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Research Article

ANTIMICROBIAL AND PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACT OF MEDICINAL FRUIT PLANTS

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ABSTRACT

Objective: This study objective was to describe the *in vitro* antimicrobial and antifungal activity of ethyl acetate extracts from leaves of papaya, pomegranate, banana, and guava. The present investigation showed that leaves extract of fruits plants are a good source of bioactive compounds which have some ethnomedicinal applications were screened for their antibacterial activity against bacterial pathogen of human.

Methods: A total of four plant extracts were used in this study to examine their antimicrobial properties and phytochemical analysis. The antimicrobial activity was evaluated for crude ethyl acetate extracts against human pathogen *Escherichia coli, Bacillus subtilis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* using an agar diffusion assay. Phytochemical analysis tests for the screening and identification of bioactive chemical constituents of extracts of the fruits leaf was performed. This study was also conducted to determine the total phenols present in leaf extract of fruit plants.

Results: The guava leaves crude extract showed minimum inhibitory concentration of 3.75 mg/ml for *B. subtilis* and *P. aeruginosa*, which showed its efficacy as a potent antimicrobial. The phytochemical analysis of the extracts revealed the presence of bioactive compound such as saponins, alkaloids, flavonoids, terpenoids, carbohydrates, and tannins. The ethyl acetate extract of banana produced the highest zone of inhibition 23 mm for *B. subtilis*. This study showed that *Punica granatum* leaf is a good source of phenolic compounds.

Conclusion: This study concludes that these fruit leaves are a potential source for bioactive metabolites and may be used in pharmaceutical industry. On the basis of the present finding leaf extract of fruits possess, the capabilities of being a good candidate in the search for a natural antimicrobial agent against infections and/or diseases caused by human pathogens.

Keywords: Leaves extract, Fruits, Antimicrobial activities, Antifungal activities, Minimum inhibitory concentration, Phytochemicals analysis.

INTRODUCTION

Diseases are the major cause of death in the developing countries and accounts to 50% of it. Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics, including the major last-resort drugs, the frequencies of the resistance are increasing worldwide [1]. Bacterial resistance to antibiotics increases mortality likelihood of hospitalization and length of stay in the hospital [2].

Recently, there has been a lot of attention focused on producing medicines and products that are natural. Several fruits and fruit extracts, as well as arrowroot tea extract and caffeine, have been found to exhibit antimicrobial activity against *Escherichia coli* O157:H7 [3]. This suggests that plants which manifest relatively high levels of antimicrobial action may be sources of compounds that can be used to inhibit the growth of forborne pathogens. The bacterial cells could be killed by the rupture of cell walls and membranes and by the irregular disruption of the intracellular matrix when treated with plant extracts [4].

Plant-based antimicrobials represent a vast untapped source. The use of the plant extract for medicinal treatment has become popular when people realized that the effective life span of antibiotic is limited and over-prescription and misuse of traditional antibiotics are causing microbial resistance [5]. Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties [6]. All medicinal plants produce important secondary metabolites such as terpenoids, flavonoids, polyphenols, chlorophylls, and betalains. Among these, phenolic compounds are considered to be the chief plant constituent because of its capacity to exhibit antioxidant, anti-cancerous, and anti-inflammatory properties [7].

In recent years, antimicrobial properties of the medicinal plants are being increasingly reported from different parts of the world [8]. At present, nearly 30% or more of the modern pharmacological drugs are derived directly or indirectly from plants and their extracts dominate in homeopathic or ayurvedic medicines [9]. Considering the vast potentiality of plants as sources for antimicrobial drugs, this study aimed to detect the antibacterial activities of some natural plant extracts and investigate the effect of some commercial antibiotics against multi-drug resistant human clinical bacterial isolates.

Musa acuminata, commonly known as banana plant, is vastly being consumed across the world. It is known for many pharmacological activities and reports show that banana leaves contain a large amount of phenolic compounds especially polyphenol oxidase which is used in the treatment of Parkinson's disorder [10].

The pomegranate (*Punica granatum*) an ancient, mystical, and highly distinctive fruit belong to Punicaceae family. Chemical constituents of the leaf extract of *P. granatum* are almost similar to those of the fruit or seed, e.g., ellagic acid, tannins (punicalin and punicafolin), and flavones glycosides including luteol in and apigenin. In previous study, it is reported that the antidiabetic and antihyperlipidemic effects of ethanolic extract of *P. granatum* in alloxan-induced diabetic rats [11].

Papaya (*Carica papaya* L.) is a member of Caricaceae Family. The papaya is especially susceptible to parasites, pests, and diseases. This fussy

plant needs a lot of water but must have good drainage and it bears most fruit in light, porous, slightly acidic soils that are rich in organic matter. It has been used to treat digestive problems and intestinal worms as well as warts, sinusitis, eczema, cutaneous tubercules and hardness of the skin. Green fruits are used to treat high blood pressure, roundworm infection, dyspepsia, constipation, amenorrhea, skin disease, general debility, and genitourinary disorders [12]. The traditional use of *C. papaya* leaves in the treatment of various ailments including urinary tract infections informed the need for this study, which was aimed at evaluating the antibacterial activity of the leaves against some agents of urinary tract infection.

Guava (*Psidium guajava*) in medicine, extracts of leaves are used to treat gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions. Guava leaves contain essential oil with the main components being α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene, and curcumene [13].

This study was carried for screening the antibacterial activity of four fruits plants used for the clinical treatment by local communities against some pathogenic bacterial strains.

METHODS

Survey and collection of plant materials

The fresh and healthy leaves of banana, pomegranate, papaya and guava were collected from different parts of Ghurdauri, Pauri, Garhwal, Uttarakhand, India. Leaves samples were brought to the laboratory and thoroughly washed in distilled water. Each plant material was labeled, numbered and information regarding date of collection, medicinal use of plant leaves and location from were collected recorded. Plant materials were identified and contaminated particles were removed. They were air-dried and ground into fine powder using mortar and pestle in the laboratory [14].

Preparation of leaves extracts

The leaves extract of total four fruits plant were air dried, chopped and again shade dried for 14 days at room temperature and then grounded leaf powder using a grinder for ease of the extraction of active compounds. The powdered plant material (15 g) was packed into a soxhlet apparatus and extracted up to 4 hrs with ethyl acetate (250 ml) at 60-80°C for defatting [15]. The extract was filtered, and the solvent was evaporated under reduced pressure using a rotary vacuum evaporator at 65°C. These purified extracts were then stored at 4°C until further use.

Antimicrobial assay

Based on their clinical and pharmacological importance, *Bacillus subtilis, E. coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were chosen for evaluating antibacterial activity. These bacterial strains obtained from Institute of Microbial Technology, Chandigarh, and fungal isolates from Forest Research Institute, Dehradun. The bacterial stock cultures were incubated at 37°C for 24 hrs on nutrient agar and fungal pathogen *Penicillium oxalicum* and *Alternaria alternate* on potato dextrose agar (PDA) at 27°C for 24 hrs followed by storage at 4°C until further use.

A total of four plant extracts were used in this study to screen their antimicrobial activity. Antibacterial susceptibility testing was done using the well-diffusion method. The plant extracts were tested on Mueller-Hinton Agar plates to detect the presence of antibacterial activity. All plates were inoculated with the test bacterium, a sterile cotton swab was dipped into the suspension, rotated several times, and pressed firmly on the inside wall of the tube above the fluid level removing excess inoculum. The surface of the agar plate was streaked over the entire sterile agar surface rotating the plate to ensure an even distribution of inoculums. After spreading the plates with bacteria, 5 mm diameter wells were punched into the medium using a sterile borer. The plates are allowed 3-5 minutes to dry the excess moisture. 50 µl aliquots of each test extract were dispensed into each well-after the inoculation of the plates with bacteria. For each bacterial strain, controls were maintained where pure solvents were used instead of the extract. The plates are sealed with parafilm, labeled, and placed in an incubator set to 37°C. After 24 hrs of incubation, each plate was examined for inhibition zones. A ruler was used to measure the inhibition zones in mm.

Determination of minimum inhibitory concentration (MIC)

MIC was determined after antibacterial activity of the plant leaf crude extracts by the standard method with minor modification. Muller-Hinton Broth was made and sterilized using autoclave. 1.0 ml of the prepared broth was dispensed into the test tubes labeled from 1 to 5 using sterile syringe and needle. A stock solution containing 60 mg/ml of the extract was prepared. Then, 1.0 ml of the solution was dispensed into the tube 1. Subsequently, from tube 1 solution was serially transferred until tube 5-1.0 ml of the solution was discarded from it. Tube 6 was used as a control for sterility of the medium and tube 7 for viability of the organisms. An overnight culture of each of the test isolates was prepared in sterile nutrient broth. 1 ml inoculum was transferred into each tube from tube 1 to tube 7 with exception of 6, to which another sterile broth was added. The final concentration of the extract in each of the test tubes numbered after dilution 60, 30, 15, 7.5, 3.75 mg/ml was incubated at 37°C for 24 hrs and examined for growth. The test tube in which growth failed to occur was the MIC of the culture.

Antifungal activity assay

PDA medium with 25% concentration of the solvent extracts of the test fruit plants was prepared. About 15 ml of the medium was poured into each Petri plate and allowed to solidify. 5 mm disc of 7-day-old culture of the pathogenic fungi was placed at the center of the Petri plates and incubated at 27±2°C for 7 days. After incubation, the colony diameter was measured in millimeter. PDA medium without the solvent extract served as control. The fungitoxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the formula

$$\% \text{ Inhibition} = dc\text{-}dt/dc \times 100$$

Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelia growth in treatment [16].

Phytochemical screening of fruits leaves crude extracts

Phytochemical analysis tests for the screening and identification of bioactive chemical constituents of extracts of the fruits leaf were performed with the standard methods with modifications [17].

Test for saponins

About 1 ml aliquots of the various plant leaf extracts were combined with 5 ml water which is at 60°C then shaken for 2 minutes as saponins are known to process frothing activity, the volume of froth produced in this experiment was observed.

Test for phenolic compounds

A value of 1 ml of test solution was treated with 10% ethanolic ferric chloride. Phenolic compounds were considered present when a color changes to dark green or bluish black.

Test for tannins

The plant leaf crude extract was treated with alcoholic 0.1% ferric chloride reagent. A bluish black color will appear.

Test for terpenoids

The plant leaf crude extract was treated with 2 ml chloroform and 3 ml sulfuric acid carefully. Reddish brown precipitate will appear if terpenoids are present.

Test for flavonoids
The plant leaf crude extract was treated with drops of 20% NaOH. Yellow appears which become colorless on adding dilute HCl.

Test for alkaloids
The plant leaf crude extract was evaporated to dryness in a boiling water bath. The residue was dissolved in 2 NHCl. The mixture was filtered and the filtrate was treated with equal amount of Wagner's reagent. The reaction shows the appearance of brown precipitate indicates the presence of respective alkaloids.

Test for carbohydrates
Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used for the detection of carbohydrates. Few drops of Benedict's reagent was added to the test solution and boiled on water bath. The formation of reddish brown precipitate indicates the presence of sugars. Depending on the concentration of the reducing sugar, the amount and color of the precipitate produced varied. A positive Benedict's test appears green, yellow, orange, or red.

Determination of total phenolic compounds
The concentration of phenolics in leave extracts was determined using spectrophotometric method [18]. The reaction mixture was prepared by mixing 0.5 ml of aqueous solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of sodium bi carbonate ($NaHCO_3$). The samples were thereafter incubated in thermostat at 45°C for 45 minutes. The absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid (0.010-0.060 mg/ml) and the calibration line was construed. Fruits leaf extracts were prepared in ethyl acetate at a concentration of 0.06 g/ml. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line, then the content of phenolics in extracts was expressed in terms of mg/g of the extracts as gallic acid [19].

RESULTS

Antibacterial activity was assessed by analyzing the diameter of the zone of inhibition and the ethyl acetate extract of leaves from four fruit plants of medicinal value. In the case of *P. aeruginosa* and *B. subtilis* isolates, the highest zone of inhibition was 23 mm which was exhibited by *Musa sapientum*. The leaf extract of *P. guajava* exhibited activity against *P. aeruginosa* and *B. subtilis* (20 mm) isolates as well as against *E. coli* and *S. aureus* (15 mm and 21 mm) isolates (Table 1, Figs. 1-4). *M. sapientum* is an antimicrobial agent against infections and/or diseases caused by *P. aeruginosa* and *S. aureus*.

MIC
All leaves extracts showing potent antibacterial activity was further determined for their MIC (Table 2). The extracts have shown MIC ranged from 30 to 3.75 mg/ml for against *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis*. Leaf extract of banana showed MIC of 7.5 mg/ml for *E. coli*, whereas Guava showed MIC of 3.75 mg/ml for *P. aeruginosa* which showed its efficacy as a potent antimicrobial. The ethyl acetate extract of papaya has shown maximum MIC of 3.75 mg/ml for *E. coli*, whereas pomegranate shows MIC of 3.75 mg/ml for *P. aeruginosa*.

Antifungal activity assay
Antifungal activities were performed against the two fungal strain including *P. oxalicum* (Fig. 5) and *A. alternate* (Fig. 6). The inhibition (%) of leaves extract against fungal pathogen is shown in Table 3. The best activities are banana ethyl acetate against *P. oxalicum* with inhibition of 40%, which on further chemical investigation will lead to isolation of antifungal chemicals.

Phytochemical Screening of leaf extract
Photochemical analysis of plant leaf extracts has shown the presence of almost all the photochemical (Table 4).

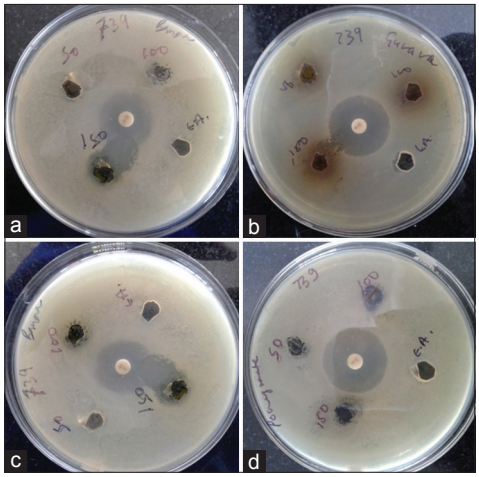

Fig. 1: Antibacterial activity shown by leaves extracts of fruits plants on pathogens against *Escherichia coli* (a): Banana (b): Guava (c): Papaya (d): Pomegranate

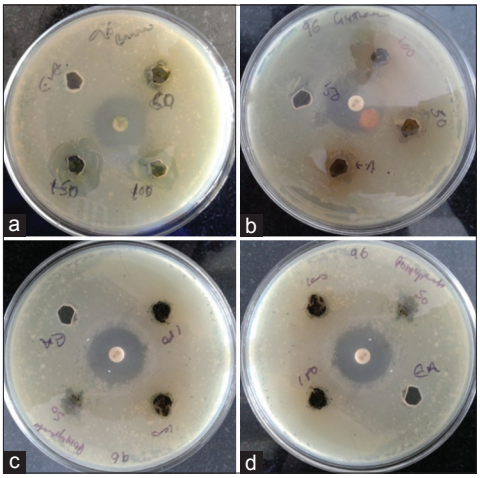

Fig. 2: Antibacterial activity shown by leaves extracts of fruits plants on pathogens against *Pseudomonas aeruginosa* (a): Banana (b): Guava (c): Papaya (d): Pomegranate

Table 1: Antimicrobial activity of medicinal plant leaf ethyl acetate extract against selected bacteria

S. No.	Plant	Mean clear zonal diameter (in mm)			
		P. aeruginasa	*E. coli*	*S. aureus*	*B. subtilis*
1	Banana	22	11	21	23
2	Guava	20	15	21	20
3	Papaya	10	10	15	12
4	Pomegranate	12	15	15	20

P. aeruginasa: Pseudomonas aeruginasa, E. coli: Escherichia coli, S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis

Table 2: Minimum inhibitory concentration of the crude ethyl acetate extract of plant leaves

Plant extract	Minimum Inhibitory concentration (mg/ml)			
	P. aeruginasa	*E. coli*	*S. aureus*	*B. subtilis*
Banana leaf	30	7.5	15	15
Guava leaf	3.75	25	15	3.75
Papaya leaf	7.5	3.75	7.5	30
Pomegranate leaf	3.75	7.5	15	7.5

P. aeruginasa: Pseudomonas aeruginasa, E. coli: Escherichia coli, S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis

Table 3: Antifungal activity of the crude ethyl acetate extract of plant leaves

S. No.	Leaves extract	Percentage of Inhibition	
		Penicillium oxalicum	*Alternaria alternate*
1	Banana leaf extract	40	10
2	Guava leaf extract	25	14
3	Papaya leaf extract	25	20
4	Pomegranate leaf extract	15	10

Table 4: Phytochemicals present/absent in plant extracts

Phyto-constituents	Banana leaf extract	Guava leaf extract	Papaya leaf extract	Pomegranate leaf extract
Saponins	+	+	+	+
Tannins	+	+	+	−
Flavonoids	+	+	+	−
Alkaloids	+	+	+	+
Carbohydrate	+	+	+	+
Terepnoid	+	+	−	+
Phenolic compound	+	+	+	+

(+): Indicates the presence of secondary metabolite, (−): Indicates the absence of secondary metabolite

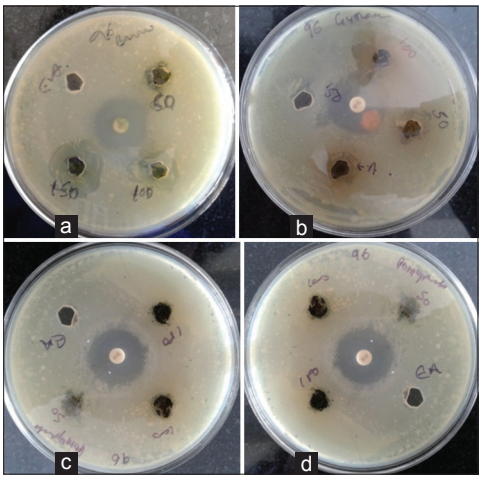

Fig. 3: Antibacterial activity shown by leaves extracts of fruits plants on pathogens against *Staphylococcus aureus* (a): Banana (b): Guava (c): Papaya (d) Pomegranate

Determination of total phenol

The amount of total phenol was determined with the Folin-Ciocalteu reagent. The calibration curve showed linearity for gallic acid in the range of 3.75-30 mg/ml, with a correlation coefficient (R^2) of 0.9992 (Figs. 6-8). Leaves of pomegranate contained the highest content of phenolics (104±2.0 mg/g), followed by banana (92.54±1.2 mg/g), guava (79.8±4.6 mg/g) and papaya (65.7±2.4 mg/g) (Fig. 7).

DISCUSSION

Guava leaf extract found good antimicrobial activity against nine different strains of *S. aureus* [20]. The antibacterial activity of guava leaf extract was tested against acne developing organisms [21]. The antimicrobial effects of pomegranate were previously studied. Indeed, it is reported that the bark, leaves, flowers and fruits of pomegranate are widely used as phytotherapeutic agents in Brazil [22]. Antibacterial activity of *P. granatum* L. stem, aqueous and different solvent extracts of stem of this plant were evaluated for antibacterial activity against Gram-positive (*Staphylococcus epidermidis* and *Bacillus megatarium*)

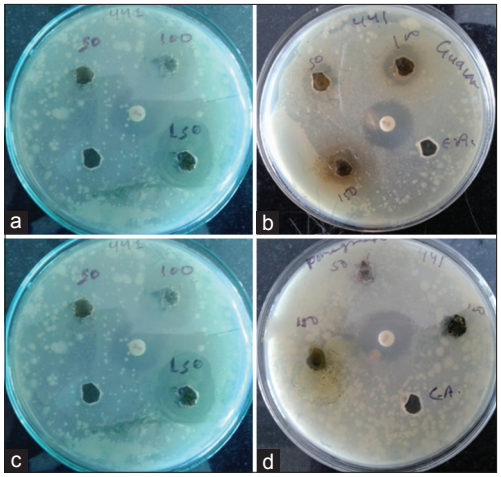

Fig. 4: Antibacterial activity shown by leaves extracts of fruits plants on pathogens against *Bacillus subtilis* (a): Banana (b): Guava (c): Papaya (d): Pomegranate

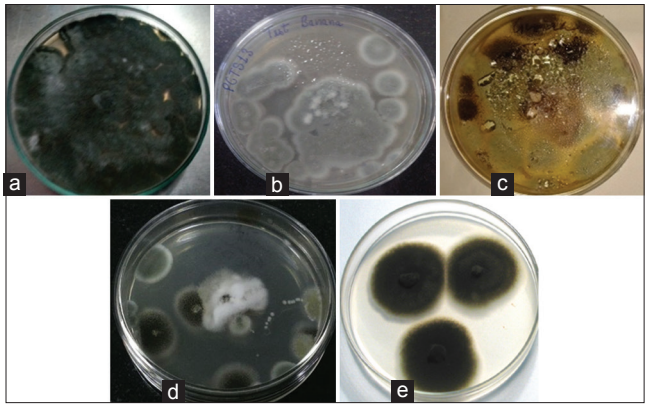

Fig. 5: Antifungal activity shown by crude extracts of medicinal fruit plants against fungal pathogen *Penicillium oxalicum* (a): Control (b): Banana (c): Guava (d): Papaya (e): Pomegranate

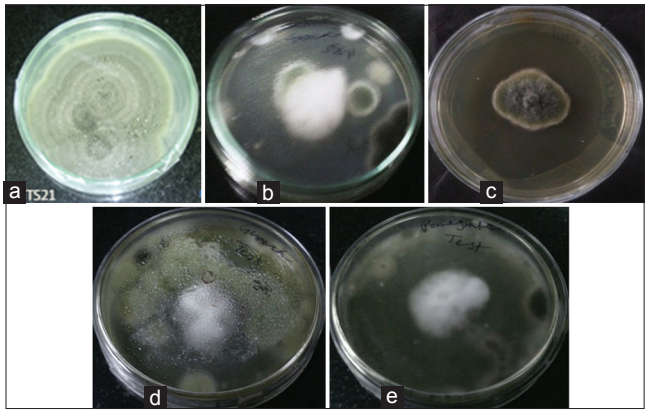

Fig. 6: Antifungal activity shown by crude extracts of medicinal fruit plants against fungal pathogen *Alternaria alternata* (a): Control (b): Banana (c): Guava (d): Papaya (e): Pomegranate

and Gram-negative (*Proteus morganii, Enterobacter aerogens* and *Alcaligenes fecalis*) bacteria of ATCC strain [23]. However, the plants differed in their antimicrobial activity against test organism at various concentrations. It is also reported that Gram-negative bacteria are more susceptible to the extracts of papaya leaf and stem [24]. According to a report the aqueous extract from leaves of *Musa paradisiacavar*

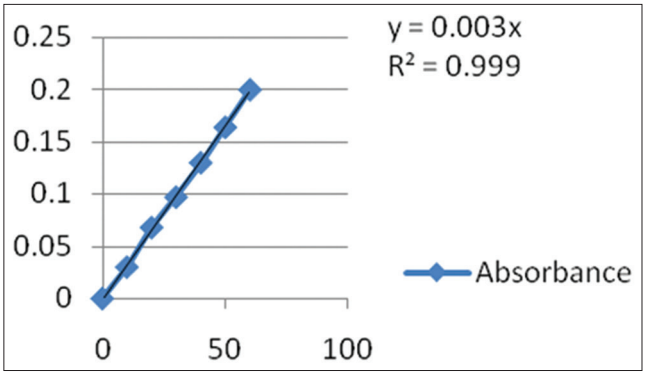

Fig. 7: Standard calibration curve of gallic acid at concentrations of 5, 10, 20, 30, 40, 50, 60 µg/ml. Spectrophotometric detection was at 765 nm

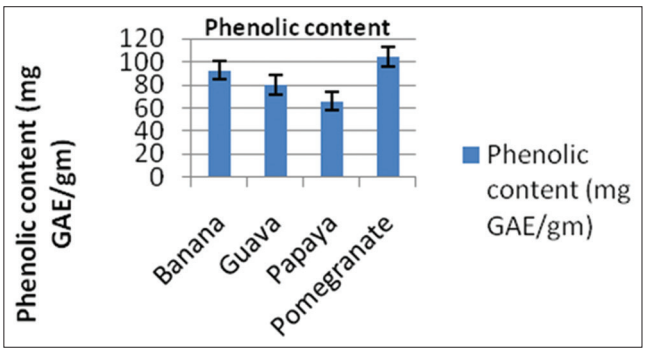

Fig. 8: Total phenolic content of fruit leaf extracts determined by the Folin-Ciocalteu assay and calculated as gallic acid equivalent in mg. Results are the average of triplicates±standard deviation

sapientum exhibited activity against pathogenic bacteria such as *Staphylococcus* and *Pseudomonas* species [25].

All active leaves extracts showing potent antibacterial activity were further determined for their MIC by a tube dilution technique against *P. aeruginosa, E. coli, S. aureus* and *B. subtilis.* The MIC of *P. granatum* aqueous leaf extract in the case of *B. subtilis*, *S. aureus* and *Salmonella typhi* were found to be 0.36 mg/ml, 0.13 mg/ml and 0.13 mg/ml respectively [26]. Ethanol extract of Guava leaves shows highest MIC of 40 µl was against *E. coli* [27]. Using aqueous extracts of *C. papaya* leaf and seeds of mature papaya fruit (2:10 weight/v) at a 10:4 v/v proportion in PDA reported no inhibition of *C. gloeosporioides* after 7 days incubation compared to control [28].

Three extracts (ethanol, petroleum ether and acetone) showed excellent antifungal activity against both *Aspergillus terreus* and *Penicillium solitum*. A host of plant extracts including ethanolic extracts of pomegranate showed antifungal activity against *Candida albicans* [29]. The previous study demonstrated that the acetone extracts of leaves of above plants showed moderate to strong activity against *Aspergillus niger, Fusarium* sp., and *C. albicans* [30].

Chemical analysis was carried out on the plant leaf extracts to determine the presence of chemical components as a prospective source for medicinal and industrial use. Their presence is an indicator that they can be exploited as precursors in the development and advancement of synthetic drugs. The active metabolites contain chemical groups such as phenols, flavonoids, terpenoids, alkaloids, tannins, carbohydrates, and saponins. Guava leaf extracts contain flavonoids, tannins, α-pinene, limonene, phenolic compounds, saponins and many other important compounds, and these compounds show very useful and important properties such as anti-diarrheal, antibacterial, anticancer, antimicrobial, antimalarial, hepatoprotective effect, anti-oxidant, anti-allergic, anti-glycemic, anti-inflammatory, wound healing, analgesic and many more [31]. The phytochemical analysis of the crude ethyl acetate extracts of plant leaf has shown the presence of almost all the phytochemicals in *C. papaya* leaves showed the presence of alkaloids, which are quite probably an important element in defense against plant pathogens [32].

All leaves extract has shown the presence of phenol as bioactive metabolite. The antioxidant activity of the phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. It is reported that the content of thyrotoxic periodic paralysis in the peel of Chinese pomegranate was 249.4±17.2 mg/g tannic acid equivalents/g fresh weight [33]. Previous reports showing that the phenolic compound content of water extract of guava leaf extract was a higher than in pure ethanol and pure methanol extracts [34,35]. It is found that exacts of M. sapientum var. Sylvester is contained a significant amount of phenols, flavonoids, and proanthocyanidins [36].

Phenolic contents are partially responsible for antimicrobial activity of fruits leaves, corroborating the relevance of fruit leaves as a healthy alimentary products as a source of antioxidant and multi-resistant bacteria drug substances. It is reported that the leaves of plantain banana (*M. sapientum var. paradisiaca*) can be used by the tribals of Western Ghats in India for bandaging cuts, blisters and ulcers [37].

CONCLUSION

The findings of this investigation suggest that the ethyl acetate extraction was suitable to identify the various phytochemicals of medicinal plants and they supported by many investigation. The crude ethyl acetate extract of fruit plant showed significant antimicrobial activity against test strains. This indicated the great potential of these plant extracts as effective antimicrobial agents that can be used as single or in combination in medicines.

This study justifies the claimed uses of leaves in the traditional system of medicine to treat various infectious diseases caused by the microbes. The obtained results may provide a support to use of the plant in the traditional medicine. Based on this further chemical and pharmacological investigations can be done to isolate and identify minor chemical constituents in the fruits plant and to screen other potential bioactivities may be recommended.

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