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Application of Plants Extracts against Bacterial Strains causing Food Poisoning for its Potential Application in Food Preservation

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Abstract : *Prevention of food from spoilage and pathogens is usually controlled by the use of chemical preservatives which have various negative impacts on human health. The chemical residues go in the food and feed chain moreover it makes the microbes more resistant to the used chemicals. Thus, the necessity to find a potentially effective, healthy, safer and natural alternative preservative. In the present study, different ethanolic plants extracts have been used to analyze their antimicrobial activity against the food borne pathogens. E.coli,Staphyloccoccus and Pseudomonas are prevalent food borne pathogens in many foods like meat, chicken and various raw foods. Various plants and herbs contain flavonoids and many other phytochemicals which are detrimental for the growth of microorganisms. In the present study ethanolic extracts of Pomegranate (Punica grantum), Thyme (Thymus vulgaris), Sweet Orange (Citrus sinensis), and Bay leaf (Laurus nobilis), Guava leaf (Psidium guajava) were checked for their antimicrobial activity against E. coli, Pseudomonas aeruginosa and Staphylococcus aureus.*

Keywords: *E. coli, Staphylococcus, Pseudomonas,* food borne, flavonoids.

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Introduction :

Food poisoning is considered as one of the most common cause of illness and death in developing countries (Doughari et al., 2007; Pirbalouti et al., 2009; Sapkota et al., 2012). Most of food poisoning reports are associated with bacterial contamination especially members of Gram negative bacteria like *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa* (Solomakos et al., 2008; Pandey and Singh, 2011).

Other Gram positive bacteria including *Staphylococcus aureus* and *Bacillus cereus* have been also identified as the causal agents of food borne diseases or food spoilage (Braga et al., 2005). Prevention of food spoilage and their etiological agentis traditionally achieved by the use of chemical preservatives (Yamamura *et al.*, 2000; Shan et al., 2007). Excess usages of chemical preservatives are leading to the acquisition of microbial resistance to the applied chemicals and unpleasant side effects of these chemicals on human health (Akinyemi et al., 2006; Bialonska *et al.*, 2010).

Plant products have also been used since ancient time for flavoring foods and beverages, and for medicinal purposes with varying success to cure and prevent diseases.The value of plants lies in some chemical substances that produce a definite action on the microbiological, chemical and sensory quality of foods, and these phytochemicals have been grouped in several categories including polyphenols, flavonoids, tannins, alkaloids, terpenoids, isothiocyanates, lectins, polypeptides or their oxygen-substituted derivatives (Cowan, 1999)

The utilization of plant extracts as antimicrobial agents for food preservation is safe (Cowan, 1999; Duffy and Power, 2001; Berahou *et al.*, 2007; Chika *et al.*, 2007, Nasar-Abbas and Kadir, 2004; Hara Kudo *et al.*, 2004; Mathabe *et al.*, 2005).

The use of natural antimicrobials such as organic acids, essential oils, plant extracts, and bacteriocins could be of great significance in food preservation. The use of plant extracts with known antimicrobial properties can be of great significance in food preservation. Natural products, from plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for control of microbial growth owing to chemical diversity.

Materials and Methods :

Isolation of food borne pathogens

(a) From Mutton : The samples were aseptically cut into thin smaller pieces using sterile knife. The analytical portions were placed in separate sterile conical flask to which 250 ml of Buffered Peptone Water was added. The flask were shaken vigorously and incubated for 5 hours at 37°C then sample rinsate was collected. (Anu et.al, 2015).

(b) From spoiled onion : The sample was weighed 1gm and dissolved in100ml of D.W for the preparation of stock solution. Serial dilution was prepared using the prepared stock solution. Then 9ml of D.W was measured and placed in each 9 test tubes i.e., serial dilution of 10^{-1},10^{-2} & 10^{-3} prepared in triplicates, (tubes was autoclaved and cooled before dispensing). 1ml of stock solution was dispensed into the 10^{-1} tube and was shaken. Then 1ml was transferred from 10^{-1} to 10^{-2} and then from $10^{-2}$to$10^{-3}$and was shaken (Mohammad et al, 2017).

Direct Plating for Culture : For cultivation of *Escherichia coli*, the sample rinsate was inoculated into double strength MacConkey Broth at 37°C for 24 hours after which it was plated onto Eosin Methylene Blue agar. For the isolation of *Staphylococcus aureus* and other non-fastidious bacteria, the rinsate was inoculated into nutrient broth at 37°C for 24 hours after which it was plated onto Mannitol Salt agar (Anu et.al, 2015). For the isolation of *Pseudomonas aeruginosa* 0.1ml of serially diluted sample was plated on *Pseudomonas* Agar Base media and then incubated at 37°c for 24 hours (Mohammad et al, 2017).

Identification of isolated bacteria : Characterization and identification of the colony isolates was achieved by initial morphological examination of the colonies in the plate

(macroscopically) for colonial appearance, size, elevation, form, edge, consistency, color, odor, opacity and pigmentation and the results were recorded. Gram's staining from the colonies provided a preliminary identification of the pathogenic bacteria. After identifying an isolates by Gram's reaction-Gram's staining, Motility determination- Soft agar stabbing (Tube Method), Catalase, Nitrate Reduction, IMVIC test, Carbohydrate Utilization, Urease production, Gelatin Hydrolysis, TSIA were performed for the confirmation of the Bacterial isolates according to the Bergey's manual.(Aneja, 2009).

Plant extracts preparation : The selected plants for extract preparation include Pomegranate *(Punica grantum*), Thyme (*Thymus vulgaris*), Sweet Orange(*Citrus sinensis*), Bay leaf (*Laurus nobilis*) and Guava leaf (*Psidium guajava*). The collected plants material was washed with water, disinfectant (Ethanol70%, $Hgcl_2$1%), rinsed with distilled water and finally dried in shade.The dried plant material of each plant species was grounded into fine powder to pass 100 mm sieve. 50 g of the fine powder was soaked in 200 ml of ethanol with stirring for 48 h., filtered through double layers of muslin, centrifuged at 9000 rpm for 10 min. And finally filtered again through Whatman filter paper No. (41) to attain a clear filtrate.The filtrates were evaporated and dried at 40°C under reduced pressure using rotatory vacuum evaporator.The extract yields were measured, stored in a small bottles in fridge at 5°C and their yield percentages were calculated using the following formula: Extract yield% = R/S100 (where R = weight of extracted plants residues and S = weight of plant raw sample).

Inoculum preparation : Each isolated bacterial strain was sub cultured overnight at 37°C on Nutrient Agar media. The bacterial growth was harvested using 5ml of sterile saline water.

Antibacterial activity of plants extract : The antibacterial effect of prepared extract was tested against the growth of gram -ve bacteria (*Escherichia coli and Pseudomonas aeruginosa*) and gram +ve bacteria (*Staphylococcus aureus)* using well diffusion method. An inoculum of each bacterial suspension was swabbed over the surface of MHA plates. A 5mm well was made on each plates using sterile cork borer. 0.15 ml of each plant extract was pipette into each well. Organic solvent (ethanol) was also introduced into well for -ve control. Disk loaded with 10 µg of Gentamycin was used as positive control. Plates were then incubated at 37°C for 24 hours. The presence of inhibition zones was measured, recorded as average diameter of the inhibition zone surrounding the wells containing extract and considered as indication for antibacterial activity.

Determination of minimum inhibitory concentrations (MIC's) of the effective plants extract : MIC can be defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after 24 h. of incubation. The most effective plant extracts which exhibiting a strong antibacterial activity at 0.15ml was used to determine their MIC using well diffusion method and evaluate their efficiency in controlling bacterial strains causing food poisoning. Different concentrations of the effective plant extract (2.5mg/ml, 5mg/ml, 10mg/ml, 12.5mg/ml, 15mg/ml, 20mg/ml) was prepared separately by dissolving extract in 5 ml of ethanol, sterilized through Millipore filter. Mueller-Hilton Agar was poured into sterile Petri dishes and swabbed with bacterial suspensions of the pathogenic strains. The well was loaded with different concentrations of the effective plant extract in Mueller-Hilton agar plates. The plates were then incubated at 37°C for 24 hours. The inhibition zones was measured and recorded against the concentrations of the effective plant extracts.

Statistical Analysis : The data recorded during the course of study was analyzed statically using Chi square test and interpretation was drawn accordingly.

Results and Discussion :

Bacterial colonies with Metallic Green Sheen were observed on the Eosin Methylene Blue Agar. Yellow colored colonies were observed on Manitol Salt Agar and on the *Pseudomonas* Base agar, Bluish- green colony was observed. (Table1). After cultural and morphological identification different biochemical test were performed, confirming that the isolated organism were *Esherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, 16 S rRNA sequencing is required for further confirmation. (Table 2)

Plant extraction yield : The ethanobotanical data of the employed plants and their extract percentage yield are illustrated in Table 3. The extract of 37 g of dried plant materials with ethanol yielded plant extract residues ranged from 1.23g to 4.20 g. The highest yield of plant extract was obtained from *Punica granatum* (4.20 g) followed by *Thymus vulgaris* (3.25 g) while *Psidium guajava* give the lowest extract yield respectively.(Table 3)

Antibacterial activity of plants extracts : Five plant species were investigated to evaluate their antibacterial activity against food poisoning bacteria including one strains of Gram positive bacteria *(S. aureus*) and two strains of Gram negative bacteria (*E. coli and P. aeruginosa*) using well diffusion method. Evaluation of antibacterial activity of these plant extracts were recorded in Table 4 and is illustrated in Fig.1 The results revealed that all plant extracts were potentially effective in suppressing microbial growth of food poisoning bacteria with variable potency. *P. granatum* was the most effective extract retarding microbial growth of all tested pathogenic bacteria at concentration of 15 mg/ml. Other plant extracts showed variable antimicrobial activity against food poisoning bacterial strains. *Laurus nobilis and Citrus sinensis* exhibited inhibitory effect against only one of the pathogenic strains (*E. coli)* whereas *T. vulgaris* was most effective against *E.coli*. Results of antimicrobial activity of the five plant extracts can suggest that *E. coli* was the most resistant strain to plant extracts. Whereas the most susceptible strains to the ethanolic plants extract was *S. aureus*. *P. granatum, P. guajava and T. vulgaris* extracts were the most effective extracts and showed a strong antibacterial activity against bacteria causing food poisoning. The organic solvent (Ethanol) was used as –ve Control but it was not showing inhibitory effect against the bacterial strains. Thus, it shows that it was the extract which showing the inhibitory effect against the bacterial strains. Hence, experiments were conducted to determine their minimal inhibitory concentration (MIC) against the most susceptible bacterial strains (*S. aureus and P .aeruginosa*).

Minimum inhibitory concentrations (MIC's) of the effective plants extract : The MIC of the most effective plant extracts (*P. granatum* and *Psidium guajava*) was employed by well diffusion method to evaluate their bacteriostatic and bactericidal properties. The inhibitory effect of *P. granatum* extract started at 5mg/ml with inhibition zones of 7mm and 6mm against *S. aureus and P. aeruginosa* while extract of *P.guajava* also suppressed bacterial growth of these strains at concentration of 5mg/ ml with inhibition zones of 9mm and 5mm respectively. (Table 5) (Fig.2)

Statistical Analysis : The data analyzed by Chi square test showed that there was an association found between the antibacterial activity of plant extracts and isolated organism.

The results of MIC's of the effective plant extracts suggested that *P. granatum, P. guajava* and *T. vulgaris* can be used to control and prevent food

borne bacteria and food poisoning diseases. Bacterial strains included in this study were chosen for their importance in food spoilage and food poisoning. *S. aureus* considered as the one of the most common source of food borne disease while *E. coli* and *P. aeruginosa* produce toxins and other metabolites that induce human gastroenteritis diseases. *P. granatum*, *P. guajava* extract suppressing microbial growth of all tested bacterial strains followed by extract of *T. vulgaris* which also appear to be potentially effective against all three bacterial strains.

These results are in accordance with those of Verma *et al.(2012),* Qader *et al. (2013)* and Mahboubi *et al. (2015).* A great variation in MIC of *T. vulgaris* extract demonstrated in several investigations may be due to considerable variation in their method of extraction, constituents as well as bacterial strains used. Also, variation in MIC of different plant extracts may arise from variation in their chemical constituents and volatile nature of their constituents. On the other hand, *P. guajava* extract was found to be effective with concentration of (15 mg/ml) against *S. aureus*, *E. coli* and *P. aeruginosa* suppressing their growth with inhibition zones of 28mm, 20mm and 28mm respectively. These results are in accordance with that of Mahfuzul Hoque *et al.(2007*) *C. sinensis* was found to be ineffective in controlling the bacterial strains and these results were contrasted with that of *B.* RajaNarendra *et al.(2013*) who reported *C. sinensis* potentially effective with inhibition of 15mm zone. On the other hand, a higher concentration of *C. sinensis* extract may be required to be effective against food spoilage bacteria and these results were coincident with that previously reported by Verma *et al.* (2012). The present study suggested that plant extracts which proved to be potentially effective can be used as natural preservatives to control food poisoning diseases and preserve food avoiding application of health hazards of chemical preservatives.

Conclusion :

The present study show that the ethanolic extracts of different plant materials exhibit antibacterial activity. These extracts exhibit a maximum zone of inhibition against all three bacteria *E. coli*, *S. aureus and P. aeruginosa.*

Food spoilage is often caused by the growth of many pathogenic bacterial strains. Prevention of food spoilage in food industry and food stuff is mainly based on the application of chemical preservatives. The adverse effect of these chemical preservatives on human health increases the demand to search for potentially effective, healthy safer and natural food preservative. The plant extracts which proved to be potentially effective as (*P. granatum, P. guajava and T. vulgaris*) can be used as natural alternative preventives.

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Table 1. Cultural and morphological characteristics of bacteria isolated on selective media

S. No.	Source	Media	Colony Morphology	Gram Reaction	Shape & Arrangement
1.	Mutton	EMB	Greenish Metallic Sheen	-VE	Rods(Bacillus) Arranged Singly or in Pairs
2.	Mutton	MSA	Circular, 2-3mm in diameter, Smooth shiny surface, Opaque, Golden yellow in color.	+VE	Cocci in clusters (staphylococcus)
3.	Onion	PBA	Greenish- blue Colonies	-VE	Rods (Bacillus),Arranged singly or paired

Table 2. Result of the biochemical characterization of the isolates

S. No.	Biochemical test	Bacteria isolated on selective media		
		EMB	MSA	PBA
1.	Motility test	+ve	–ve	+ve
2.	Indole production	+ve	–ve	–ve
3.	Methyl red	+ve	+ve	–ve
4.	Voge'sproskauer	–ve	+ve	–ve
5.	Citrate utilization test	–ve	+ve	+ve
6.	Catalase	+ve	+ve	+ve
7.	Urease	–ve	+ve	–ve
8.	Carbohydrate fermentation • Glucose • Fructose	 +ve +ve	 +ve –ve	 +ve –ve
9.	Gelatin liquefaction	–ve	+ve	+ve
10.	Cetrimide test	_	_	+ve
11.	TSIA(Triple Sugar Ion agar)	+ve	_	alkali(red)
12.	Nitrate Reduction test	+ve	+ve	+ve
	Bacteria Isolated	***Esherichia coli* (putative)**	***Staphylococcus aureus* (putative)**	***Pseudomonas aeruginosa* (putative)**

+ve = positive, -ve = negative

*However 16 S rRNA sequencing will be required to confirm species of isolated organism.

Table 3. Ethanobotanical data of employed plant species and their extract yield percentage

Plant species	Family	Local name	Plant part used	Extract pH	Extract yield %
Punica granatum	Lythraceae	Pomegranate	Peels	4.7	11.35
Thymus vulgaris	Lamiaceae	Thyme	Leaves	6.8	8.78
Citrus sinensis	Rutaceae	Sweet orange	Peels	4.4	9
Laurus nobilis	Lauraceae	Bay leaf	Leaves	6.5	6.35
Psidium guajava	Myrtaceae	Guava	Leaves	5.5	3.32

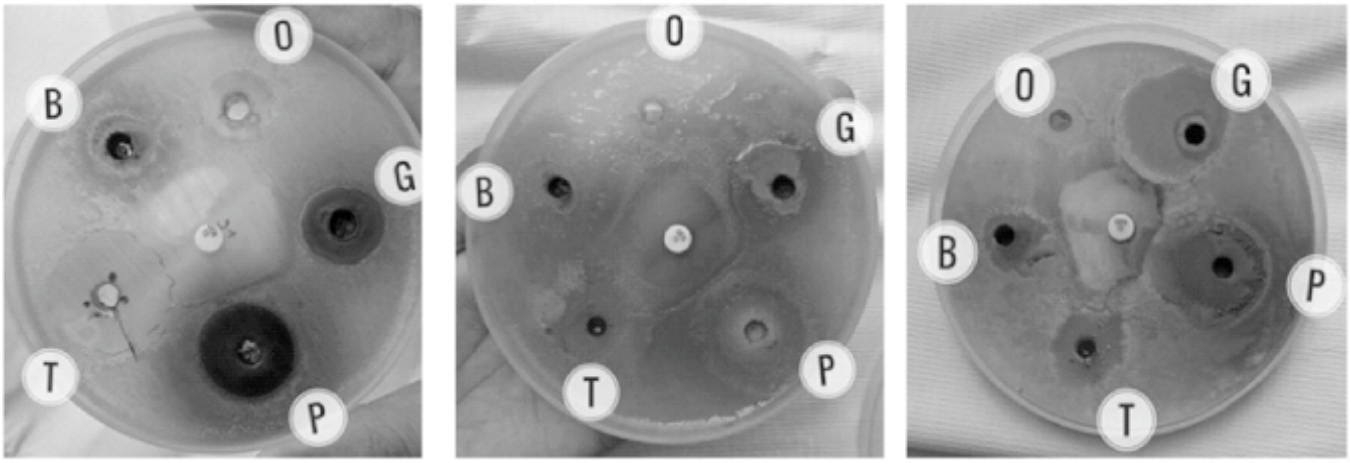
Table 4. Growth inhibition of some food poisoning bacterial strains caused by plant extracts

Plant species	Inhibition zone(mm) Gram +ve bacteria	Gram –ve bacteria	
	S. aureus	***E. coli***	***P.aeruginosa***
Punica granatum	30mm	24mm	30mm
Thymus vulgaris	21mm	28mm	19mm
Laurus nobilis	15mm	16mm	14mm
Citrus sinensis	13mm	17mm	12mm
Psidium guajava	28mm	20mm	28mm
Gentamycin	24mm	25mm	22mm

Data are means of two replicates (n = 2) * Cavity diameter = 5mm

Table 5. MIC's of the most effective plant against *S. aureus and P. aeruginosa*

S. No	Plant extract	Conc. mg/ml	Inhibition zone (mm) Gram+ve bacteria	gram –ve bacteria
			S. aureus	***P. aeruginosa***
1.	*Punica granatum*	2.5	00mm	00mm
		5	7mm	6mm
		10	16mm	12mm
		12.5	20mm	16mm
		15	30mm	30mm
		20	38mm	32mm
2.	*Psidium guajava*	2.5	00m	00mm
		5	9mm	5mm
		10	19mm	14mm
		12.5	20mm	17mm
		15	21mm	19mm
		20	30mm	21mm



Escherichia coli ***Staphylococcus aureus*** ***Pseudomonas aeruginosa***

*P- *P. granatum*, T- *T. vulgaris*, B- *L. nobilis*, O- *C. sinensis*, G- *P. guajava* +C- +VE control *(gentamycin)*

Fig. 1. Antimicrobial screening test of ethanolic plant extract (15mg/ml) against some bacterial strains of food poisoning

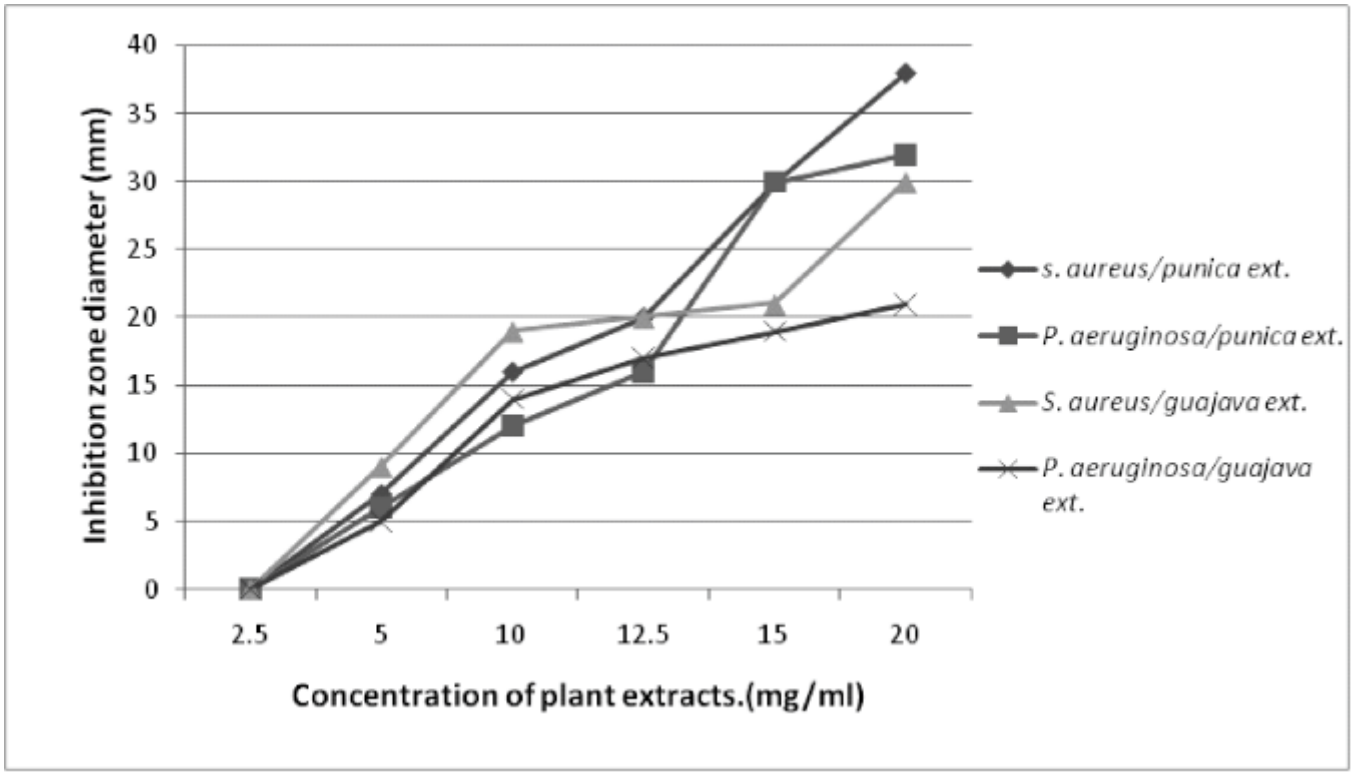


Fig. 2. MIC of effective plant extract against *S.aureus* and *P. aeruginosa*

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