaloids, saponins and tannins in the plant extracts. The toxicity of saponins to parasite worms (anthelmintic activity), insects (insecticidal activity), fish (piscidal activity), molluscs (molluscicidal), their antibacterial, antifungal and antiviral activities are well known [63,64]. The membranolytic properties of saponins are involved in their antibacterial effects instead of simply changing the surface tension of extracellular medium, therefore get affected by population density of microbes [65]. Flavonoids, and tannins present in the extract have been associated with antimicrobial effects in various studies using plant extracts [66]. Plant derived alkaloids are usually found to have antimicrobial potential [67]. Alkaloids may be helpful against AIDS associated intestinal infections [68] as well as HIV infection [69]. Persistent opportunistic fungal infections have turned out to be a main factor for mortality and morbidity in immunocompromised patients [70]. The common fungal infections are mostly caused by the *Candida* and *Aspergillus* species. Recent trends in epidemiology have pointed out a shift towards infection caused by *Aspergillus* and non-albicans *Candida* [71].

### Conclusion

The range of antimicrobial activities showed by these extracts could perhaps be explained by the presence of flavonoids, tannins, alkaloids and saponins. The microbes may inhibit more potently by purified components. Our results signify the potential of *P. aphylla* as medicinal agent's sources, which may give leads in the constant exploration for antimicrobial agents from plants. Further studies on the purification of bioactive components and phytoconstituents can disclose the precise potential of the plant to restrain numerous pathogenic microorganisms. Thus, antimicrobial activity displayed by the extracts against different bacterial and fungal strains that are linked with different infectious diseases, may provide scientific validation for the ethnomedicinal uses of these plants.

### Declarations

**Conflict of interest:** The authors declare no conflict of interest.

**Funding:** This research work is funded by Higher Education Commission of Pakistan (Indigenous Scholarship, Batch IV).

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