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EVALUATION OF THE COMBINATIONAL ANTIMICROBIAL EFFECT OF *PRUNUS PERSIA* AND *ANNONA SQUAMOSA* SEEDS METHANOLIC EXTRACT ON STANDARD MICROBIAL STRAINS

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ABSTRACT
All living organisms are continuously exposed to potential pathogens but the disease is the exception rather than the rule. The present investigation was conducted to evaluate the antibacterial efficacy of two traditional plants that is *Prunus Persia* and *Annona Squamosa* against various clinical pathogenic strains. The coming out of medicine conflicting pathogens is one of the most vital threats to active treatment of bacterial diseases. Mode of action of Methanolic extracts likely involves moderately a lot of targets in the cell due to huge number of active components and also their hydroplillicity assists them to screen in the cell membrane, depicting them permeable , leading to seepage of cell contents. This calls for a transformed effort to classify agents efficient against disease causing bacteria to present antimicrobials. Seed extracts of two different plants *viz. Prunus Persia* and *Annona squamosa*, were prepared by methanol extraction method at the ratio of 1:2 using 100ml volume of methanol and stock concentration of 50mg/ml in dimethyl sulfoxide (DMSO) of each extract was made. The extracts and fractions were tested for antimicrobial activity against standard microbial strains of, *Staphylococcus aureus* (gram-positive), *Escherichia coli* (gram-negative), *Klebsiella pneumoniae* (gramnegative) *Salmonella typhi* (gram-negative), *Enterococcus faecalis* (gram positive), *Pseudomon aerugenosa* (gram-negative), and *Salmonella paratyphi* (gram-negative) by means of Agar-Disc Diffusion Method and minimal inhibitory concentration (MIC) was noted. *The test culture of standard microbial cultures was 2 X 105 CFU/ml, and standard antibiotic used is Amoxycillin and Ampicillin.* In this context, two extract from traditional plants, Custard Apple (*Annona squamosa*) and Peach (*Prunus Persia*) were used alone or in combination to evaluate their antimicrobial effectiveness against both Gram negative and Gram positive bacterial clinical isolates. Antimicrobial test was accomplished by agar disc diffusion method. Although, both extract were found to be efficient in inhibiting pathogens to varying measures to the tested organisms, the *Annona squamosa* extract is found to be more effective than *Prunus Persia*. When both extracts were used in combination, they have shown strong synergistic effect against all the pathogens tested in the present study except for the *P. aerugenosa* and *S. Paratyphi*. Bactericidal abilities shown by the seed extract resulting their notable potential for exploration for effective natural antimicrobial agents against standard pathogenic bacteria. The extracts have shown the synergistic effects even at their MIC against *E. fecalis*, indicating that with further researches these extracts can be used for treating enteric diseases.

KEYWORDS: *Annona squamosa , Prunus Persia*, antibacterial activity, agar disc diffusion method, synergistic effect and minimum inhibitory concentration.

INTRODUCTION

Herbal medicines can treat the diseases where chemicals and other drugs have failed. These include common illnesses, which lead to drowsiness and other side effects when treated through the regular medicines. Herbal remedies have the capacity to bring a certain amount of effect in the body and prove to be effective in treating health problem. The use of herbal medicine is popular in several local communities in India, Nigeria as well as other developing countries. Prominent among the reasons is poverty among the population as well as lack of basic primary health care system. *Annona squamosa* (sugar apple) is commonly cultivated in tropical South America but not often in Central America, very frequently in southern Mexico, West Indies. Cultivation is most extensive in India where the tree is exceedingly popular. The sugar apple is one of the most important fruits in the interior of Brazil. Sugar apple tree ranges from 10 to 20 ft (3 – 6m) in height with open crown of irregular branches and zigzag twigs. A branch tips opposite the leaves, the fragrant flowers are borne single or in groups of 2 to 4. The fruit is nearly round, oval or conical, long its thick rind composed of knobby segments, separating when the fruit is ripe and revealing a conically segmented, creamy – white delightfully fragrant juice sweet delicious flesh Crushed leaves of the plant are used in India to overcome hysteria and faint spell while leaf decoction is used in the

case of dysentery (Kirtikar *et al*., 1968). Throughout tropical America, a decoction of leaves is imbibed as tonic cold remedy, digestive or to clarify urine whereas the crushed ripe fruit mixed with salt is applied on tumors while the bark and root are both highly astringent. The traditional claim that concoctions of *A. squamosa* can be used in the treatment of bacterial diseases need to be substantiated with scientific facts that could either support or negate this claim which necessitates the need for this study. Herbal medicines can treat the diseases where chemicals and other drugs have failed. These include common illnesses, which lead to drowsiness and other side effects when treated through the regular medicines. Herbal remedies have the capacity to bring a certain amount of effect in the body and prove to be effective in treating health problem. The use of herbal medicine is popular in several local communities in India, Nigeria as well as other developing Countries (Kotkar *et al.*, 2001). Prominent among the reasons is poverty among the population as well as lack of basic primary health care system (Encyclopedia of Cultivated Palms). *Annona squamosa* (sugar apple) is commonly cultivated in tropical South America but not often in Central America, very frequently in southern Mexico, West Indies.. Cultivation is most extensive in India where the tree is exceedingly popular. The sugar apple is one of the most important fruits in the interior of Brazil. Sugar apple tree ranges from 10 to 20 ft (3 – 6m) in height with open crown of irregular branches and zigzag twigs. A branch tips opposite the leaves, the fragrant flowers are borne single or in groups of 2 to 4. The fruit is nearly round, oval or conical, long its thick rind composed of knobby segments, separating when the fruit is ripe and revealing a conically segmented, creamy – white delightfully fragrant juice sweet delicious flesh Crushed leaves of the plant are used in India to overcome hysteria and faint spell while leaf decoction is used in the case of dysentery. Throughout tropical America, a decoction of leaves is imbibed as tonic cold remedy, digestive or to clarify urine whereas the crushed ripe fruit mixed with salt is applied on tumors while the bark and root are both highly astringent (Padhi *et al*., 2011). The traditional claim that concoctions of *A. squamosa* can be used in the treatment of bacterial diseases need to be substantiated with scientific facts that could either support or negate this claim which necessitates the need for this study. The peach, *Prunus persica*, is a deciduous tree, native to North-West China, in the region comprised between the Tarim basin and the north slopes of the Kunlun Shan Mountains, where it was first domesticated and cultivated. It bears an edible juicy fruit also called a peach (Saddiq, 2010). The species name *persica* refers to its widespread cultivation in Persia, whence it was transplanted to Europe. It belongs to the genus *Prunus* which includes the cherry and plum, in the family Rosaceae. The peach is classified with the almond in the subgenus *Amygdalus*, distinguished from the other subgenera by the corrugated seed shell. Peaches and nectarines are the same species, even though they are regarded commercially as different fruits (Irobi *et al*., 1996). Nectarines have an orange center and faint fuzz, while peaches have white centers and very fuzzy skin; genetic studies suggest nectarines are produced due to a recessive allele, whereas peaches are produced from dominant allele for fuzzy skin *Prunus persica* grows to 4–10 m (13–33 ft) tall and 6 in. in diameter. The leaves are lanceolate, 7–16 cm (2.8–6.3 in) long, 2–3 cm (0.79–1.2 in) broad, pinnately veined.The flowers are produced in early spring before the leaves; they are solitary or paired, 2.5–3 cm diameter, pink, with five petals. The fruit has yellow or whitish flesh, a delicate aroma, and a skin that is either velvety (peaches) or smooth (nectarines) in different cultivars (Cellular Methods in Biology). The flesh is very delicate and easily bruised in some cultivars, but is fairly firm in some commercial varieties, especially when green. The single, large seed is red-brown, oval shaped, approximately 1.3–2 cm long, and is surrounded by a wood-like husk. Peaches, along with cherries, plums and apricots, are stone fruits (drupes). There are various heirloom varieties, including the Indian peach, which arrives in the latter part of the summer (Bauer *et al*., 1966).

MATERIALS & METHOD

Plant material and extract preparation

Friut seed of ripe *Annona squamosa* and *Prunus persia* procured from local market of Bangalore and seeds were removed and separated from them and it is washed with tap water first and then thoroughly with deionized water, hot air oven dried temperature 45 degree Celsius for 4 hours, which can further be increased to 30 minutes to 1 hour depending upon the moisture content of the seeds following by, grinding and converted into fine powder in Murphy Richards grinder (Janssen *et al*., 1897). The dried powdered materials (50 g) were extracted successively with 100 ml of solvents *viz.* Methanol, according to their increasing polarity by using Soxhlet apparatus for 36 h. The obtained extracts were then filtered by using Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) and then concentrated under vacuum at 40°C by using a rotary evaporator[2]. The extract was then lyophilized (CHRIST Frost lyophilizer, Eurofins Pvt Ltd) to powdered form at -55°C under vacuum conditions. A stock concentration of 50mg/ml in dimethyl sulfoxide (DMSO- Sigma-Aldrich, Germany) of each extract was made and stored in freezer for further experimentation (Dickert *et al*., 1981).

Test microorganisms

Six bacterial cultures *Salmonella typhi, Escherichia coli, Staphylococcus aeurus, Pseudomonas aeruginosa,* and *Enterococcus faecalis, Klebshiella pneumoniae* were used in the present study*;* all the tested strains were obtained from Sagar Apollo Hospital, Karnataka, India. Bacterial cultures were grown in nutrient broth (Himedia, M002) at 37^{o}C and maintained on nutrient agar slants at 4^{o}C[37] (Cowan MM 1999)

Agar-Disc Diffusion Method And Preparation of Microbial Cultures: 2X $10^{5\ CFU}$/ml

The test was carried out by agar disc diffusion method. About 25 to 30 ml of Nutrient agar medium was poured in the sterilized petri dishes and permited to solidify. Bacterial strains were cultured overnight in Nutrient agar (HiMedia, Mumbai) at 37±2°(Suresh *et al*., 2008). Overnight grown culture of microorganisms was used for inoculums preparation. A loopful of isolated colony was

inoculated in 4ml of Peptone water (HiMedia, Mumbai) at 37°C for 2h. The turbidity of resulting suspension was compared to 0.5 McFarland turbidity standards. The level of turbidity was corresponding to approximately 2.0×10^5 cfu/ml. The Nutrient Agar media (HiMedia, Mumbai) which is solidified it was then inoculated with microorganism suspended in peptone water, using sterile swab stick, standardized inoculate of each isolate was swabbed onto the surface of Nutrient Agar in separate Petri dishes (Chandrashekar *et al*., 2011). Discs of the extracts were positioned to the surface of the inoculated media. The plates were inverted and allocated to stand for 30 mins for the extract to diffuse into the agar after which the plates were incubated. The experiment was was carried out in triplicates to get rid of any error (Chavan *et al*., 2011). The Petri dishes were incubated for 24 h at 37±2°C for bacteria. The antimicrobial activity was calculated by measuring the diameter of zone of inhibition in millimeters around the well of the discs, Shown in table -1, Fig-1A below.

TABLE 1: Antimicrobial activity was calculated by measuring the diameter of zone of inhibition in millimeters

Organism Name.	Extract 1: *Annona squmosa* Zone of inhibition in (mm)-Conc-50mg/ml				Extract 2: *Prunus Persia* Zone of inhibition (mm)- Conc-50mg/ml			
	1µl	2.5 µl	5 µl	10 µl	1µl	2.5 µl	5 µl	10 µl
E.coli	16	18	20	30	09	11	16	18
S .typhi	18	20	22	31	12	15	18	20
S.aeurus	15	17	19	32	18	23	25	25
E. faecalis	22	24	26	27	17	20	23	23
P. aeruginosa	11	13	15	24	11	14	18	23
K.pneumoniae	10	12	14	20	10	09	13	13

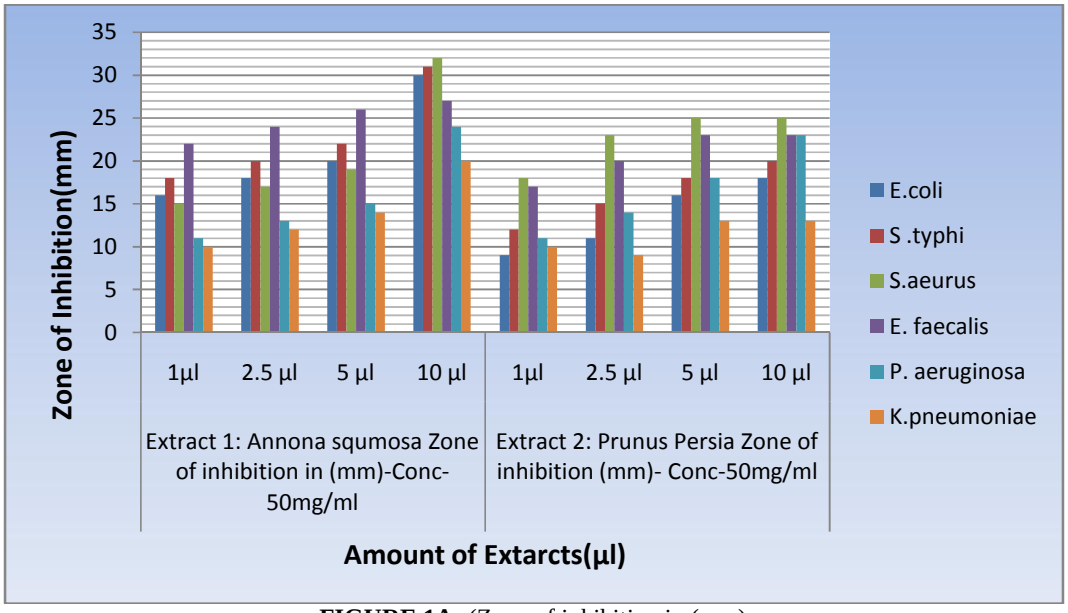

FIGURE 1A: (Zone of inhibition in (mm)

However the strong combinational effect of the extract can be seen more effective in some of the Microorganism such as *E. coli*, *S. typhi*, *S. aeurus*, *E. faecalis* and *K. pneumoniae* as mentioned in the table -2, Fig-2A, but to the P. *aeruginosa* and the combinational effect does not affect co- dominantly.[All the microbial strains are highly sensitive to *Amoxycillin and Ampicillin* acid proving their pathogenicity] (Drug today).

TABLE 2: (Synergistic effect of *Annona squamosa* and *Prunus Persia*)

Organism Name	Extract 1: *Annona squmosa* Zone of inhibition in (mm))	Extract 2: : *Prunus Persia* Zone of inhibition (mm)	Mixed Ext:1 and Ext:2 Zone of inhibition (mm)
	5.0 µl	5.0 µl	5.0 µl
E.coli	20	16	25
S .typhi	22	18	28
S.aeurus	19	25	30
E.fecalis	26	23	34
P. aeruginosa	15	18	18
K.pneumoniae	14	13	18

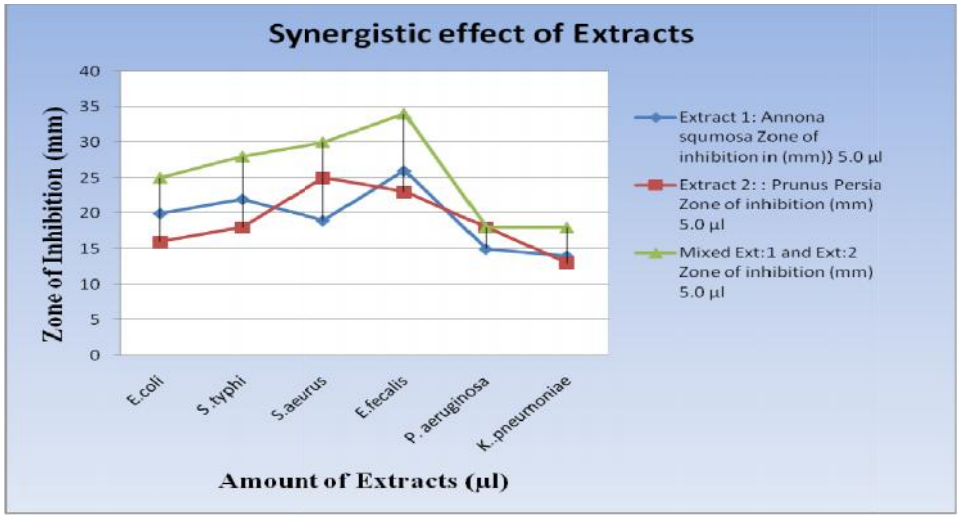

Fig-2A

Determination of minimal inhibitory concentration (MIC)

In the present experiment, extracts which showed positive result were further evaluated for determination of MIC a minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after 18 to 24 h. The extracts that confirm antibacterial activity by agar well diffusion method were subjected to serial micro broth dilution technique to establish their minimum inhibitory concentration by using microscopic evaluation (Eloff *et al.*, 1998). In this MIC was determined by the liquid dilution method, dilution series were set up with 180µl of nutrient broth medium, to each microtitre well 10µl of standard suspension of bacterial colony was added and 10µl of diluted extract was added and incubated at 37 for 24 hours (Epino *et al*., 1993). The minimal concentration which did not show any growth for the tested bacteria after microscopic evaluation was determined as MIC. Based on the assessment *Pseudomonas aeruginosa* is showing the MIC at 1:15 dilution while the one which was being inhibited most is *Enterococcus faecalis* that is at 1:40 dilution. The below mentioned table-1 shows the individual minimal inhibitory concentration (MIC) of the microorganisms, mentioned in table -3

TABLE 3: Individual minimal inhibitory concentration (MIC) of the microorganisms

Dilution with MilliQ Water Extract: Water Conc-50mg/ml	Test Organism name					
	E. coli	*S. typhi*	*S. aureus*	*E. fecalis*	*P. aeruginosa*	*K. pneumoniae*
1:05	×	×	×	×	×	×
1:10	×	×	×	×	×	×
1:15	×	×	×	×	×	×
1:20	×	×	×	×	√	×
1:25	√	×	×	×	√	√
1:30	√	×	×	×	√	√
1:35	√	√	√	×	√	√
1:40	√	√	√	×	√	√
1:45	√	√	√	√	√	√
1:50	√	√	√	√	√	√

RESULTS & DISCUSSION

Annona squamosa and Prunus Persia, tested bacterial strains showed different prototype of inhibition zone. Evaluations were verified in tabular form (Table 1). The Methanolic extracts of *Annona squamosa* showed more antimicrobial activity than Prunus Persia. The result of antibacterial screening by agar disc diffusion method (Table 1, Fig. 1A) designates that elevated zone of inhibition was shown by the methanol extract *Annona squamosa* for *Salmonella typhi* 31mm/10 µl and lowest for *Klebsiella pneumoniae* 20mm /10 µl. The Prunus Persia extract highest zone of inhibition for *Staphylococcus aureus* 25mm/10 µl and lowest for the *Klebsiella pneumoniae* 13mm/10 µl. Strong combinational effect were observed, when the extract were used in combination for the *Salmonella typhi* 28mm , *Enterococcus faecalis* 34mm & *Staphylococcus aureus* 30mm though for the microbial strain of *Pseudomonas aeruginosa* there is no considerable combinational effect (Table-2) . A more commonly precise method of evaluation is the broth dilution method. In this study, therefore, the broth dilution technique was used in determining the activities measured as MIC by microscopic evaluation. The choice of MIC values for all the microbial strains interrelated well with the results obtained by using the agar disc diffusion method. The minimum inhibitory concentration is highest

for the *Enterococcus faecalis* that was its MIC is 1:40 Dilution and lowest for the *Pseudomonas aeruginosa i.e*. 1:15 dilution (Table-3). Therefore due to the antimicrobial activities of these plants there are several reasons that people employ plants for medication. This comprise advancement of health following herbal treatment, low cost of the drugs, non availability of synthetic drugs particularly in the countryside areas, where available were either sham or expired drugs and in some cases the people are more familiarized to and at ease with traditional healing.

CONCLUSION

In the current study, based on preceding reports we were found that the methanolic extracts of *annona squamosa*, and *Prunus Persia* seed or cotytledon extracts illustrated extensive range of antibacterial activity. Additional investigations should be conceded out in finding other activities of the extracts of root and seed cotyledon. The revelation of activity against the different isolates by the seed extract of *annona squamosa*, and *Prunus Persia* form the basis of its use in traditional medicine and antimicrobial activities of the methanolic extract of the plant as an effective antibacterial agent. The Extract of *Annona squmosa* and *Prunus Persia* has shown antibacterial effect against all the pathogens tested. Each of the extract had differential inhibitory effect against specific pathogens. These extracts have also shown strong synergistic effect even at their MIC indicating their different target sites. Both the extract was highly effective against *E. fecalis*, indicating that these extract can be used for treating enteric diseases.

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