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The *in vitro* evaluation of antibacterial and antifungal activities of ripe fruit extracts (pericarp and seed) of *Bunchosia armeniaca* (Cav.) DC.

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Abstract: Plants are rich sources of phytoconstituents that have the ability to contribute biological activities. Indigenously prepared crude extracts of medicinal plants have been used as medications to treat infections caused by pathogenic microorganisms by people of various ethnic origins for many centuries in folkloric medicine. The research in discovering new and innovative antimicrobial compounds from plants has received increased concern due to the global rise of antimicrobial resistance. The present study aims to evaluate the *in vitro* antimicrobial potential of pericarp and seed extracts of ripe fruits associated with a native South American plant specimen known as *Bunchosia armeniaca*. The antibacterial and antifungal activities of aqueous and ethanolic crude extracts with concentrations ranging from 1000 to 125 µg/ml were determined against bacterial pathogens of *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Streptococcus pyogenes*, viridans group streptococci, and fungal pathogens *Candida albicans* and *Aspergillus fumigates* by performing the agar well diffusion assay. The pericarp extract did not exhibit any antimicrobial activity while the aqueous seed extract was only active against *Escherichia coli, which* indicated a mean inhibition zone diameter of 16.2 mm at its highest concentration. The minimum inhibitory concentration of the seed extract was 250 µg/ml. The qualitative phytochemical analysis indicated the presence of flavonoids, phenols, tannins, alkaloids, phytosterols, and saponins in both aqueous crude extracts. The findings of this investigation justifies that seeds of *Bunchosia armeniaca* ripe fruit have the ability to function as a novel, potent antibacterial therapeutic agents against multi-drug resistant *Escherichia coli.*

Keywords: *Bunchosia armeniaca*; Antibacterial activity; minimum inhibitory concentration; agar well diffusion assay

INTRODUCTION

Medicinal plants are valuable, indispensable, and potentially renewable sources of natural products that have made significant contributions towards the enhancement of human health. The use of herbs in folkloric medicine and their medicinal importance have been recorded since antiquity

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(Abeysinghe, 2010). Medicinal plants are rich and diverse sources of biologically active phytochemical compounds known as secondary metabolites. Some of these secondary metabolites can induce promising antimicrobial activity (Khanam, Wen and Bhat, 2015). The level of antimicrobial potential can vary from one anatomical region to another of a plant. Most synthetically produced antimicrobial drugs are derivatives of naturally occurring antimicrobial compounds. Recently, a great deal of interest has been shown by scientists towards research that focus on the isolation of antimicrobial compounds from natural sources like plants due to the global rise of antimicrobial resistance and worrisome side effects caused by synthetic antimicrobial drugs which have become major public health issues (Hakemi *et al.*, 2015). According to the World Health Organization (WHO), even as of today, about 80% of individuals that reside in developing countries rely on traditional herbal medicine for their primary health care needs (Aneja, Sharma and Joshi, 2012). The following study aims to investigate and evaluate antibacterial and antifungal potentials of ripe fruits and their seeds associated with a tropical plant specimen found in Sri Lanka called *Bunchosia armeniaca,* also known as "the peanut butter fruit". *Bunchosia armeniaca* is species of shrubs that belongs to the family Malpighiaceae (Lim, 2012). These evergreen perennial shrubs originated from South America, where they can be predominately found in Amazonia, Atlantic Forest, and Pantanal (Queiroz *et al.*, 2014). This species of *Bunchosia* can grow up to 5 m in height (Lim, 2012). Currently, there are no reports of *Bunchosia armeniaca* being applied in orthodox medicine. However, recent literature indicates that according to a research conducted at the Federal University of Santa Catarina in Brazil, leaves of *Bunchosia armeniaca* have excellent antibacterial and anti-inflammatory activities (Queiroz *et al.*, 2014). A research recently carried out locally indicated that ripe fruits of *Bunchosia armeniaca* have the ability to induce potent antioxidant activity. Studies indicate the presence of a wide variety of phytochemical compounds like flavonoids alkaloids, tannins, saponins, triterpenoids, steroids, phenols, and glycoside in the extracts of *Bunchosia armeniaca* fruits and leaves (Premathilaka and Silva, 2016). It has been justified that flavonoids predominantly act as compounds responsible for mediating antibacterial activity in these plants (Queiroz *et al.*, 2014).

MATERIAL AND METHODS

Collection and preparation of bacterial and fungal isolates: A total of nine microbial specimens were used in this experiment. Three Gram-positive bacteria that include *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (clinical sample), viridans group streptococci (clinical sample), and four Gram-negative bacteria that include *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (clinical sample), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (clinical sample) collected from the Medical Research Institute of Sri Lanka. Two fungal specimens that include *Candida albicans* (clinical sample) and *Aspergillus fumigates* (clinical sample) were collected from Lanka Hospitals Diagnostics (Sri Lanka). All microbial specimens were prepared in suspensions that were adjusted to 0.5 McFarland turbidity standards, which correspond to a microbial cell count of 1.5×10^8 CFU/ml.

Collection and Identification of plant material: Reddish fully ripe fruits of *Bunchosia armeniaca* were freshly plucked and collected from a garden tree situated at Avissawella region of Colombo district, in Sri Lanka (GPS coordinates: 6°57′11″N 80°13′06″E) in October 2016. Samples of the whole plant and fruits were sent for taxonomic authentication to the National Herbarium of Peradeniya in Sri Lanka.

Preparation aqueous plant extracts: Fruits were gently rinsed with distilled water and their

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pericarps were separated from seeds. The pericarps and seeds were separately grinded and freeze-dried at (- 40°C) for 24 hours. The dried plant material was pulverized to a coarse powder using mortar and pestle. Thirty grams of powdered pericarp and seeds were separately dissolved in 30 ml of sterile distilled water (1000 µg/ml). The stock solution was incubated at room temperature for 24 hours. The incubated aqueous stock solution was sieved through a muslin cloth and filtered with Whatman Grade 1 filter paper and followed by 0.45 µm membrane filter. The filtrates were stored in airtight containers at 4°C for further use. The pure aqueous stock solution was serially diluted to obtain different concentrations of 500, 250, and 125 µg/ml.

Preparation of ethanolic extracts: Another 30 g of freeze-dried and powdered pericarps and seeds were separately macerated in 100 ml of 95% ethanol at room temperature for 48 hours with occasional shaking. The macerated solutions were sieved through a muslin cloth and filtered with Whatman Grade 1 filter paper. The ethanol content of the filtrate was removed using the rotary evaporator under vacuum at 40°C for about half an hour. The dried filtrates were reconstituted with 30 ml of 20% dimethyl sulfoxide (DMSO) (1000 µg/ml). The reconstituted filtrates were further subjected to filtration through a 0.45 µm membrane filter and stored in airtight containers at 4°C for further use. The ethanolic stock solution was serially diluted to obtain different concentrations of 500, 250, and 125 µg/ml.

Assay for antibacterial activity: The *in vitro* agar well diffusion assay was used to evaluate the antibacterial and antifungal activities of the fruit extracts. The antibacterial assay was performed on Muller-Hinton agar. Muller-Hinton agar was supplemented with 5% sheep blood in case of *Streptococcus* spp. 1 ml of the 0.5 McFarland bacterial or fungal suspensions were transferred to 24 ml of Muller-Hinton agar and Sabouraud dextrose agar respectively and poured in to petri dishes of standard size in order to obtain an uniform agar depth of 4 mm. After pouring, the agar medium was allowed to solidify and the plates were inverted and left to dry for about 15 minutes. A sterilized steel cork borer of 8 mm in diameter was used to punch wells on the agar surface. All extracts were brought to room temperature prior to be used in the antimicrobial assay. Aliquots containing 100 µl of extract from the 1000 µg/ml stock solutions were inoculated to the wells. Other aliquots containing 100 µl of sterilized distilled water or 20% DMSO were inoculated to other wells and considered as negative controls for aqueous and ethanolic extracts respectively. The antibiotic disks gentamicin 10 µg were used as positive controls in the antibacterial assays. The plates were allowed to stand for about 1 hour at room temperature for pre-diffusion of the extract in to agar and incubated at 37°C for 24 hours for bacteria. All antibacterial assays were conducted in triplicates. The diameter of the zone of inhibition for each well and disk were read and measured in "mm".

Assay for antifungal activity: The *in vitro* agar well diffusion assay was used to evaluate the antifungal activities of the fruit extracts. The antifungal assay was performed on Sabouraud dextrose agar. One milliliter of 0.5 McFarland fungal suspensions was transferred to 24 ml of Sabouraud dextrose agar respectively and poured in to petri dishes of standard size in order to obtain an uniform agar depth of 4 mm. After pouring, the agar medium was allowed to solidify and the plates were inverted and left to dry for about 15 minutes. A sterilized steel cork borer of 8 mm in diameter was used to punch wells on the agar surface. All extracts were brought to room temperature prior to be used in the antimicrobial assay. Aliquota containing 100 µl of the extract from the 1000 µg/ml stock solutions were inoculated to the wells. Other aliquots containing 100 µl of sterilized distilled water or 20% DMSO were inoculated to other wells and considered as the negative control for aqueous and ethanolic extracts, respectively. The antifungal drug disks voriconazole 1 µg were used as positive controls in the antifungal assay. The plates were allowed to stand for about 1 hour at room temperature

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for pre-diffusion of the extract in to agar and incubated at 28°C for 48-72 hours for fungi. All assays were conducted in triplicates. The diameter of the zone of inhibition for each well and disk were read and measured in "mm".

Determination of the minimum inhibitory concentration and minimum microbicidal concentration: Likewise, the agar well diffusion assay was carried out to determine the minimum inhibitory concentration for different concentrations of 1000, 500, 250, and 125 µg/ml. All antimicrobial assays were conducted in triplicates. The diameter of the zone of inhibition for each well and disk were read and measured in "mm". A sample from the zone of inhibition produced for each concentrations ranging from 1000 - 125 µg/ml were aseptically pre-elevated and separately plated on fresh agar plates and observed for the formation of bacterial or fungal colonies after overnight incubation to determine the minimum microbicidal concentration.

Screening for qualitative phytochemical analysis: The aqueous seed and pericarp extracts of *Bunchosia armeniaca* ripe fruits were qualitatively screened for the presence of flavonoids, alkaloids, phenols, phytosterols, saponins, and tannins by the application of alkaline reagent test, Wagner's test, Liebermann–burchard test, Salkowski's test, froth test, and Braymer's test respectively. One to 5ml from the 1000µg/ml stock solution were taken for phytochemical analysis.

Test for flavonoids (alkaline reagent test): Approximately 1 ml of the seeds and pericarps filtrates were individually treated with 6 consecutive drops of 10% sodium hydroxide solution and followed by 6 consecutive drops diluted hydrochloric acid. The disappearance of the intense yellow coloration after the addition of diluted hydrochloric acid is an indication of flavonoids present in the extract (Somkuwar and Kamble, 2013).

Test for alkaloids (Wagner's test): Approximately 1ml of the seeds and pericarps filtrates were individually treated with 6 consecutive drops of diluted hydrochloric acid and followed by the of addition 6 consecutive drops of Wagner's reagent (iodine and potassium iodide). The formation of a reddish brown precipitate is an indication of alkaloids present in the extract (Somkuwar and Kamble, 2013).

Test for phenols: Approximately 1ml of the seeds and pericarps filtrates were individually treated with 6 consecutive drops of ferric chloride solution. The formation of dark brown to black colour is an indication of phenols present in the extract (Somkuwar and Kamble, 2013).

Test for phytosterols (Salkowski's test): Approximately 1ml of the seeds and pericarps filtrates were individually treated with chloroform and followed by the addition of 6 consecutive drops of concentrated sulphuric acid. The formation of double layers with red and golden is an indication of phytosterols present in the extract (Somkuwar and Kamble, 2013).

Test for saponins (Froth test): Approximately 5 ml of the seeds and pericarps filtrates were individually subjected to vigorous shaking for 15 minutes. A foamy generation of at least 1cm in height is an indication of saponins present in the extract (Somkuwar and Kamble, 2013).

Test for tannins (Braymer's test): Approximately 1ml of the seeds and pericarps filtrates were individually treated with 6 consecutive drops of 10% alcoholic ferric chloride solution. The formation of green color is an indication of tannins present in the extract (Somkuwar and Kamble, 2013)

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RESULTS AND DISCUSSION

Many medicinal plants used in traditional medicine are a rich source of natural antimicrobial products. Their antimicrobial properties have been used to treat infectious diseases for many years. The efficiency and universality of plants used in traditional medicine is evident from their constant harnessing and use (Khan *et al*., 2015). According to Premathilaka and Silva (2016) the plant species *Bunchosia armeniaca* has been used in traditional herbal medicine for treating several human medical conditions and disorders in countries associated with South America.

Neither aqueous or ethanolic extracts of pericarp exhibited any observable antibacterial or antifungal activities while the aqueous seed extract was only active against *Escherichia coli* with bacteriostatic action which indicated a mean inhibition zone diameter of 16.2 ± 0.03 mm at its highest concentration of 1000 µg/ml (figure 1 and Table 1). Moreover, none of the ethanolic extracts exhibited any observable antibacterial or antifungal activities. The minimum inhibitory concentration of the aqueous seeds extract was 250 µg/ml which produced a mean inhibition zone diameter of 13.2 ± 0.04 mm (figure 2 and Table 2). The aqueous seeds extract at 1000 mg/ml did not induce bactericidal effect since the presence of miniature colonies of *Escherichia coli* were detected on agar after incubation. The qualitative phytochemical analysis test revealed the presence of flavonoids, phenols, tannins, alkaloids, phytosterols, and saponins in both aqueous crude extracts.

The aqueous seeds and pericarps extracts of *Bunchosia armeniaca* ripe fruit were qualitatively screened for the presence of bioactive secondary metabolites like flavonoids, phenols, tannins, alkaloids, phytosterols, and saponins. The results for phytochemical analysis of *Bunchosia armeniaca* ripe fruit extracts are summarized in (figures 4 – 11 and Table 2).

Queiroz *et al*. (2014) noted that flavonoids were the primary phytochemical compounds that mediated antibacterial activity of *Bunchosia armeniaca* leafs. Being polyphenolic secondary metabolites, these flavonoids have the ability to induce bacterial growth inhibition by mainly involving the traumatisation of bacterial DNA.

CONCLUSION

In conclusion, the present finding of this study provides evidence that the seeds of *Bunchosia armeniaca* ripe fruit have narrow spectrum or semi-narrow spectrum bacteriostatic action against bacteria and qualifies as a decent candidate to be recognized as a novel antibacterial agent with potent therapeutic ability to treat infections caused by multi-drug resistant *Escherichia coli.* Further studies are recommended to determine the antibacterial mode of action of lead phytochemical molecules associated with seeds and the antibacterial effect of crude extract of *Bunchosia armeniaca* seeds on animal models.

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Figure 1: Shows the investigated plant specimen and its parts. Top - *Bunchosia armeniaca* tree with ripe fruits from the site of collection. Below left – Ripe fruit of *Bunchosia armeniaca.*. Below right– Seed of *Bunchosia armeniaca* fruit.

ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
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Table 1: Antibacterial activities and minimum inhibitory concentrations of the aqueous seeds extract of *Bunchosia armeniaca* ripe fruits against *Escherichia coli*.

	IZD (mm)	
Concentration (µg/ml)	Seed extract	Gentamicin (10 µg)
125	NI	14.1±0.06
250	13.2±0.04	14±0.06
500	14.3±0.13	14±0.13
1000	16.2±0.03	14.1±0.04

The data obtained for dose dependent antibacterial activity were presented as follows:
IZD = mean inhibition zone diameter of three replicates (mm) ±SEM (n=3), NI = no inhibition,
NA = no data available.

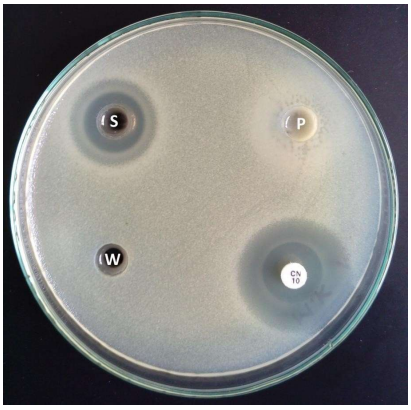

Figure 2: Antibacterial activities of aqueous crude extracts of the ripe fruits of *Bunchosia armeniaca* against *Escherichia coli* by using the agar well diffusion assay. The antibacterial activity is expressed by the zone of inhibition. Abbreviations: S - Seeds extract, P - Pericarps extract, W - Sterile distilled water (negative control), and CN - Gentamicin 10 µg disk (positive control).

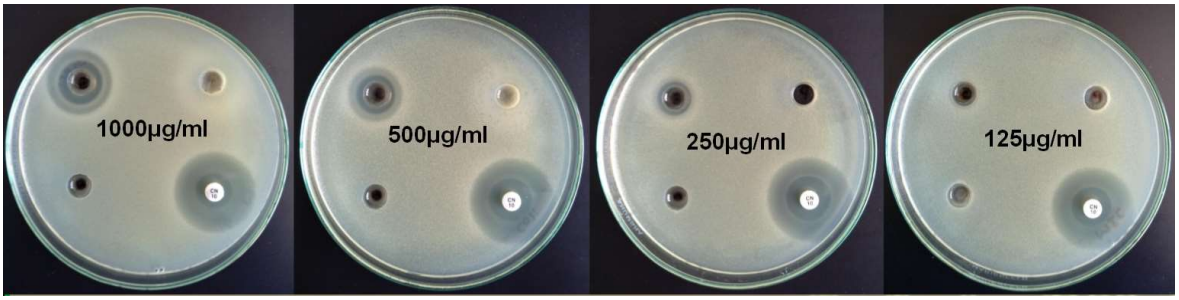

Figure 3: Minimum inhibitory concentration assay for aqueous crude extracts of the ripe fruits of *Bunchosia armeniaca* against *Escherichia coli* by using the agar well diffusion assay. The antibacterial activity is expressed by the zone of inhibition. Abbreviations: Top left of plates: Seed extract; top right of plates: pericarp extract; below right of plates: sterile distilled water (negative control), and below left of plates - gentamicin 10 µg disk (positive control).

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Table 2: Phytochemical profile of seeds and pericarp extracts of *Bunchosia armeniaca* ripe fruit.

Phytochemical compound	Test used for detection	Presence in seed	Presence in pericarp	Observation
Flavonoids	Alkaline reagent test	+	+	Moderate yellow coloration turned colorless
	Lead acetate test	+	+	Formation of a yellowish precipitate
	Sulfuric acid test	+	+	Mild yellow to orange coloration
Alkaloids	Wagner's test	+	+	Formation of a reddish brown precipitate
Phenols	Liebermann–burchard	+	+	Dark brown coloration
Phytosterols	Salkowski's test	+	+	Two layers of red and golden colour formed
Saponins	Froth test	+	+	A foam of nearly 1cm in height was produced
Tannins	Braymer's test	+	+	Light green coloration

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(3):20-28

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