*International Research Journal of Biological Sciences* _____ ISSN 2278-3202
Vol. **2(5),** 68-73, May **(2013)** *Int. Res. J. Biological Sci.*

# Evaluation of the Combinational Antimicrobial Effect of *Annona Squamosa* and *Phoenix Dactylifera* Seeds Methanolic Extract on Standard Microbial Strains

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**Available online at: www.isca.in**
Received 6th March 2013, revised 18th April 2013, accepted 30th April 2013

## Abstract

*In recent times, there is an extensive interest in these Alcoholic extracts due to the emergence and spread of new drug resistant human pathogens to existing antimicrobials. The emergence of medicine opposing pathogens is one of the most critical threats to booming treatment of bacterial diseases. Mode of action of Methanolic extracts likely involves fairly a lot of targets in the cell due to huge number of active components and also their hydroplillicity helps them to screen in the cell membrane, rendering them permeable , leading to leakage of cell contents. This calls for a transformed effort to identify agents efficient against disease causing bacteria to present antimicrobials. Seed extracts of two different plants viz. Phoenix dactylifera 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 Klebsiella pneumoniae (gram-negative), Staphylococcus aureus (gram-positive), Escherichia.coli (gram-negative), Salmonella typhi (gram-negative) , Enterococcus faecalis (grampositive), 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 3 X $10^5$ CFU/ml, and standard antibiotic used is Ampicillin with clavulanic acid. In this context, two extract from traditional plants, Custard Apple (Annona squamosa) and Dates (Phoenix dactylifera) were used alone or in combination to assess their antimicrobial efficacy against both Gram negative and Gram positive bacterial clinical isolates .Antimicrobial test was completed by agar disc diffusion method. Although, both extract were found to be effective in inhibiting pathogens to varying degrees to the tested organisms, the Annona squamosa extract is found to be more effective than Phoenix dactylifera.When both extracts were used in combination, they have shown strong synergistic effect against all the pathogens tested in the present studyexcept for the P.aerugenosa and S. Para typhi. Bactericidal abilities displayed by the seed extracts signified their remarkable 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, Phoenix dactylifera, antibacterial activity, agar disc diffusion method, synergistic effect and minimum inhibitory concentration.

## Introduction

Based to the assorted health organization surveys such as WHO, IHO, almost 70-80% populations living in the developing countries rely almost solely on conventional medicine for their basic health care needs[1]. Investigation of the chemical constituents of the plants and pharmacological test may endow with us the basis for developing the progress of new agents[2].The importance of conventional medicines in solving the best of health problem solutions is invaluable on a global pharmaceutical market. Natural products have been a significant source of marketable medicines and drug source. Nearly 61% of drugs marketed worldwide can be outlined to natural products[3,4.] Finding on medicinal plants has exaggerated and information on these plants has been exchanged. This research will go a long way in the scientific discovery of therapeutic plants for the assistance of man and is likely to diminish the belief on artificial drugs[5]. Annonaceae is one of the biggest families, which comprising about 130 genera over 2000 species are *Annona*, with 150 species, genera, the species of *Annona squamosa* is a small evergreen tree attaining 6-7.5 meters (20-24 ft) tall, is usually found in deciduous forests, grown all over India and other countries. It is generally called as custard apple; it is inhabitant of West Indies. The plant is rationally used for the dealing of epilepsy, dehydration, heart related problem, nematode infection, stomach imbalance, hemorrhage, antibacterial infection, fever, and stomach acidity. It also has defiantfertility, anticancer and abortifacient properties[6,7,8]. Numerous activities have been considered on the plant of *Annona squamosa* like antimutagenic9, Anthelmintic[10] Antidiabetic[11], Antithyroid[12] and Antimicrobial activity[13].

*Phoenix dactylifera* (Date palm) a diploid chromosome number with 2n = 36, is a member of the monocot family Arecaceae classified as a dioecious giant evergreen tree[14]. Date palms have been grown in the Middle East since at least 6000 BC[15]. It is main crop of the countries such as around the Arabian Gulf[16,17]. The different parts of this plant are majorly used in conventional medicine for the treatment of various disorders which include memory instability, fever, pain, stammering, nervous disorders[18]. Ehthanolic extract of *P. dactylifera* fruit and seeds showed greatly proven anti-wrinkle efficiency and anti oxidant scavenging properties is being incorporated as an ingredient of D'Orientine™ S composition which is used to guard the skin from ecological sources of aging and wrinkling (World Intellectual property organisation, 2009). *Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated, sugar fermenting, greatly facultative anaerobic, rod shaped. It is also found in the normal flora of the mouth, skin, and small and large intestines[19]; it can cause harsh changes to human lungs if breathed .*Salmonella typhi* is a Gram-negative facultative rod-shaped bacterium in the alike proteobacterial family as *Escherichia coli*, the family Enterobacteriaceae. *Salmonella* are the cause of more than one diseases called salmonellosis, enteric fever (typhoid), resulting from bacterial raid of the bloodstream, and acute gastroenteritis, mostly from a foodborne infection. *Staphylococcus aureus* is a Gram-positive extracellular bacterium that is the most frequent cause of skin and soft tissue infections,such as respiratory tract infection, impetigo, and folliculitis[20,21]. *E.faecalis* may contaminate food and causes food poisoning[22]. *E. coli* greatly influence gastroenteritis, urinary tract infections, and neonatal meningitis. In rare cases, virulent strains are also cause for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and pneumonia[23]. *Pseudomonas aeruginosa* is a prospected pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacterecemia and a range of systemic infections, specifically in sufferers of brutal burns, cancer and AIDS victims who are immunosuppressed. *Pseudomonas aeruginosa* is seldom a pathogen of plants, as well[24]. Plant seed extracts have been reported by numerous researchers for their antimicrobial activity[25]. Current study was directed at exploration on antipathogenic (antimicrobial) activity of *dactylifera* (Palmae) and *Annona squamosa* (Annonaceae) seed extracts[26,27].

## Material and Methods

**Plant material and extract preparation:** Friut seed of ripe Annona squamosa and Phoneix dactylifera procured from local market of jayanagar Bangalore and seeds were separated from them and it is washed through tap water first and subsequently, thoroughly washed with deionized water, and then it is dried in the hot air oven with a temperature of about 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[28]. The desiccated powdered material (50 g) was extracted consecutively with 100 ml of solvents *viz.* Methanol, according to their ever-increasing polarity by means of Soxhlet apparatus for 36 h. The obtained extracts were then filtered by using Whatman No. 1 filter paper (Whatman International Ltd, England) and then determined under vacuum at 40°C by using a rotary evaporator[29]. 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 both extract was made and preserved in freezer for advance experimentation.

**Test microorganisms:** Seven bacterial cultures *Escherichia.coli, Salmonella typhi, Staphylococcus aeurus, Enterococcus faecalis, Pseudomonas aeruginos**a**, Salmonella paratyphi* and *Klebshiella pneumoniae* were used in the present study*;* all the tested strains were obtained from Sagar Apollo Hospital, Karnataka, India. Bacterial strains were cultivated in nutrient broth (Himedia, M001) at 37oC and preserved on nutrient agar slants at 4°C[30]

**Agar-Disc Diffusion Method And Preparation of Microbial Cultures: 3X $10^5$ CFU/ml:** The test was done by agar disc diffusion technique. About 25 to 30 ml of Nutrient agar medium was placed in the sterilized petri dishes and left to harden or solidify[31]. Bacterial cultures were cultured overnight in Nutrient agar (HiMedia, Mumbai) at 37±2°. Overnight grown culture of microorganisms was used for inoculums making. Ring full of isolated colony were inoculated in 4ml of Peptone water (HiMedia, Mumbai) at 37°C for 2 to 2.30 h. The turbidity of resulting suspension was compared to 0.5 McFarland turbidity values. The level of turbidity was corresponding to approximately $3.0 \times 10^5$ cfu/ml[32]. 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. Discs of the extracts were placed to the surface of the inoculated media. The Petri plates were inverted and left to stand for 30 min for the extract to diffuse into the agar after which the plates were incubated. The experiment was carried out in triplicates to get rid of any error[33,34]. The Petri dishes were incubated for 24 h at 37±2°C for bacteria. The antimicrobial activities were calculated by measuring the diameter of zone of inhibition in millimeters around the periphery of the discs[35,36], Shown in table -1, figure-1 below.

However the robust 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, but to the other two organism such as P. *aeruginosa* and S. para typhi the combinational effect does not affect co-dominantly.

[All the microbial strains are highly sensitive to Ampicillin with clavulanic acid proving their pathogenicity][37,38,39].

**Determination of minimal inhibitory concentration (MIC):** A minimum inhibitory concentration (MIC) is the lowest attentiveness of an antimicrobial culture that stops the growth of a microorganism after 18 to 24 h[40-42]. The extracts that gives antimicrobial activity by agar disc diffusion method was subjected to serial micro broth dilution technique to establish their minimum inhibitory concentration by using microscopic evaluation. In this minimum inhibitory concentration was noted by the liquid broth dilution method, dilution sequence were set up with 180µl of nutrient broth medium, to each micro titer well 10µl of standard suspension of bacterial colony was added and 10µl of diluted extract was supplemented and incubated at 37 for 24 hours[43,44]. The lowest concentration which did not show any growth for the experienced bacteria subsequent to microscopic assessment was resolute as minimum inhibitory concentration[45-48]. Based on the evaluation *Pseudomonas aeruginosa* is showing the MIC at 1:15 dilution while the one which is being inhibited most is *Enterococcus faecalis* that is at 1:40 dilution[49,50]. The below mentioned table-1 shows the individual minimal inhibitory concentration (MIC) of the microorganisms, mentioned in table -3.

**Table-1**
**Zone of inhibition of both the plants extracts in (mm)**

| Organism Name | Extract 1: *Annona squmosa* Zone of inhibition in (mm)-Conc-50mg/ml | | | | Extract 2: *Phoneix dactylifera* 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* | 23 | 24 | 27 | 30 | 09 | 11 | 16 | 18 |
| *S .typhi* | 31 | 31 | 31 | 31 | 12 | 15 | 18 | 20 |
| *S.aeurus* | 27 | 28 | 30 | 32 | 18 | 23 | 25 | 25 |
| *E. faecalis* | 23 | 23 | 23 | 23 | 18 | 20 | 23 | 23 |
| *P. aeruginosa* | 22 | 23 | 24 | 24 | 19 | 21 | 23 | 23 |
| *S. para typhi* | 22 | 27 | 28 | 30 | 14 | 16 | 16 | 17 |
| *K.pneumoniae* | 11 | 15 | 17 | 20 | 10 | 13 | 13 | 13 |

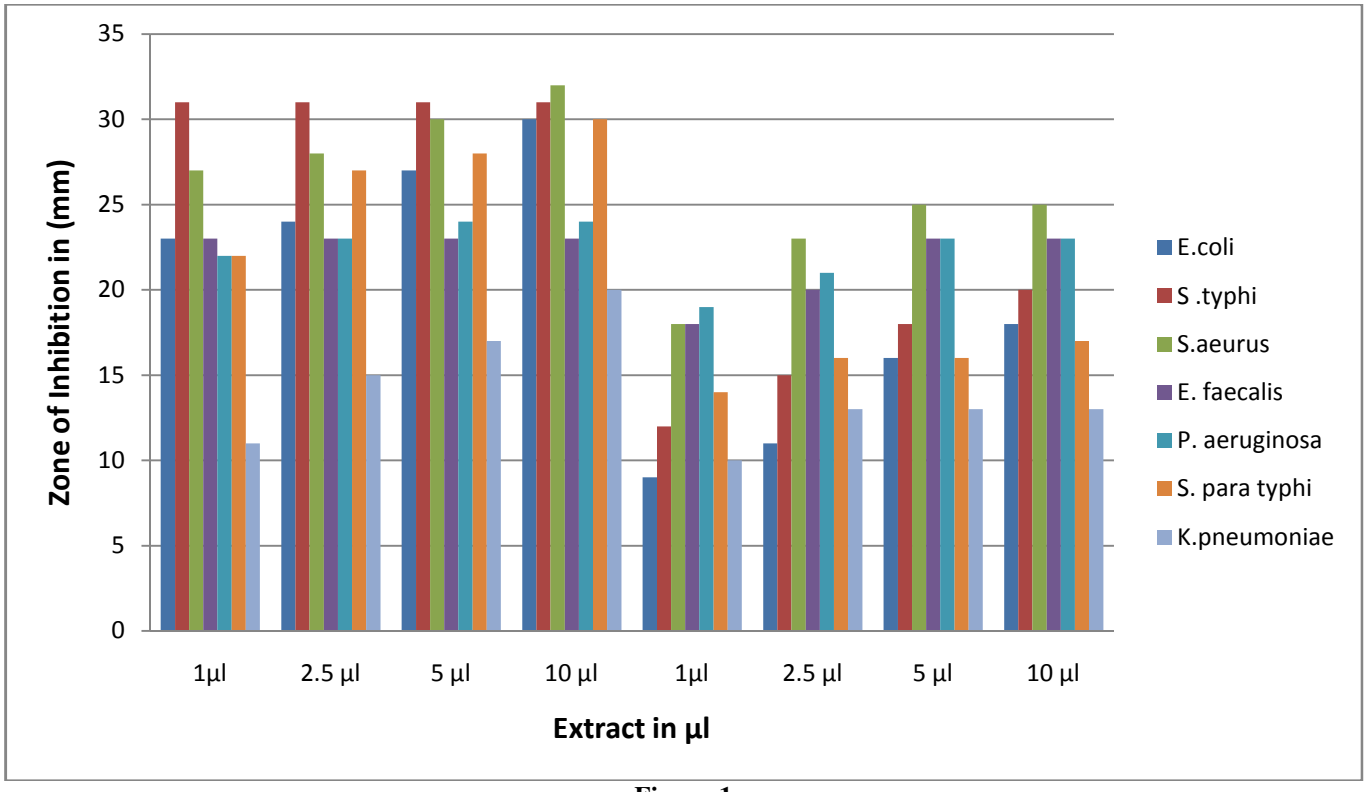


**Figure-1**
**Zone of inhibition of both plants on different microorganism in mm**

**Table-2**
**Synergistic effect of *Annona squamosa* and *Phoneix dactylifera***

| Organism Name | Extract 1: *Annona squmosa* Zone of inhibition in (mm)) | Extract 2: *Phoneix dactylifera* 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* | 27 | 16 | 30 |
| *S .typhi* | 31 | 18 | 34 |
| *S.aeurus* | 30 | 25 | 32 |
| *E.fecalis* | 23 | 23 | 34 |
| *P. aeruginosa* | 24 | 23 | 24 |
| *S. para typhi* | 28 | 16 | 27 |
| *K..pneumoniae* | 17 | 13 | 18 |

**Table-3**
**Minimum inhibitory concentration**

| Dilution with MilliQ Water<br><br>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 and Discussion

*Annona squamosa* and *Phoenix dactylifera* have the immense medicinal value. The tested bacterial strains showed different pattern of inhibition zone. Readings were recorded in tabular form (table 1). The Methanolic extracts of *Annona squamosa* showed more antimicrobial activity than *Phoenix dactylifera*. The outcome of antibacterial selection by agar disc diffusion method (table 1, figure 1) specify that highest zone of inhibition was revealed by the methanolic extract *Annona squamosa* for *Salmonella typhi* 31mm/10 µl and lowest for *Klebsiella pneumonia* 20mm/10 µl. The Phoneix dactilefera extract highest zone of inhibition for *Staphylococcus aureus* 25mm/10 µl and lowest for the *Klebsiella pneumoniae* 13mm/10 µl. Robust combinational effect were observed, when the extract were used in combination for the *Salmonella typhi* 34mm, *Enterococcus faecalis* 34mm and *Staphylococcus aureus* 32mm however for the microbial strain of *Pseudomonas aeruginosa* and *Salmonella paratyphi* there is no significant combinational effect (table-2). A more general and precise method of consideration is the broth dilution technique. In this study, the broth dilution method was used in determining the activities calculated as MIC by microscopic evaluation. The array of MIC values for all the microbial strains interrelated well with the results acquired by using agar disc diffusion method. The minimum inhibitory concentration is highest for the *Enterococcus faecalis* that is, its MIC is 1:40 Dilution and lowest for the *Pseudomonas aeruginosa* ie 1:15 dilution (table-3). Therefore due to the antimicrobial activities of these plants there are plenty of reasons that people use these plants for traditional medication. This conclude progress of health following conventional herbal treatment, squat cost of the drugs, non accessibility of artificial drugs particularly in the countryside areas, where obtainable were also counterfeit or expired drugs and in some cases the people are more comfortable to the conventional healing.

## Conclusion

In the present experiment, we have found that the alcoholic extracts of *Annona squamosa*, and *Phoenix dactylifera* seed or cotyledons extracts showed wide range of antibacterial activity. Further investigations should be carried out in finding other activities of the extracts of root and seed cotyledon. The demonstration of activity against the different isolates by the cotyledon extract of *Annona squamosa* and *Phoenix dactylifera* is the basis for its use in traditional medicine. Antimicrobial activities of the methanolic extract of these plants are quite

effective as an antibacterial agent. The Extract of *Annona squmosa* and *Phoneix dactylifera* 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.

## Acknowledgement

The authors are awfully thankful to Dr. Hemechandra Sagar (Chairman), Dr. Premchandra Sagar (Vice Chairman), Dr. Krishna Gowda, Director, Dayananda Sagar College of Biological Sciences, Dr.C.D. Sagar Centre for Life Sciences, Bangalore-560078, INDIA, for their colossal guidance and support for this project.

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