Bulletin of Pharmaceutical Research 2021;11(1-3):173
An Official Publication of Association of Pharmacy Professionals
ISSN: 2249-6041 (Print); ISSN: 2249-9245 (Online)
DOI: 10.21276/bpr.2021.11.2

RESEARCH PAPER

ANTIBACTERIAL ACTIVITY STUDIES ON PAPAYA LEAVES AND ORANGE PEELS AGAINST COMMON BACTERIAL SPECIES

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Received: Dec 28, 2020 / Revised: May 06, 2021 / Accepted: May 09, 2021

The present study is aimed to observe and describe in detail the synergy between natural plant extracts and standard antibiotics combating bacterial infections. Bacterial resistance is now a very common problem caused due to the resistance developed by the bacterial strains to a particular antibiotic. This study was done to improve the effectiveness of such antibiotics which have or in near future will develop resistance towards various bacteria. The antibacterial activity of *Citrus sinensis* (orange) and *Carica papaya* (papaya) was studied against five bacterial strains: *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Staphylococcus aureus*. The antibiotics used in this study were Gentamicin and Amikacin. The combination of papaya leaf extract and orange peel extract showed inhibitory activity against all the bacterial strains. Both of these extracts were combined along with the antibiotics and tested against the bacterial strains. Most effective response was shown by the combination with antibiotic gentamycin.

Key words: Microorganisms, antibiotics, Zone of Inhibition, extraction, *Citrus sinensis*, *Carica papaya*.

INTRODUCTION

Healing potential of plants has been known from thousands of years. Medicinal properties of plants can be attributed to biologically active substances [1-5]. They can have a remarkable effect to other plants, microorganisms and animals from their immediate or wider environment. All these organic compounds are defined as biologically active substances, and generally represent secondary metabolites. These occur as an intermediate or end products of secondary plant metabolism.

Infectious diseases caused by bacteria and fungi affect millions of people worldwide. Throughout the history of mankind, infectious diseases have remained a major cause of death and disability. Today, infectious diseases account for one-third of all deaths in the world. The World Health Organization estimates that nearly 50,000 people die each day throughout the world from infectious diseases. The discovery of antibiotics was an essential part in combating bacterial infections. Antibiotics are one of the most important weapons in fighting bacterial infections and have greatly benefited the health related quality of human life since their introduction. Different antibiotics exercise their inhibitory activity on different pathogenic organisms [6].

However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed but generally extends to other compounds of the same class. Bacterial resistance and its rapid increase is a major concern of global public health and is emerging

as one of the most significant challenges to human health. Treating bacterial infections by antibiotics is beneficial but their indiscriminate use has led to an alarming resistance among microorganisms as well as led to re-emergence of old infectious diseases. Plant antimicrobials have been found to be synergistic enhancers, though they may not have any antimicrobial properties alone, but when they are taken along with standard drugs they may tend to enhance the effect of that drug [7,8].

Therefore, there is a direct need to search for new classes of antibacterial substances, especially from natural sources. Unlike synthetic drugs, antimicrobials of plant origin are not associated with side effects and have a great therapeutic potential to heal many infectious diseases [6]. Sometimes the use of single antibiotic does not produce the desired effective inhibitory effect and to overcome this, a combination of drugs is often used with plant extracts for their synergistic effect which surpasses their individual performance. Synergism is defined as a positive interaction created when two agents are combined and together they exert an inhibitory effect that is greater than the sum of their individual effects. One approach to treat infectious diseases is the use of plant extracts individually or as an alternative approach is the use of combination of plant extracts with antibiotics. This latter approach *i.e.* combination therapy or synergistic therapy against resistant microorganisms may lead to new ways of treating infectious diseases and probably this represents a potential area for further future investigations. Combination therapy can be useful to restore the antibacterial potential of the antibiotics and help in reducing the resistance developed by the bacteria [9,10].

MATERIALS

Plant description

Papaya: Name of plant: Papaya (Leaves); Biological source: *Carica papaya*; Family: Caricaceae; Synonyms: papaw or pawpaw.

Description of plant

It is widely grown plant in tropical and subtropical agro-climatic regions across the globe. In India, papaya rank 5th with regards to area and production of the papaya. Papaya plant is considered as tree although it appears to be palm like trunk, up to 8 meters (26 feet) tall and is not as woody as the designation generally implies. The plant is crowned by deeply lobed leaves, which are sometimes 60 cm (2 feet) across, borne on leaf stalk (60 cm) long. Papaya leaves contain the alkaloid named carpaine which kills microorganisms that often interfere with digestive juice (**Figure 1**).

(a) (b) (c)

Fig. 1. a) Whole plant of Papaya, b) Papaya fruit, c) Large lobed leaves

Chemical constituents

Besides carpaine, *C. papaya* leaves contained phenolic compounds such as *p*-coumaric acid, 5,7-dimethoxycoumarin, caffeic acid, kaempferol, quercetin and chlorogenic acid. It also contained components like papain, chymopapain, cystain, tocopherol, ascorbic acid, flavonoids and cyanogenic glucoside.

Uses

Consuming papaya helps in reducing risk of heart diseases, cancer, diabetes, aiding in digestion, improving blood glucose control in people with diabetes, improving wound healing and lowering blood pressure. It is used in treatment of arthritis and numerous diseases like corns, sinuses, dyspepsia, amenorrhea, warts, glandular tumor, eczema and constipation.

Studies done on the plant

In addition to antibacterial, antioxidant, immuno-stimulant properties, papaya leaf juice was associated with an accelerated rate of

increase of platelet count and are also enriched with anti-malarial properties, making it the best home remedy to fight against dengue fever and other illnesses [11]. Besides it, each part of the papaya like root, stem, leaf, flower, fruit, seed, rinds, and latex has its own nutraceutical properties. It serves as food, cooking aid, and ethnomedicine to prevent and treat wide-range of diseases and disorders. It has also been traditionally used as appetite enhancer, meat tenderizer, purgative, medicinal acne, abortifacient and vermifuge. It helps in the management of sickle-cell anaemia, HIV, heart diseases and digestional disorders too.

Orange: Name of the plant: Orange (peels); Biological source: *Citrus sinensis*; Family: Rutaceae; Synonyms: Sweet orange.

Description of plant

It is found to be most cultivated fruit tree in the world. The fruits of orange tree can be eaten or processed to have its juice or fragrant peel. Sweet orange trees often reaches up to 6 meters (20 feet) height. They have medium sized and ovate, broad, glossy evergreen leaves with petioles having narrow wing. Its usual shape and color is round and pulp orange but there are variations also (**Figure 2**).

(a) (b)

Fig. 2. a) Sweet orange fruit; b) White five-petaled flower

Chemical constituents

It consist of essential constituents such as terpenes (carveol, carvone, menthol, perillyl alcohol and perillaldehyde). In addition, it provides vitamin C, folic acid, potassium and pectin.

Uses

It is used to reduce weight, for better digestion, to cure hangover, treats bad breath, fights infection, cold and flu and also to cure asthma.

Studies done on the plant

Antioxidant, anti-oxidative burst, and phytotoxic activities [12].

Chemicals used

Nutrient Broth: It was used for inoculating the bacterial cultures and for dilutions to match the MacFarlane scale. The total quantity of nutrient broth used was 6 g; Nutrient Agar: It was used for the preparing solid growth media for the bacteria. This was poured on petri plates and solidified. The total quantity used was 90 g; Ethanol: It was used to sterilize the working tables and also for maceration of papaya leaves. The total quantity used was 600 ml; Methanol: It was used for the maceration of dried orange peels. The total quantity used was 200 ml; Dimethyl Sulfoxide (DMSO): It was used as an inert substance to prepare extract dilutions. The total quantity used was 600 ml; Barium chloride and concentrated sulfuric acid: These were used to prepare Macfarlane Scale; Antibiotics used: Gentamycin, Amikacin; Microorganisms used: *E. coli*, *B. subtilis*, *K. pneumonia*, *S. typhi*, *S. aureus*.

METHODOLOGY

Method of extraction of papaya leaves by ethanolic maceration method

The fresh leaves of papaya were collected and thoroughly rinsed in tap water and then rinsed with sterile distilled water. The leaves were dried in the hot air oven at 40°C for 3 days. The dried leaves were collected and crushed with hand then, were subjected to sterile laboratory mixer or blender to obtain a coarse powder. This powder was stored in airtight containers and protected from sunlight until the next day for maceration. The powder was then, added to 90% ethanol in stoppered conical flask and kept for 72 h for maceration with intermediate shaking. After 72 h, this extract was first filtered through muslin cloth followed by filter paper. Once the

filtrate was ready, it was subjected to hot water bath at 60°C until all the ethanol was evaporated. The total amount of yield obtained was 924 mg. The required amount (300 mg) was then weighed and dissolved in dimethyl sulfoxide [13].

Method of extraction of orange peels by methanolic maceration method

Kinoo oranges purchased from local market were peeled and the peels were washed thoroughly with tap water and then, with sterile distilled water. The peels were then, kept in hot air oven at 32°C for 4 days. After 4 days, the peels had completely dried and were cut into small pieces of approximately 3 cm size. These pieces were then, blended into coarse powder with the help of sterile laboratory mixer or blender. The powder was then, mixed with methanol and kept for maceration in stoppered conical flask for 72 hours with intermediate shaking. Once the maceration was complete, the extract was filtered through muslin cloth followed by filter paper. This filtrate was subjected to evaporation at 40°C until all the methanol was evaporated. The total amount of yield obtained was 879 mg. The required amount (300 mg) was then, weighed and dissolved in dimethyl sulfoxide [14].

Antimicrobial test

Antibacterial activity of Papaya leaves and Orange peel with some standard antibiotics was assessed against pure culture of pathogenic bacteria by using agar disc diffusion method.

Sterilization of glass wares and nutrient media

All the glass wares were sterilized by dry heat sterilization. The required quantity of nutrient broth, nutrient agar and other nutrient media were sterilized by autoclaving at 121°C for at least 15 min by using saturated steam under at least 15 psi of pressure.

Preparation of plates

The Petri plates were prepared by pouring 20 ml of sterilized nutrient agar seeded with 1 ml test culture containing 1 × 108 CFU/ml as McFarland 0.5 turbidity standard. Plates were then, allowed to solidify.

Preparation of dried filter paper discs

Whatman filter paper no. 1 was used to prepare discs approximately 6 mm in diameter, which were placed in a Petri dish and sterilized in hot air oven. Sterile filter paper discs (6 mm) were impregnated with 20 µl of each drug separately and allowed to saturate for 30 min.

Preparation of test solutions

The stock solutions of given antibiotics and plant extracts were prepared in dimethyl sulfoxide (DMSO) and stock solutions were diluted to obtain the working solutions.

Procedure

All the glassware were sterilized by dry heat sterilization. Required quantity of nutrient agar and nutrient broth was prepared. Filter paper disc were prepared by punching of holes of approximately 6 mm diameter of Whatman filter paper and sterilized in hot air oven. Seed culture of given microorganism was prepared and transferred to 1 ml of inoculum in 10 ml nutrient broth. 1 ml of seed culture was poured in a sterile petri plate and semi hot nutrient agar was added and allowed to solidify. Stock solution of given antibiotic in sterile distilled water was prepared and diluted according to the required concentration. The dilutions were added to sterilize Petri plates labeled with the respective concentrations. Once transferred, sterilized discs were placed (punched from a Whatman filter paper no. 1 each having a diameter of 6 mm) in the Petri plates and allowed it to soak for 5 min. As the discs were being soaked, it was made sure that the agar was solidified. After 5 mins, a disc was picked with the help of sterilized forceps, excess solution was drained and was placed gently on the solidified agar. This was repeated until all the 4 discs were placed at equidistance from each other in the petri plates. Petri plates were incubated at 37 °C for 24 h. After incubation, the diameter of zone of inhibition (ZOI) was measured for each of the antibiotic disc. The values obtained were compared with those on the Disk Diffusion Zone Diameter Chart to determine the susceptibility level to the antibiotics used.

RESULTS AND DISCUSSION

All the results are reported as mean±SEM. Synergistic effects resulting from the combination of antibiotics with Papaya and Orange plant extracts is studied and experimented. Antibiotics were tested on microorganisms as shown in **Table 1** which represent that all the microorganisms are susceptible to Amikacin showing maximum ZOI that is 24 mm in disc concentration of 15 µg/ml

and 15 mm in disc concentration 10 µg/ml and resistant to Gentamicin showing maximum ZOI of 15 mm in disc concentration of 15 µg/ml and minimum ZOI of 9mm in disc concentration of 10 µg/ml. Microorganisms which were most susceptible to Amikacin were *Salmonella typhi* and *Staphylococcus aureus* with ZOI of 15 mm in disc concentration of 10 µg/ml and 24 mm ZOI in disc concentration 15 µg/ml. Same microorganisms were most resistant to Gentamicin with ZOI of 9 mm in disc concentration 10 µg/ml and 12mm in disc concentration 15 µg/ml. As microorganisms were resistant to Gentamicin, this antibiotic was used along with individual herbal extract as well as with combination of herbal extract.

Table 1. Antibacterial activity of antibacterials

Microorganism	Antibiotic	Disk conc. (µg/ml)	Petri plate no. (P)	Diameter of ZOI			Average of ZOI	Interpretation (R-I-S)
				1	2	3		
Bacillus subtilis	Gentamicin	10	P-1	9	10	10	10	Resistant
		15	P-2	14	13	13	13	Resistant
	Amikacin	10	P-21	18	19	17	18	Susceptible
		15	P-22	24	24	25	24	Susceptible
Salmonella typhi	Gentamicin	10	P-3	9	9	9	9	Resistant
		15	P-4	13	12	11	12	Resistant
	Amikacin	10	P-23	20	20	21	20	Susceptible
		15	P-24	25	24	24	24	Susceptible
Klebsiella pnuemoniae	Gentamicin	10	P-5	11	11	10	11	Resistant
		15	P-6	12	14	10	12	Resistant
	Amikacin	10	P-25	15	14	15	15	Susceptible
		15	P-26	21	19	20	20	Susceptible
Escherichia coli	Gentamicin	10	P-7	11	10	09	11	Resistant
		15	P-8	15	16	15	15	Intermediate
	Amikacin	10	P-27	17	15	16	16	Susceptible
		15	P-28	20	20	22	21	Susceptible
Staphylococcus aureus	Gentamicin	10	P-9	10	9	9	9	Resistant
		15	P-10	14	11	12	12	Resistant
	Amikacin	10	P-29	22	20	20	20	Susceptible
		15	P-30	24	25	23	24	Susceptible

When Gentamicin was used with ethanolic extract of Papaya leaves, it was observed that its synergistic effect showed that microorganisms were no more resistant to antibiotic. And same synergistic effect was observed when the antibiotic was used with methanolic extract of orange peel presented in **Table 2** and **Table 3**.

Table 2. Synergistic activity of ethanol extract of Papaya with different standard antibiotic (Gentamycin) against bacteria

Microorganism	Petri plate no. (P)	Diameter of ZOI (mm)			Average of ZOI (mm)	Interpretation (R-I-S)
		1	2	3		
Bacillus subtilis	P-85	18	18	18	18	Intermediate
Salmonella typhi	P-85A	15	14	17	15	Intermediate
Klebsiella pnuemoniae	P-86	17	15	17	16	Intermediate
Escherichia coli	P-86A	15	14	16	15	Intermediate
Staphylococcus aureus	P-87	14	15	15	15	Intermediate

As papaya leaves extract showed better activity in ethanol solvent and orange peel extract showed better activity in methanol solvent. So, these specific solvents were used during the study. Further on, explaining both tables, it was noticed that the synergistic effect of papaya

extract with Gentamicin antibiotic showed *Bacillus subtilis* was more sensitive than other microorganisms which resulted in greater ZOI of 18 mm.

Table 3. Synergistic activity of methanol extract of Orange peel with different standard antibiotic (Gentamycin) against bacteria

Microorganism	Petri plate no. (P)	Diameter of ZOI (mm)			Average of ZOI (mm)	Interpretation (R-I-S)
		1	2	3		
Bacillus subtilis	P-87A	17	16	17	17	Intermediate
Salmonella typhi	P-88	16	15	16	16	Intermediate
Klebsiella pnuemoniae	P-88A	17	15	16	16	Intermediate
Escherichia coli	P-89	16	16	15	16	Intermediate
Staphylococcus aureus	P-89A	18	17	17	17	Intermediate

And the synergistic effect of Orange peel extract with Gentamicin antibiotic showed that *Bacillus subtilis* and *Staphylococcus aureus* were more sensitive than other microorganisms which resulted in greater ZOI of 17 mm.

To make sure the susceptibility of micro-organism toward this antibiotic (Gentamicin), it was used along with combination of Papaya leaves and Orange peel extract against bacteria.

Table 4 indicated that, after the use of antibiotic with combination of herbal extracts resulted in susceptibility of microorganisms which were previously resistant against antibiotic. The synergistic effect of this showed that microorganisms were susceptible and most susceptible microorganisms were *Klebsiella pneumoniae* and *Staphylococcus aureus* with greater ZOI of 22 mm.

Table 4. Synergistic activity of combination of Orange peel and papaya extract with antibiotic (Gentamicin) against bacteria

Microorganism	Petri plate no. (P)	Diameter of ZOI (mm)			Average of ZOI (mm)	Interpretation (R-I-S)
		1	2	3		
Bacillus subtilis	P-90	21	20	21	21	Susceptible
Salmonella typhi	P-91	22	20	20	21	Susceptible
Klebsiella pnuemoniae	P-92	23	21	22	22	Susceptible
Escherichia coli	P-93	20	21	23	21	Susceptible
Staphylococcus aureus	P-94	21	23	21	22	Susceptible

Thus, the study showed that there was an increase in activity of combination of orange peel and papaya leaves extract with antibiotic (Gentamicin). Based on ZOI (**Figures 3,4**), it was observed that antibacterial activity of combination of herbal extract with antibiotic was more susceptible than individual antibacterial activity of herbal extract.

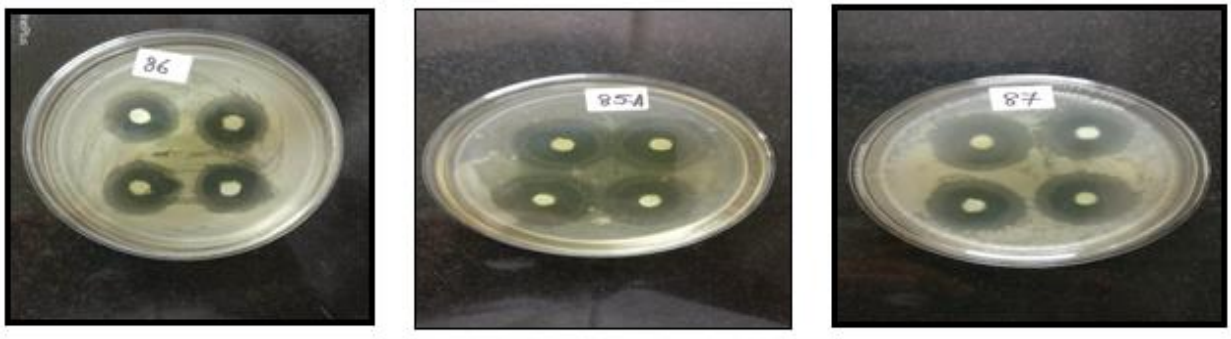

Fig. 3. Antibacterial activity against microorganisms *K. pneumonia*, *S. typhi*, *S. aureus*

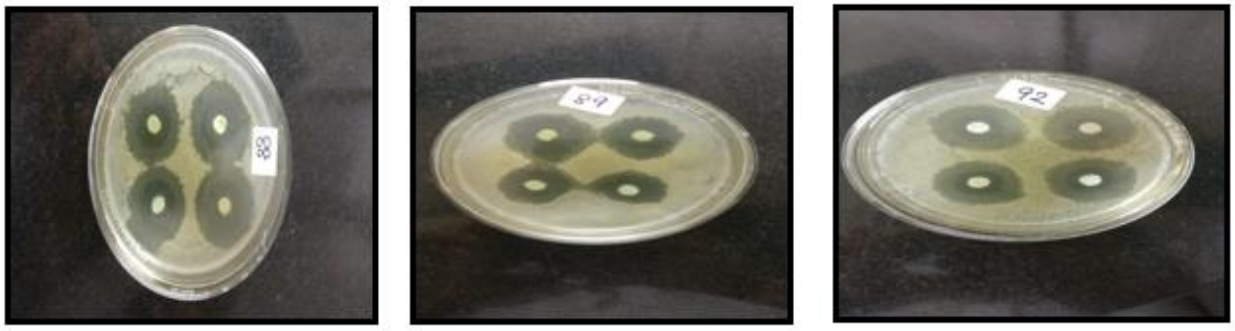

Fig. 4. Antibacterial activity against microorganisms *S. typhi*, *E. coli*, *K. pneumonia*

CONCLUSION

Combinations of the antimicrobials that demonstrate an *in vitro* synergistic effect against infecting strains result in successful therapeutic result. In addition, combinations of agents that exhibit synergy or partial synergy could potentially improve the outcome for patients having difficulty in treating infections. Thus, evidence of *in vitro* synergism could be useful in selecting most favorable combinations of antimicrobials for the practical therapy of serious bacterial infections. Our results reveal that the combined use of plant extracts and antibiotics could be useful in treatment of infectious diseases and useful in fighting emerging drug resistance problem.

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