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Original Article

Phytochemical analysis and antibacterial activity of organic extract of *Catharanthus Roseus* L. flower against gram-positive and gram-negative bacteria

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A B S T R A C T

Plant extracts especially medicinal plants used as alternative treatment due to minimal side effect and danger to human health. The present study focuses on the phytochemical analysis and antimicrobial properties of medicinally recognized known plant of *Catharanthus roseus*. In this research, the bioactive secondary metabolites and antimicrobial activities of organic extracts of *C. roseus* flowers parts were investigated. The phytochemical screening of both ethanol and ethyl acetate plant extracts revealed the presence of various secondary metabolites such as alkaloids, flavonoids, protein, phenol, saponins and other phytochemicals. However, agar well diffusion method was used for antimicrobial testing of organic extracts against multidrug resistant (MDR) food-borne Gram-positive (*Staphylococcus hominis*) and Gram-negative (*Citrobacter freundii*, *Escherichia coli* and *Aeromonas caviae*) bacteria. The study revealed that the flower extracts of *C. roseus* showed highest (15.5± 0.50 mm) antibacterial activity against *Citrobacter freundii* BTGE-K_4 but the lowest (5.67± 0.58 mm) antibacterial activity against *S hominis* BTGE-K_11. Moreover, the lowest minimum inhibitory concentration (MIC) value was 125 µg/mL which showed against the tested MDR bacteria. Moreover, this study supports the importance of using medicinal plants as an alternative source for the treatment of bacterial diseases and other pharmaceutical purposes such as preservatives due to minor side effects, cost effectiveness and development of resistance to antibiotics.

Introduction

Medicinal plants containing therapeutic properties of its parts or whole body which can be used for synthesis of useful drugs. The secondary metabolites of medicinal plants vary significantly in their quality and quantity in different plant parts (Vijayalakshmi *et al.* 2014). From ancient period, Bangladesh has known to be rich repository of medicinal plants. Many plants with great medicinal value are born here and there around us because of our soil fertility (Bangladesh Department of Environment, 2015). But the pharmacological actions of most of the plants are not properly explored or analyzed in depth (Bardhan *et al.*, 2018). Being a part of developing country like Bangladesh, the proper exploration of pharmacological potential of these plants can play a vital role in the field of economy and medicine (Bangladesh Department of Environment, 2015; Bardhan *et al*., 2018).

According to WHO (World Health Organization), a recent estimation shows that 80% worldwide people rely on herbal medicines for some aspect of their primary health care needs (World Health Organization, 2019). Besides this, the estimated around 21,000 plant species can be used as complementary or traditional medicinal plants. Traditional herbal medicines are getting significant attention in global health debates. In China, traditional herbal medicine played a significant role in treat severe acute respiratory syndrome (SARS) (Chinese, 2004) and COVID-19 (Yang *et al.*, 2020; Luo *et al*. 2020). The developed countries such as United States of America (USA), plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as India and China, the contribution is as much as 80% (Zamiska, 2006). In this perspective, even Bangladeshi medicinal plants can contribute greatly to both medicinal and economic purposes.

Catharanthus roseus, is a species of flowering plant in the family Apocyanaceae (Padmaa Paarakh *et al.,* 2019) which produce more than 130 medicinally important and antibacterial terpenoid indole alkaloids (TIAs). Among them, vinblastine and vincristine are commercial TIAs and they have strong antibacterial properties (Pan *et al.,* 2016). *C. roseus* has been used traditionally in folk medication to cure diarrhoea, blood pressure and diabetes. These plants were selected on the basis of folk medicine. Thus the therapeutic medicinal plant is of great interest of potential alternative treatment against resistant bacteria (dos Santos *et al*. 2015). In modern medicine, chemotherapeutic agents and alkaloids from *C. roseus* are known for their anti-cancer and analgesic properties (Arora *et al.,* 2010). Several studies show that *C. roseus* is one of the powerful medicinal plants with various essential biochemicals.Various scientific studies reported that they posses the antidiabetic(Tiong *et al.,* 2013); antihypertensive (Ara, Rashid, and Amran 2009), antifungal (Balaabirami and Patharajan, 2012), anticancer,cytotoxic and wound healing activity (Puspita *et al.,* 2013). Moreover, there are commonly two varieties of *C. roseus* on the basis of flower colour such as pink (flowered rosea) and white (flowered alba) (Kumar *et al.,* 2012). However, medicinal plants are being used widely for management of diseases all over the world; more than 50% of modern clinical drugs have originated from plant or plant based products (Thakur, Singh, and Jain 2012). Over the past two decades, the use of herbal medicine has increased tremendously because of its low cost and side effects, however, the lack of adequate research data still remains in this area. So, the present investigation was carried out for the evaluation of phytochemical screening and antibacterial activity testing of *C. roseus* flowers extract to explore more insight into this medicinally important plant.

Materials and Methods

Plant sample collection and processing

The fresh and mature flowers of *C. roseus* were collected from various locations in Kushtia Sadar and campus area of Islamic University, Bangladesh. The flowers were washed carefully 2-3 times with running tap water and finally washed with sterile water for removing dust and surface microbes. Then the flowers were dried about 20-30 days under the shading place for collecting flowers extracts. The dried flowers were crushed to become fine powder and kept separately in airtight polyethylene zipper bags for further use in experimental purpose Figure 1. (A-D).

Solvent extraction

The extraction of samples were prepared by soaking 5 g of dried flower powder in 50 mL of each solvent such as ethanol, ethyl acetate, chloroform, and petroleum ether and kept them overnight in a rotary shaker. The solution was left for 72 hours at room temperature and then it was filtered with the help of sterilized Whatsman No.1 (5mm diameter) filter paper. The filtrate solution samples were concentrated using water bath and stored at 4°C for further use.

Phytochemical analysis

In order to phytochemical analysis, we prepared three kinds of reagent solution such as Mayer's reagent: it is used for the detection of alkaloids. Solution (A) was made by dissolving 0.68g mercuric chloride in 30 mL of distilled water. Solution (B) was made by dissolving 2.5 g of potassium iodide in 10 mL of distilled water. Solution A & B were mixed and the volume was adjusted to 100 mL with distilled water. 1% Sodium hydroxide (NaOH) solution: 1g pellets of NaOH was weighted and dissolved in 100mL distilled water. It was used in detecting the presence of quinine.

Mercuric chloride solution ($HgCl_2$): the amount of 7.4 g of mercuric chloride was dissolved in 100 mL of distilled water at about 20 °C temperature. This solution was used for protein test.

Figure 1. The complete plant of *Catharanthus roseus* L with raw and dried flower image. A. a complete flower plant of ***C. roseus***; **B.** Collected flower was kept in a vessel and washed in water; and **C.** Completely dried flower was kept in airtight polyethylene zipper bags for further use in experimental purpose **D.** The semidried flower was spread in a shedding place.

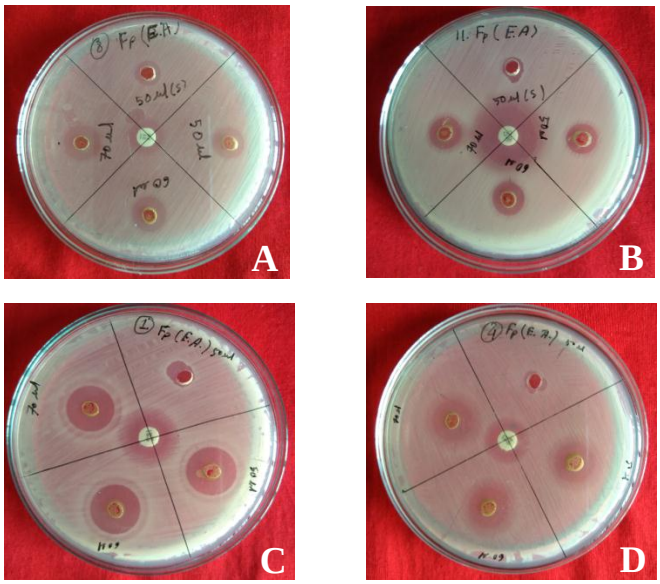

Figure 2. Antimicrobial effects of ethyl acetate extract of *C. roseus* against four different multidrug resistant bacteria (MDR) such as the three MDR species were Gram-negative **A**. *E. coli* BTGE-K_8; **C.** *Aeromonas caviae* BTGE-K_1; and **D.** *Citrobacter freundii* BTGE-K_4 and one was Gram-positive **B.** *Staphylococcus hominis* BTGE-K_11. In the central position of each plate, standard reference antibiotic Doxycycline (30 µg/disc) was used as control for

all the tested bacteria. The zones of inhibition around the wells were measured accurately using a metric ruler to the nearest millimetre.

Different qualitative chemical tests can be performed for establishing profile of methanol and aqueous extract for its chemical composition. The following tests were performed on extracts to detect various phyto-constituents present in them.

Detection of Alkaloids: Previously isolated 1 mL extraction from *C. roseus* flowers was added to few drop of Iodine solution which formed yellow colour precipitate indicates the presence of alkaloids (Kabesh *et al*., 2015).

Detection of Protein*:* Similarly 1 mL of extraction of *C. roseus* flowers was added few drop of mercuric chloride and formation of yellow colour indicates the presence of protein (Kabesh *et al*., 2015).

Detection of Steroid: The one (1) mL of extraction of *C. roseus* flowers mixed with 1 mL of chloroform and concentrated with sulphuric acid ($H_2SO_{4)}$ sidewise. A red colour presence at the lower chloroform layer indicates presence of steroids (Kabesh *et al*., 2015).

Detection of Flavonoids (Alkaline reagent test): The extraction amount (2mL) of *C. roseus* flowers was treated with few drops of 20% sodium hydroxide (NaOH) solution and the mixtures solution was created acute yellow colour which disappeared on addition of 2 drops dilute hydrochloric acid (HCL) indicates the presence of flavonoids (Gul *et al*., 2017).

Detection of Phenols: (Ferric chloride test): The amount (2 mL) of *C. roseus* flowers extracts was treated with 0.5 mL of aqueous 5% ferric chloride ($FeCl_3$) and observed for formation of deep blue or black colouration, which confirms the presence of phenols (Hema *et al*., 2012).

Detection of Saponins (Foam test): Distilled water (6 mL) was added in 2 mL of *C. roseus* flowers extract. The mixture was shaken thoroughly in graduated cylinders for 15 minutes and observed for the formation of constant foam to confirm the presence of saponins (Dubey, 2014).

Detection of Terpenoids (Salkowki's test): Chloroform (2 mL) was added to 2 mL of extract and added a few drops of concentrated H_2SO_4. The mixture was shaken well. A reddish brown precipitate produced immediately indicates the presence of terpenoids (Amin et al., 2013).

Detection of Quinines**:** To 1 mL of extract added 1 mL of 1% NaOH and mixed well. Appearance of blue green or red indicates presence of Quinines **(**Kabesh *et al*., 2015).

Inoculums preparation

Each bacterial strain was sub-cultured overnight at 35 °C in Nutrient agar slants. The bacterial growth was harvested using 5 ml of sterile saline water, its absorbance was adjusted at 580 µm and diluted to attain viable cell count of 10^7 CFU/ml using spectrophotometer.

Antibacterial analysis of plant extract

In this study, ethanol, ethyl acetate, petroleum ether and chloroform flower extracts of *C. roseus* were tested for their antimicrobial effects against four different (one Gram-positive and three Gram-negative) MDR food-borne pathogenic bacteria. The studied microorganisms included MDR clinical strains of Gram-negative bacteria (*Escherichia coli* BTGE-K_8, *Aeromonas caviae* BTGE-K_1, *Citrobacter freundii* BTGE-K_4) and and Gram-positive bacteria (*Staphylococcus hominis* BTGE-K_11) were collected from Microbiology laboratory, Department of Biotechnology and Genetic Engineering, Faculty of Biological Sciences, Islamic University, Kushtia-7003, Bangladesh. A antibacterial assay was performed by agar well diffusion method (Chaman, Sharma, and Reshi 2013). Petri dishes were prepared by pouring 20 ml of Nutrient Agar medium and allowed to solidify. Plates were solidified and 100 µL of bacterial culture was poured and uniformly spread and the inoculum was allowed to dry for 5 minutes. Agar well of 5 mm in diameter were prepared with the help of a sterilized stainless cork borer. The wells were labelled appropriately and to each well were loaded with 500µg/50µl, 250µg/60 µl and 125µg/70 µl plant extract, and corresponding solvent (50µl) of the extract using a micro-pipette. Standard reference antibiotic Doxycycline (30 µg/disc) was used as controls for the tested against bacteria. The plates were incubated at 37 °C for 24 hours. After incubation for 24 hours, the zones of inhibition around the wells were measured accurately using a metric ruler to the nearest millimetre.

Measurement of minimum inhibitory concentrations (MIC's) of the C. roseus L. flower plants extract

The minimum inhibitory concentrations (MIC) is defined as the lowest concentration of plant extract that inhibit bacterial growth within a certain time (24 h) incubation (Mostafa *et al*. 2018). It was necessary to prepared different concentration of plant extracts. The serial dilution technique of plant extracts was as follows: mother stock was prepared by dissolving 5 mg dry extract into 10 mL of corresponding solvent. Thus the concentration of mother stock became 500 µg/mL. Then the autoclaved eppendorf conical tubes (PolyLab, India) were taken for serial dilution. From mother stock, 5 mL solution was taken into first eppendorf tube and same amount (5 mL) of corresponding solvent was added to make the final volume 10 mL and final concentration was become 250 µg/mL. Then, 5 mL aliquot was transferred from second into third eppendorf tube and same amount of corresponding solvent was added to make the final volume 10 mL and the final concentration was become 125 µg/mL. This process was repeated for several times and made solution of 62.50, 31.25, 15.62, 7.813, 3.906 and 1.953 µg/mL concentrations and the final one was only respective solvent. We made four wells on each petri dish with borer. Among four wells, three were filled with different concentration of plant extract and one was filled with only corresponding solvent extraction. The extract concentrations of 500, 250, and 125 µg/mL were added in each well and the amount of volume of 50, 60 and 70 µl, respectively. The corresponding solvent of the extract was added in the well which volume was 50 µl. Finally one antibiotic disk of Doxycycline (30 µg/disc) was applied in the middle position of cultured medium of each petri dish. Similar procedure was followed for each of solvent extract of *C. roseus.*

Determination of minimum bacterial concentrations (MBC'S)

The minimum bacterial concentrations (MBC'S) were measured according to the procedure of Mostafa *et al*. 2018. We selected the three lowest concentration of plant extract Petridishes which exhibited invisible growth (inhibition zone of MIC plates) and sub-cultured onto sterile Nutrient a gar (NA) plates. The plates were incubated at 37 °C for 24 h. and then examined for bacterial growth in corresponding to plant extract concentration. MBC was taken as the concentration of plant extract that did not exhibiting any bacterial growth on the freshly inoculated agar plates.

Statistical analyses

Each experiment was completed in triplicate and antimicrobial activity of different extracts were evaluated by measuring the diameter of zones of inhibition in mm against all tested bacteria. Statistical analyses were performed One-way analysis of variance (ANOVA) using IBM SPSS Statistics version 21. Post Hoc (Duncan's multiple range test, DMRT) Tests were done by SPSS and differences were considered as significant at the level of $p < 0.05$. All the data are expressed as means ± standard deviation ($n = 3$).

Results and Discussion

The phytochemical screening of ethanol, ethyl acetate petroleum ether and chloroform plant extracts revealed the presence of various secondary metabolites. In this study, eight different phytochemicals/ secondary metabolites (alkaloids, flavonoids, steroid, protein, phenol, saponins, terpenoid and quinine) screening of flower extracts of *C. roseus* Linn with the four different solvent ethanol, ethyl acetate, petroleum ether and chloroform were investigated. Three phytochemicals (alkaloids, flavonoids, and steroid) were presence in all four solvent extraction but the phytochemical quinine and saponine were presence in only ethanol extraction. Proteins and phenols are present in ethanol and ethyl acetate extracts and terpenoids are present in ethyl acetate and petroleum ether extracts, respectively (Table 1). Different research reports suggest that plant secondary metabolites and their derivatives showed the antimicrobial properties. Among the secondary metabolites, alkaloids and polyphenols have shown strong antimicrobial activity and Polyphenols have the antioxidant properties which provide the basis for antimicrobial effects (Othman *et al*., 2019). The antibacterial activity of the plant extracts varied significantly depending upon the plant parts. Research data demonstrates that the antibacterial activity of plant parts depend upon the extraction procedure, solvent category, and tested bacterial strains (Nascimento *et al.*, 2000).

Different solvent extracts of medicinal important plants were investigated the antibacterial activity with gram positive and gram negative bacteria (Mostafa *et al*., 2018) and they have significant scope to develop a novel broad spectrum of antibacterial herbal medications (Mizanur *et al.* 2017). In this study, the highest (10.83± 0.29 mm) antimicrobial effect flower extract of *C. roseus* with ethanolic extract against *Escherichia coli* BTGE-K_8 strain and lowest (5.67± 0.58 mm) antimicrobial effect against *Staphylococcus hominis* BTGE-K_11 was observed (Table 2). There was a statistical significant difference ($p < 0.05$) of zone of inhibition with 125, 250 µg/ml concentration compare to 500 µg/ml concentration of flowers extract of ethanol and ethyl acetate. In case of ethyl acetate, the highest (15.5± 0.50 mm) antimicrobial effect antimicrobial effect of flower extract of *C. roseus* against multidrug resistant bacteria (MDR) of *Citrobacter freundii* BTGE-K_4 was revealed (Figure 1). In this study, ethyl acetate and ethanol were found to be a more suitable solvent on the basis of the maximum extraction of active metabolites and result of antimicrobial effect (Table 1, 2 and 3) and other two solvent i.e. petroleum ether and chloroform showed less activity (<5 or nil) zone of inhibition (data not shown). Gram-negative bacteria were found more susceptible as compared to Gram-positive species. The extra outer membrane of Gram-positive cell wall might be responsible for selective permeability of samples which resulted in ability to inhibit the tested three gram negative bacteria due to the presence of active antimicrobial compounds without penetrating into the cell (Akhtar *et al*., 2018). In this study, we observed that alkaloids, steroid, and flavonoid were extracted from all organic solvents which exhibiting antibacterial activities. Antibacterial activities also depend on phytochemical extraction process and their preparation dose. Therefore, the efficacies of tested flower plant extracts were less than the standard reference antibiotic (Table 2 and 3). In a study, flower extract of *C. roseus* did not show the significant ($p < 0.05$) inhibition of microbial strains (*E. coli, Bacillus subtilis* and *S. aureus*) (Goyal *et al*. 2008). Similarly petroleum ether and chloroform showed no zone of inhibition. Sukumar (1987) reported that the alkaloids compounds showed maximum zone of inhibition (15mm) against *S. aureus* (Pushpam and Sri, 2012). In this study, alkaloids presence in the flower extract of *C. roseus* which might showed the antibacterial properties. In another research, antimicrobial activity against *S. aureus* zone of inhibition 8 mm by flower extract. Similarly, our result was showed the highest zone of inhibition 8.50 ± 0.50 mm with the concentration 500 µg/ml *C. roseus* with ethanol solvent. However, the overall antibacterial result of the *C. roseus* flowers can be considered moderate, the results of this study are interesting taking in account the fact that the tested bacterial strains were MDR (Kabesh *et al*., 2015) proved that the antibacterial activity against gram negative bacteria with ethanol and methanol leaf extract of *C. roseus.* But, in this study flower extracts of *C. roseus* with ethanol showed the antibacterial activity and other solvent extracts (chloroform, and petroleum ether) did not show any antibacterial activity (data not shown). In a research, organic extracts showed the potent antibacterial activity compared to aqueous extracts. Even different parts (leaves, flowers and roots) of *C. roseus* plants showed the various degree of efficiency to inhibit bacterial growth. In addition, the ethanol extract was found to be most active against the tested bacterial species compare to other organic solvents (Goyal *et al*. 2008). But this study, ethyl acetate showed more active to ethanol extract (Table 1 and 2). The different studies also suggest that by using of organic solvents in the preparation of plant extracts were more readily extracted compared to aqueous extracts. The polarity of antibacterial compounds make them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species (Thongson *et al*. 2004; Goyal *et al*. 2008).

Table 1. Information on eight different phytochemical analysis of flower extracts of *C. roseus* Linn

Sl. No	Tested phytochemical Compound	Name of Reagent/test	Observation	Result (presence and absence of chemical compound in flower extracts of *C. roseus* (L.)			
				Ethanol	Ethyl acetate	Petroleum ether	Chloroform
1	Alkaloid	Iodine sol^{n}	Yellow colour precipitate	+	+	+	+
2	Protein	Mercuric chloride sol^{n}	Yellow colour	+	+	_	_
3	Steroid	chloroform and concentrated with sulphuric acid (H_2SO_4)	Red colour	+	+	+	+
4	Flavonoid	Alkaline reagent test	Yellow colour	+	+	+	+
5	Phenol	Ferric chloride test	Deep blue or black colour	+	+	_	_
6	Saponin	Foam test	Formation of constant foam	+	_	_	_
7	Terpenoid	Salkowki's test	A reddish brown precipitate	_	+	+	_
8	Quinine	Sodium hydroxide sol^{n}	Blue green or red indicates	+	_	_	_

Table 2. Antimicrobial effect of flower extract of *C. roseus* (L.) with ethanol solvent multidrug resistant bacteria (MDR) of Gram positive and Gram negative bacteria

Sl. No	Extract Con^{c}	Extraction amount	Zone of inhibition in mm (mean± SD)			
			Multidrug resistant (MDR) Gram positive bacteria	Multidrug resistant (MDR) Gram negative bacteria		
			Staphylococcus hominis BTGE-K_11	*Escherichia coli* BTGE-K_8	*Aeromonas caviae* BTGE-K_1	*Citrobacter freundii* BTGE-K_4
1	500 µg/ml	50µl	^{a}8.50 ± 0.50	^{a}10.83± 0.29	^{a}8.67± 0.58	^{a}8.17± 0.28
2	250 µg/ml	60µl	^{ab}8.17± 0.29	^{ab}10.17± 0.76	^{ab}8.50± 0.50	^{ab}7.33± 0.29
3	125 µg/ml	70µl	^{b}5.67± 0.58	^{b}0.00± 0.00	^{b}7.17± 0.29	^{b}7.00± 0.00
4	62.5 µg/ml	80µl	^{a}0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
5	Positive control (Doxycycline)	30 µg/disc	^{C}18.17± 0.29	18.83± 0.76	15.5± 0.87	18.83± 0.76
6	Negative control (only solvent)	50µl	_	_	_	_

#Disk diffusion method: consider as susceptibility > 5 mm, "-" No zone of inhibition; "C"= Control
Means followed by different letters (a, ab, and b) in each coloum are significantly different according to Duncan's multiple range comparisons (DMRTs),
The mean difference is significant at the 0.05 level.
Data are means of three replicates (n = 3) ± standard deviation

Table 3. Antimicrobial effect flower extract of *C. roseus* (L.) with ethyl acetate solvent against multidrug resistant bacteria (MDR) of Gram positive and Gram negative bacteria

Sl. No	Extract Concentration	Extract amount	Zone of inhibition in mm (mean± SD)			
			Multidrug resistant (MDR) Gram positive bacteria	Multidrug resistant (MDR) Gram negative bacteria		
			Staphylococcus hominis BTGE-K_11	*Escherichia coli* BTGE-K_8	*Aeromonas caviae* BTGE-K_1	*Citrobacter freundii* BTGE-K_4
1	500 µg/ml	50µl	^{a}11.17 ± 0.28	^{a}12.83± 0.76	^{a}10.33± 1.15	^{a}15.5± 0.50
2	250 µg/ml	60µl	^{ab}10.83± 0.29	^{ab}11.66± 0.58	^{ab}9.66± 0.58	^{ab}14.16± 0.29
3	125 µg/ml	70µl	^{b}9.67± 0.58	^{b}10.33± 0.57	^{b}9.33± 0.57	^{b}13.66± 0.59
4	62.5 µg/ml	80µl	$^{\#}$0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
5	Positive control (Doxycycline)	30 µg/disc	^{C}17.5± 0.87	18.83± 0.76	15.83± 0.79	18.5± 0.87
6	Negative control (only solvent)	50µl	_	_	_	_

#Disk diffusion method: consider as susceptibility > 5 mm, "-" No zone of inhibition; "C"= Control
Means followed by different letters (a, ab, and b) in each coloum are significantly different according to Duncan's multiple range comparisons (DMRTs),
The mean difference is significant at the 0.05 level.
Data are means of three replicates (n = 3) ± standard deviation

Conclusion

The present research demonstrates that the antibacterial activity of plant parts depend upon the plant parts, extraction procedure, solvent category, and tested bacterial strains. In a few cases (ethanol and ethyl acetate), the experimental plant extracts were active against MDR bacteria under very low concentration (125 µg/ml), thus minimizing the possible toxic effects. The results of our present study showed the extracts have potent antibacterial activity against MDR pathogenic strains such as *E. coli* BTGE-K_8, *A. caviae* BTGE-K_1, and *C. freundii* BTGE-K_4 and *S. hominis* BTGE-K_11. Therefore, our results could be used to control MDR bacteria, which are becoming a threat to human health.

Conflict of interest

The authors have no conflict of interest or financial relationships that could be constructed as a potential conflict of interest.

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