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Research Article
Discovery of Antibacterial and Antifungal Activities of South American *Vaccinium macrocarpon* Fruit: An Ethnomedicinal Plant

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Abstract

Background and Objective: The antibacterial and antifungal activities of ethnomedicinal plants has been recognized for many years. The leaves of the plant produce many chemical compounds that may be significantly helpful in the defence against various pathogens. In this research, the crude ethanol, n-hexane, chloroform, n-butanol and aqueous soluble extracts of South American *Vaccinium macrocarpon* was investigated for its antibacterial and antifungal activates against various Gram-positive and Gram-negative pathogens such as *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Aspergillus niger. The* results were also compared with the conventional medicine such as ciprofloxacin for antibacterial defence while Fluconazole for antifungal defence. **Materials and Methods:** Two methods were utilized for this purpose, 96-wells microplate method and agar wells diffusion method regarding percentage inhibition, MIC value and zone of inhibitions. **Results:** The results showed that in antibacterial activities, the first method resulted that n-butanol extract had highest, crude ethanol extract had lowest while n-hexane and chloroform had no antibacterial activities. In the 2nd method the result showed that crude ethanol extract had highest, n-hexane extract had lowest while chloroform had no activities. In the case of antifungal activities, the results of agar wells diffusion method showed that crude ethanol had highest, n-hexane had lowest while chloroform and dimethyl sulfoxide had no antifungal activities. **Conclusion:** Overall the plant extracts found to be more effective against Gram-negative pathogens than Gram-positive.

Key words: Antibacterial, antifungal, fluconazole, ethnomedicinal plant, agar wells diffusion method, Gram-negative pathogens, *Vaccinium macrocarpon* fruit

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Throughout the history of mankind, the alternative medicines extracted from the medicinal plants i.e., spices, herbs and their components have been used for the effective treatment of venereal infectious diseases and conditions. Venereal infectious diseases and conditions mean that are highly responsive to traditional treatment and also referred to sexually transmitted diseases (STDs)[1]. According to the World Health Organization (WHO) estimation, 340 million new cases of STDs reported globally in 1999 including at least 111 million cases with young people under 25 years of age. The highest number of these new infections are reported in South and Southeast Asia, followed by sub-saharan Africa and Latin America and the Caribbean[2]. Scientific experimentation in the late 19th century revealed that some spices, herbs and their components have pharmaceutical, antioxidant, antibacterial and antifungal properties which have been the source of 25% of useful therapeutic agents[3]. The development and acceptance of traditional medicines extracted from medicinal plants as an alternative medicine for better healthcare have led researchers to investigate the pharmaceutical, antioxidant, antibacterial and antifungal properties of medicinal plants[4,5]. In the view of these facts and figures, there has been rising interest in the discovery of new biologically active compounds having pharmaceutical, antioxidant, antibacterial and antifungal properties through the systematic and experimental based screening of medicinal plants against the following bacteria and fungus[6-8].

Salmonella typhi is an important rod-shaped intercellular pathogen exclusively pathogenic for humans causing life-threatening systemic infections and typhoid fever. In humans, two kinds of infections are observed; typhoid or paratyphoid which is enteric fever and non-typhoidal gastroenteritis[9]. The *Salmonella typhi* found globally in the developing regions i.e., Africa, Asia and South America with South Asia at the highest risk of these infections. Globally, each year 20 million cases of *Salmonella typhi* infections are reported including 200,000 deaths[10].

Staphylococcus aureus has been recognized as a globally versatile micro-organism that can cause by the ingestion of contaminated food and water and may lead to illness from mild range requiring no treatment to severe and potentially fatal infections[11,12]. Approximately 30% of healthy people are infected by *Staphylococcus aureus* due to its colonialization in the human body as a part of normal flora.

Escherichia coli is a non-spore forming, rod-shaped pathogen usually found in raw meat, raw milk, non-chlorinated water and contaminated food and vegetables. It may or my no be mobile and can survive at freezing temperature, grows between 45 to 114°F with growth time 30-87.6 min at optimum pH of 6.0-8.0 in the presence as well as the absence of air and pathogenic to humans and animals[13,14]. *Escherichia Coli* is a common inhabitant that suppress the growth of harmful bacteria by synthesizing an appreciable number of vitamins. However, within the species, 4 strains are harmful and cause life-threatening infections.

Pseudomonas aeruginosa is one of the leading problematic nosocomial pathogens which is responsible for 10-15% of nosocomial infections worldwide[15]. Due to the naturally intrinsic resistance of spices and the remarkable ability to acquire further mechanisms of resistance to multiple groups of structurally unrelated antimicrobial agents its treatment is difficult and expensive[16].

Bacillus subtilis are a Gram-positive, rod-shaped, endospore-forming aerobic or facultatively anaerobic pathogen that exhibits a wide range of physiological abilities that allow them to grow in every natural environment. This pathogen is transmitted to a human with direct contact from infected herbivores or indirectly via their products. This syndrome is characterized by nausea, vomiting, slower-onset diarrheal syndrome which may lead to severe eyes, ears, wounds, urinary tract, respiratory tract and gastrointestinal tract infections[17].

Aspergillus niger is a mould fungus that rarely reported as a cause of pneumonia with 5% frequency in invasive infections and thermally less stable[18]. The ideal growth temperature and pH are 30-34°C and 4.0, respectively making difficult germination at human body temperature of at least[19] 37°C.

In order to make remarkable developments in the treatment of above mentioned life-threatening diseases, many researchers devoted their efforts for *in vitro* and experimental investigation of antibacterial and antifungal activities of many plants such as Moroccan Plants[20], Indonesian Ethnomedical Plants[21], Tunisian Santolina Chamaecyparissus[22], Cichorium Intybus[23], Indian Yarrow[24], Monodora Tenuifolia[25], Backhousia Citridora[26], Tropical and Temperate Woods[27], Mexican Tarragon[28], Cabbage Seeds[29], Japanese Cedar[30], Chinese Cabbage[31], Mexican and Chilean Plants[32].

This research aimed to experimentally investigate the antibacterial and antifungal activities of South American Cranberry (Vaccinium Macrocarpon) and comparison with Ciprofloxacin, an antibiotic used for the treatment of bacterial infections. The antibacterial and antifungal activities were investigated against bacteria; *Salmonella typhi, Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* and fungus; *Aspergillus niger*.

MATERIALS AND METHODS

Sample preparation and fractionation

Solvents: All solvents such as Ethanol (C_2H_5OH), Methanol (CH_3OH), n-Hexane (C_6H_{14}), Chloroform ($CHCl_3$) and n-Butanol (C_4H_9OH) were purchased from Merck, Germany and used without any further purification.

Plant material: The plant material Cranberry (Vaccinium Macrocarpon) powder was provided by YAXX International USA for research purposes.

Maceration: Vaccinium macrocarpon (1 kg) dried powder was soaked in ethanol solvent (5 L) for 15 days with continuous agitation after regular interval of time during maceration until the powder was dissolved. After 15 days, the material was pressed and filtered through muslin cloth first and then using filter paper to separate the course material from the solvent. The filtrate was about 2 L. The marc remained was then again re-soaked in ethanol and process was repeated and again to extract all constitutes from plant material. At the end the filtrate was found to be 2.5 L.

Drying of filtrate: All the filtrate samples collected from repeated maceration process were mixed and subjected to the drying process. For drying purpose rotary evaporator (Yamato, Rotary Evaporator and Model-RE 801) was used at reduced pressure, temperature 35°C and agitation speed of 90-120 rpm. After the evaporation of the solvent, the weight of a dried crude extract of Vaccinium Macrocarpon was found to be 170 g.

Fractionation: The obtained 170 g of methanol crude extract was suspended in water (1 L). Then it was successfully extracted by different polar and non-polar solvents such as Ethanol (C_2H_5OH), n-Hexane (C_6H_{14}), Chloroform ($CHCl_3$) and n-Butanol (C_4H_9OH) respectively. All extracted crude was filtered to remove the particulate matter and then particle free crude extract was dried completely using rotary evaporator (Yamato, Rotary Evaporator and Model-RE 801) at a temperature of 40°C under reduced pressure to obtained dry extracts. The residue was re-extracted twice repeating the same procedure to obtain all crude extracts.

Antibacterial activities

Test organisms: The anti-bacterial activities of crude extracts were tested on following bacteria; Bacillus subtilis (NCTC 8236), Staphylococcus aureus (NCTC 6571), *E. coli* (NCTC 10418), *Pseudomonas aeruginosa* (ATCC 10145) and *Salmonella typhi* (NCTC 10787). The bacteria were provided in the form of stock culture agar by the Department of Biological and Biomedical Sciences, The Aga Khan University, Karachi, Pakistan.

96-wells microplate method

Preparation of culture and extract solution: Nutrient broth (8 g) Merck, Germany was dissolved in distilled water (1 L) and sterilized in an autoclave at a temperature of 121°C for 15 min at a pressure of 15 Psi. After that 50 mL of nutrient broth and 50 µL of stock culture bacteria were added in Erlenmeyer flasks and mounted on a horizontal shaker at a speed of 200 rpm for 24 h at 37°C. After 24 h, the bacterial culture was diluted with nutrient broth to attain the optical density between 0.12-0.19 according to the 0.5 McFarland standards. This assay is called broth dilution method because the bacterial culture was diluted with nutrient broth. For an extract solution, 5 mg of the extract was dissolved in methanol (1 mL). For MIC50 serial dilution was done.

Methodology of 96-wells microplate method: In this method, the antibacterial activities were investigated in sterilized 96-wells microplates in a laminar flow hood to attain aseptic environmental conditions. Optical density was elevated when there was no bacterial amplification in log phase. Total volume in a well was 200 µL including 20 µL of methanol extract and 180 µL suspension of bacterial culture. At 540 nm, the initial absorbance was measured. After that, the plates were incubated at 37°C for 16-24 h and after that again final absorbance was measured at 540 nm to calculate the percentage inhibition as an index of bacterial growth. All readings were taken as triplicate and results are presented as means values. Ciprofloxacin was used as a positive inhibitor and methanol was added in the assay as a negative control. The inhibition (%) was calculated by the following formula:

$$\text{Inhibition}(\%) = \left[\frac{X - Y}{X}\right] \times 100$$

where, X is the absorbance in negative control with bacterial culture and Y is the absorbance in test samples with bacteria. The results were taken by the average of three experiments.

Minimum inhibitory concentration (MIC): Serial dilutions of the test samples were made to calculate the minimum inhibitory concentration by the following protocol as described above using EZ-Fit Enzyme Kinetics by Perrella Scientific Software.

Agar wells diffusion method for the antibacterial activities: Initially in this method, agar was prepared. For this purpose, Mueller Hinton Agar (28 g) was dissolved in distilled water and sterilized at 121°C for 15 min in an autoclave. After that protocol followed with slight modification. Petri dishes were sterilized in a hot air oven at 150°C and then put into laminar flow head for aseptic environmental conditions. Sterilized Mueller Hinton Agar (20 mL) was transferred into petri dishes and solidified. Bacterial culture was streaked on the agar surface and allowed to dry. Then on each Petri dish, 4 holes with diameter 6 mm were made in dried agar using sterile cork borer. Out of 4 holes, one hole was filled by standard ciprofloxacin (20 µL) and other three holes were filled by extract solution (20 µL) using a micro pipette. All these Petri plates were put into an incubator for 18-24 h at 370°C. After the incubation (18-24 h), the zones of inhibitions were measured in order to estimate the antibacterial activity. The results were taken by the average of three experiments.

Antifungal activities

Preparation of culture: Sabouraud Dextrose Broth (8 g) was dissolved in distilled water (1 L) and sterilization of broth was carried out in an autoclave at 121°C and 15 Psi for 15 min. In order to impede the bacterial growth, ciprofloxacin (50 mg) was added into the broth. The sterilized broth and stock culture of *Aspergillus niger* (Fungus) were mixed in Erlenmeyer Flasks. The flasks were mounted on a shaker at room temperature for 24 h.

Agar wells diffusion method for antifungal activities: Initially in this method, Sabouraud Dextrose Broth was mixed with distilled water and sterilized in an autoclave at 121°C and 15 Psi for 15 min. After this in sterilized Petri dishes, agar (20 mL) was poured and solidified in an aseptic environment and fungus culture was streaked. Then on each petri dish, holes with a diameter of 6 mm were made with the help of flamed sterile borer. Extract solution (15 µL) of 5 mg mL^{-1} in methanol and fluconazole (15 µL) as standard in 1 mg mL concentration were poured in holes and kept the Petri dishes at 25°C for 24-36 h and after that zone of inhibition was measured.

RESULTS AND DISCUSSION

The antibacterial and antifungal activities of *Vaccinium macrocarpon* South American Fruit against *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Aspergillus niger* presented in Table 1 which agreed with the previously reported antibacterial and antifungal activities of ethnomedicinal plants[33-36]. It was evaluated that the effect of plants extracts on the growth of various Gram-positive (*Staphylococcus aureus, Bacillus subtilis* and *Aspergillus niger*) and Gram-negative bacteria (*Salmonella typhi, Escherichia coli and Pseudomonas aeruginosa)* by utilizing two different methods 96-wells microplate method and 96-agar wells diffusion method. To author's knowledge, this is the first report presenting the experimental studies instead of *in vitro* studies on the antibacterial and antifungal activities of *Vaccinium macrocarpon* South American fruit. It is well known that the chemical characterization of the plant's extracts will provide a better understanding towards their antibacterial and antifungal activities[37,38].

In 96-wells microplate methods, two parameters percentage of inhibition and MIC values were determined to examine the antibacterial activities of the plant. In the case of *Salmonella typhi*, which was a Gram-negative bacteria responsible for life-threatening infections in humans. The n-butanol soluble fraction showed the highest percentage of inhibition (86.00±2.91) which was comparable to ciprofloxacin (90.71±1.05). The MIC values for the aqueous soluble fractions (10.57±1.41) were even higher than ciprofloxacin (7.93±0.98). The n-hexane soluble fraction and chloroform soluble fraction showed no inhibition and MIC against *Salmonella typhi.* In the case of *Staphylococcus aureus*, a Gram-positive bacterium caused severe and potentially fatal infections. The n-butanol soluble fractions showed considerable percentage inhibition (64.50±2.10) and remarkable MIC (11.17±1.37) compared with the ciprofloxacin inhibition (91.29±1.74) and MIC (8.51±1.21) respectively while the n-hexane soluble fraction, chloroform soluble fraction and aqueous soluble fraction showed no inhibition and MIC against *Staphylococcus aureus.* In case of *Escherichia coli* which was a Gram-negative pathogen and caused traveler's diarrhea. The crude ethanol extract had significant inhibition (84.13±1.53) while n-hexane had significant MIC value (9.28±0.81) comparable with the ciprofloxacin inhibition (90.11±0.75) and MIC value (8.25±0.37) respectively. The chloroform-soluble fractions showed no inhibition and MIC against *Escherichia coli* pathogen. In case of *Pseudomonas aeruginosa* which was a Gram-negative nosocomial pathogen and caused severe therapeutic infections. The crude ethanol extract showed both remarkable inhibition 86.09±2.93 as well as MIC value of 9.86±1.35 which was comparable with the ciprofloxacin inhibition 91.83±0.21 and MIC value of 7.67±1.90, respectively while

Table 1: Antibacterial activities of plant extracts against various pathogens via 96-wells microplate method

Bacteria	Samples	Inhibition (%)	MIC (µL)
Salmonella typhi	Crude ethanol extract	85.68±2.23	9.81±1.91
	n-hexane soluble fraction	-	-
	Chloroform soluble fraction	-	-
	n-butanol soluble fraction	86.00±2.91	9.04±0.96
	Aqueous soluble fraction	82.27±2.01	10.57±1.41
	Ciprofloxacin	90.71±1.05	7.93±0.98
Staphylococcus aureus	Crude ethanol extract	51.35±1.55	12.25±0.87
	n-hexane soluble fraction	-	-
	Chloroform soluble fraction	-	-
	n-butanol soluble fraction	64.50±2.10	11.17±1.34
	Aqueous soluble fraction	-	-
	Ciprofloxacin	91.29±1.74	8.51±1.21
Escherichia coli	Crude ethanol extract	84.13±1.53	8.82±0.81
	n-hexane soluble fraction	75.06±3.37	9.28±0.81
	Chloroform soluble fraction	-	-
	n-butanol soluble fraction	81.43±1.63	8.96±1.12
	Aqueous soluble fraction	80.06±3.37	9.09±0.81
	Ciprofloxacin	90.11±0.75	8.25±0.37
Pseudomonas aeruginosa	Crude ethanol extract	86.09±2.93	9.86±1.35
	n-hexane soluble fraction	-	-
	Chloroform soluble fraction	-	-
	n-butanol soluble fraction	85.09±0.43	9.07±1.35
	Aqueous soluble fraction	-	-
	Ciprofloxacin	91.83±0.21	7.67±1.90
Bacillus subtilis	Crude ethanol extract	77.08±1.67	13.22±1.06
	n-hexane soluble fraction	-	-
	Chloroform soluble fraction	-	-
	n-butanol soluble fraction	75.67±1.08	10.67±0.96
	Aqueous soluble fraction	-	-
	Ciprofloxacin	92.15±0.79	7.66±0.79

the n-hexane soluble fraction, chloroform soluble fraction and aqueous soluble fractions didn't show any inhibition and MIC against *Pseudomonas aeruginosa* pathogen. In the case of *Bacillus subtilis* which was a Gram-positive and an anaerobic pathogen and caused severe urinary tract, respiratory tract and gastrointestinal tract infections. The crude ethanol extract showed remarkable inhibition and MIC which was comparable with the ciprofloxacin. The crude ethanol extract showed inhibition of 77.08±1.67 and MIC value of 13.22±1.06 while the ciprofloxacin showed inhibition of 92.15±0.79 and MIC value of 7.66±0.79. The n-hexane soluble fraction, chloroform soluble fraction and aqueous soluble fractions did not show any inhibition and MIC against *Bacillus Subtilis* pathogen. All the results were strongly in agreement with the previously reported researches[1,39-42]. Overall, the plants extract especially n-butanol soluble fraction showed more inhibition against Gram-negative bacteria than the Gram-positive bacteria which is in agreement with the previously reported studies[42]. It may be an indication of the presence of an antibiotic compound in a broad spectrum or simply general metabolic toxins. The n-butanol soluble fraction showed the highest inhibition and MIC values in all cases while the crude ethanol soluble fraction showed the lowest inhibition and MIC values in all cases. The n-hexane soluble fractions and chloroform soluble fraction showed no inhibition and MIC values in most of the cases.

In agar wells diffusion method the parameter zones of inhibition are used in order to evaluate the antibacterial and antifungal activities of plant extract against various Gram-positive and Gram-negative bacteria. The results of antibacterial activities and antifungal activities against various pathogens were tabulated in Table 2 and 3, respectively. The results of antibacterial activities via agar wells diffusion method showed that in case of all pathogens, the crude ethanol fraction showed remarkable zone of inhibition when compared with ciprofloxacin antibacterial activity like in case of *Salmonella typhi* (20 mm), *Staphylococcus aureus* (16 mm), *Escherichia coli* (20 mm) and *Bacillus subtilis* (15 mm) while the ciprofloxacin had in the range of (19-22 mm).

However, in the case of *Pseudomonas aeruginosa*the n-butanol soluble fractions showed a significant zone of inhibition (20 mm) comparable with the ciprofloxacin (19 mm). Finally, the crude ethanol extract showed the highest zone of inhibition in all cases while n-hexane soluble fractions

Table 2: Antibacterial activities of plant extracts against various pathogens via agar wells diffusion method

Bacteria	Samples	Zone of inhibition
Salmonella typhi	Crude ethanol extract	20
	n-hexane soluble fraction	-
	Chloroform soluble fraction	-
	n-butanol soluble fraction	14
	Aqueous soluble fraction	12
	Ciprofloxacin	21
	Ethanol	-
Staphylococcus aureus	Crude ethanol extract	16
	n-hexane soluble fraction	6
	Chloroform soluble fraction	-
	n-butanol soluble fraction	-
	Aqueous soluble fraction	-
	Ciprofloxacin	22
	Ethanol	-
Escherichia coli	Crude ethanol extract	20
	n-hexane soluble fraction	14
	Chloroform soluble fraction	-
	n-butanol soluble fraction	18
	Aqueous soluble fraction	14
	Ciprofloxacin	21
	Ethanol	-
Pseudomonas aeruginosa	Crude ethanol extract	18
	n-hexane soluble fraction	-
	Chloroform soluble fraction	-
	n-butanol soluble fraction	20
	Aqueous soluble fraction	-
	Ciprofloxacin	19
	Ethanol	-
Bacillus subtilis	Crude ethanol extract	15
	n-hexane soluble fraction	-
	Chloroform soluble fraction	-
	n-butanol soluble fraction	12
	Aqueous soluble fraction	-
	Ciprofloxacin	20
	Ethanol	-

Table 3: Antifungal activities of plant extracts against *Aspergillus Niger* via agar wells diffusion method

Fungus	Samples	Zone of Inhibition
Aspergillus niger	Crude ethanol extract	18
	n-hexane soluble fraction	11
	Chloroform soluble fraction	-
	n-butanol soluble fraction	16
	Aqueous soluble fraction	12
	Fluconazole	22
	Dimethyl sulfoxide	-

showed the lowest zone of inhibition in all cases. The chloroform-soluble fraction showed no activity against any pathogens tested in this study. The plant's extracts overall showed high activity against gram-negative bacteria as compared to the gram-positive bacteria which was in agreement with the previously reported researches[42]. In case of antifungal activity against *Aspergillus niger* in tabulated in Table 3. The results of plants extract were compared with the activity of conventional medicine used for the medication of this infection known as Fluconazole. The results showed that crude ethanol extract showed the highest zone of inhibition (18 mm) compared to Fluconazole (22 mm). The n-hexane soluble fraction showed lowest while crude ethanol fraction showed the highest antifungal activity while the chloroform soluble fraction and dimethyl sulfoxide showed no antifungal activity. This study showed the presence of various compounds such as phenolic compounds, oils, flavonoids and alkaloids etc. Which may act as antibacterial and antifungal agents against many pathogens. In the light of the above study, this preliminary results may lead to the discovery of novel antibacterial and antifungal drugs for the promotion and effective management of medication against infectious diseases[43,44].

As observed from the results that the Vaccinium Macrocarpon fruit extracts were found to be highly efficient against Gram-negative pathogens as compared to Gram-positive pathogens due to their effective antifungal and antibacterial properties. Moreover, the synergistic effects from the use of conventional medicine in combination with the plant extracts lead to a new direction for the effective treatment of infectious diseases. This may also help especially in rural communities and informal settlements.

CONCLUSIONS

It was concluded that the extracts of ethnomedicinal plants had great potential of defence against various bacteria and fungus which can cause life-threatening infectious diseases. Overall the plants extracts were found to be more active against Gram-negative pathogens as compared to Gram-positive pathogens. The synergistic effects from the use of conventional medicine in combination with the plant extracts lead to a new direction for the effective treatment of infectious diseases. This may also helpful in rural communities and informal settlements.

SIGNIFICANCE STATEMENT

The antibacterial and antifungal activates of Vaccinium Macrocarpon fruit was investigated the first time against various Gram-positive and Gram-negative pathogens. The synergistic impact of conventional medicine in combination with the plant extracts are also reported. Moreover, the results are further compared with conventional medicine such as ciprofloxacin for antibacterial defence while Fluconazole for antifungal defence.

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