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An Ethnobotanical Comparitive Analysis of the Pharmacological Aspects of *Annona reticulata, Annona squamosa* and *Annona muricata*, with Mathematical Modeling.

Sandeep Suryan, Nagananda G.S*, Hamsapriye, Padmashree Anand, and Swetha Seshagiri.

Centre for Incubation, Innovation, Research and Consultancy (CIIRC), Jyothy Institute of Technology, Tataguni, Off Kanakapura Road, Bangalore 560082, Karnataka, India.

ABSTRACT

Cancer is a major cause of morbidity and mortality worldwide. Since Annonacea members possess lot of medicinal properties such as cytotoxic, immune modulator, etc., they are being used as a natural remedy for a wide range of illnesses. The study accounts for the pharmacological applications of Annona reticulata, Annona squamosa, Annona muricata leaf extracts individually, and in combination. Annona squamosa extract exhibited highest antioxidant activity compared to the other two extracts which has been validated mathematically. The combination of all the three extracts has been found effective against both Bacillus cereus and Streptococci sp.. Annona squamosa significantly inhibited the growth of Staphylococcus aureus. Annona squamosa has been found to be effectual against Candida albicans, Aspergillus niger and Cryptococcus. The combination of all three samples exhibits higher apoptosis of cancer cells compared to Annona muricata extract.

Keywords: *Annona reticulata, Annona squamosa, Annona muricate,* Ethnobotanical Comparitive.

Corresponding author

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INTRODUCTION

Increasing globalization and industrialization have profound impact on the life style of majority of the world population [1]. The change in the lifestyle in turn leads to the emergence of various chronic diseases which are the main threat to the population. Cancer is found to be one of the major causes of morbidity and mortality worldwide. Exposure to cancer risk factors are prevalent in industrialized as well as developing countries alike. The cause and the type of cancer vary globally and in the year 2000, approximately seven million deaths occurred from cancer over the 10 million cases of cancer [2]. By the end of 2020, number of cancer cases is expected to increase largely. Lungs, breast, stomach and liver cancer are some of the common types of cancer in developing countries [1]. Beneficial collaboration between researchers in both the group of countries can often help in improving the well-being of local as well as global populations struggling against cancer. As the allopathic drugs that are available for the cancer treatment are usually expensive and have umpteen side effects, the demand for the non-allopathic medicines is in great demand. Hence there is a need for the discovery of the alternative drugs that minimizes the risk factors.

Currently, folk medicines have acquired an important place in most of the developing countries. Several plants having medicinal properties have been identified and used throughout human history. Ethnobotany, a branch of science which deals with the traditional knowledge of people concerning plants and their medical and religious uses is being recognized as an effective way to discover the future medicine. The exploitation of herbs in treating various diseases is widespread in non-industrialized societies. Most of the times various vital components present in the herbs have a synergistic activities or helps in buffering the toxic effects. The bioactive components present are directly associated with the prevention of diseases [3]. Annonacea harbors various species which has lot of medicinal properties [4]. They are being used as a natural remedy for a wide range of illnesses. They are basically antiparasitic, antispasmodic, antidiarrheal, antiulcer, analgesic and sedative in nature. They also possess vermifugal effects. Some of the members of Annonaceae family are Annona reticulata (Ramphala), Annona squamosa (Sitaphal), Annona muricata (Lakshmanaphal), Annona Montana etc [4]. Annona muricata (A. muricata) is popularly known as soursoup and can be used for various therapeutic purposes [5]. Several reports on the pharmacological applications of A. muricata are available in the literature. Annona squamosa (A. squamosa) has exhibited antioxidant, antidiabetic, anti-infective and anti dyslipidemic properties. Various parts of Annona reticulata (A. reticulata) plant has demonstrated various therapeutic activities as anticancer, CNS depressant, analgesic, anti-hyperglycemic, anti-inflammatory, anti-proliferative, wound healing and antiulcer activity [6,7]. All three members of Annonaceae family A. reticulata, A. squamosa and A. muricata possess excellent pharmacological properties. Although few reports on the pharmacological aspects of all three members are available in the literature, studies on the combination of all three are not available [8-10]. Thus the present study has been undertaken to determine the pharmacological applications of A. reticulata, A. squamosa and A. muricata leaf extracts individually along with its combination.

MATERIALS AND METHOD

Sample Collection: Fresh leaves of A. reticulata, A. squamosa and A. muricata were collected in and around Chikmagalur District, Karnataka, India.

Sample Extraction: The collected leaves were dried under shade for three consecutive days. The dried leaves of A. reticulata, A. squamosa and A. muricata were grounded into coarse powder and were subjected to soxhlet extraction using ethanol as the solvent for 48 hours individually. Further the extracts were concentrated using rotary vacuum evaporator. The concentrated samples were then reconstituted in Dimethyl sulfoxide (DMSO) 1mg/ml [11]. Equal amount of each A. reticulata, A. squamosa and A. muricata extract were blended together to make the fourth sample i.e. combination for the comparative study.

Antioxidant Activity of Annona reticulata, Annona squamosa and Annona muricata and its combination: Two methods namely 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were employed to study the antioxidant activity.

DPPH Method: Different aliquots of all four extracts were taken for the study. Five milliliter of a 0.1mM methanolic solution of DPPH was added to all the samples and was shaken thoroughly. The tubes were allowed to stand for 20 min at 27˚C. The absorbance of the samples was measured at 517 nm using ascorbic

acid as standard (1mgml^{-1}). Methanol was taken as blank [12]. The experiment was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100$$

ABTS Method: The reaction mixture contained different aliquots of leaf extracts along with 300 µL of ABTS solution and the volume was made up to 1 ml with ethanol. The reaction mixture was incubated at room temperature for 30 minutes in dark condition. The absorbance was measured at 745nm using ascorbic acid as the standard. ABTS radical cation ($ABTS^{+}$) was produced by the reaction of 7 mM ABTS with 2.45 mM Ammonium persulfate [13]. The de-colorization in the tubes were observed and further were expressed as the inhibition percentage of the cation present in the sample and was further calculated using the formula:

$$ABTS\ radical\ scavenging\ Activity\ (\%) = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100$$

Antimicrobial Activity of Annona reticulata, Annona squamosa and Annona muricata and its combination:

Antimicrobial activity of A. reticulata, A. squamosa and A. muricata leaf extracts were determined by the agar well diffusion method [13]. Clinical isolates such as Streptococci, Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus, Serratia marcescens and Escherichia coli were used in this study. The plates were inoculated with 100 µL of test pathogens (Both Gram positive and Gram negative bacteria) on Muller Hinton agar. Further 100 µL of the sample extracts were loaded in 6mm diameter wells on the agar plate. The plates were then incubated at 37 °C for 24 h and zone of inhibition around the wells were measured (in millimeters). The DMSO was used as a test control. Similarly fungal cultures such as Aspergillus niger, Aspergillus flavus, Penicillium pupura, Candida albicans and Cryptococcus sp. were also used in the study. 100 µL of fungal spore suspension was inoculated on to Muller Hinton agar and all four extracts were loaded in 6mm well. Further the plates were incubated at room temperature for three days and the effects were compared with that of standard antibiotic (amoxicillin) and antifungal (fluconazole) at a concentration of 1 mgmL^{-1}. All assays were performed in triplicate.

Anti-cancerous Activity of Annona muricata and its combination: The effect of A. muricata as well as the combination of all the three extracts on the growth and proliferation of Human colon carcinoma (HCT-116), Human lung carcinoma (A549), Human keratinocyte carcinoma (HaCaT), Human liver cancer cell line (HepG2) and Human breast adenocarcinoma cell line (MCF7) was tested by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide) (MTT) cell viability assay. MTT assay was performed with HeLa, Hep G2 and human lymphocytes. The cells were seeded in 96-well flat-bottom micro titter plates and incubated in a CO_2 incubator at 37 °C with 5 % CO_2 and 95 % air overnight for cell adhesion. Various concentrations of the leaf extracts (2.5, 5, 10 and 20 µg mL^{-1}) were added in quadruplicates to the check for 24, 48 and 72 hrs. Upon incubation, 100 µL of MTT solution was added. The culture was then incubated for 3 hours in dark. After the incubation, the supernatant was aspirated and 100 µL of DMSO was added to dissolve the formazan. The absorbance was measured at 540 nm with the help of an ELISA plate reader [14]. The percentage viability was calculated using the formula:

$$\text{Percentage Viability } (\%) = \frac{A540\ of\ the\ test\ Sample}{A540\ of\ the\ control} \times 100$$

The effect of both the samples on the normal cells was tested by treating them on healthy lymphocytes. Lymphocyte isolation was carried out based on the method as described by Nadumane et al., [15] cytotoxicity was analyzed by MTT assay as mentioned above.

Statistical Analysis: The obtained values were expressed as Mean ± SEM. All statistical analysis has been performed at 1 % significance level. The effect of the sample extract concentration on antioxidant activity of all the three extracts taken independently and collectively has been analyzed using linear fit model. Similarly, the correlation between pairwise extracts have been computed and analyzed.

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RESULTS AND DISCUSSION

Antioxidant Activity of Annona reticulata, Annona squamosa and Annona muricata and its combination

The radical scavenging activity of all the four samples was found to be lower compared to the standard in both DPPH as well as ABTS$^+$ method. IC_{50} values for all the samples are as depicted in Table. 1. A. squamosa showed significantly higher percentage of DPPH scavenging activity compared to the samples followed by the combined sample. Figure 1a represents the percentage of DPPH scavenging activity of all the four samples along with the standard. A. reticulate and A. muricata showed lesser antioxidant activity. Similarly with respect to ABTS$^+$ method, A. squamosa illustrated higher scavenging activity compared to other samples (Fig. 1b). The combined sample represented good scavenging activity but, the scavenging activity of A. reticulate and A. muricata were not that promising. The potency of the samples in scavenging the radicals is due to the availability of the number of free radicals donated by the hydroxyl groups [16]. The scavenging activity is directly proportional to the phenolic content of the sample (higher the phenolic content, higher is the scavenging activity). The free radicals present elicit a number of degenerative diseases; therefore samples possessing free radical scavenging activity can be a potent medical importance.

Table 1: IC_{50} value of different extracts against two different assays

Extracts	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)
Standard	21.04^a	55.66^b
Annona reticulata,	74.21^e	100.02^e
Annona squamosa	42.92^b	46.7^a
Annona muricata	50.88^c	60.98^c
Combination	52.87^d	64.73^d

Mean of 15 replicates. Mean values with different superscripts (a, b, c, d, e) differ significantly at P<0.01 by Tukey (HSD) test

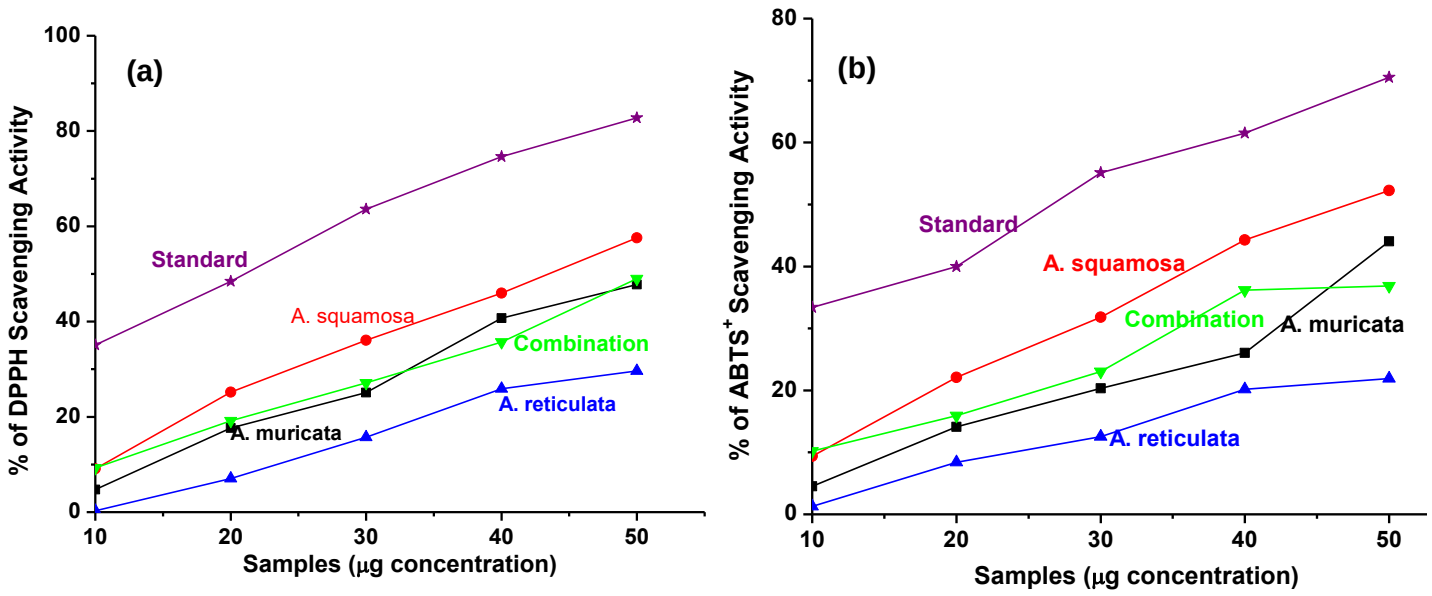

Figure 1a and b: DPPH and ABTS$^+$ Scavenging Activity of A. reticulata, A. squamosa, A. muricata and their combination at different concentrations

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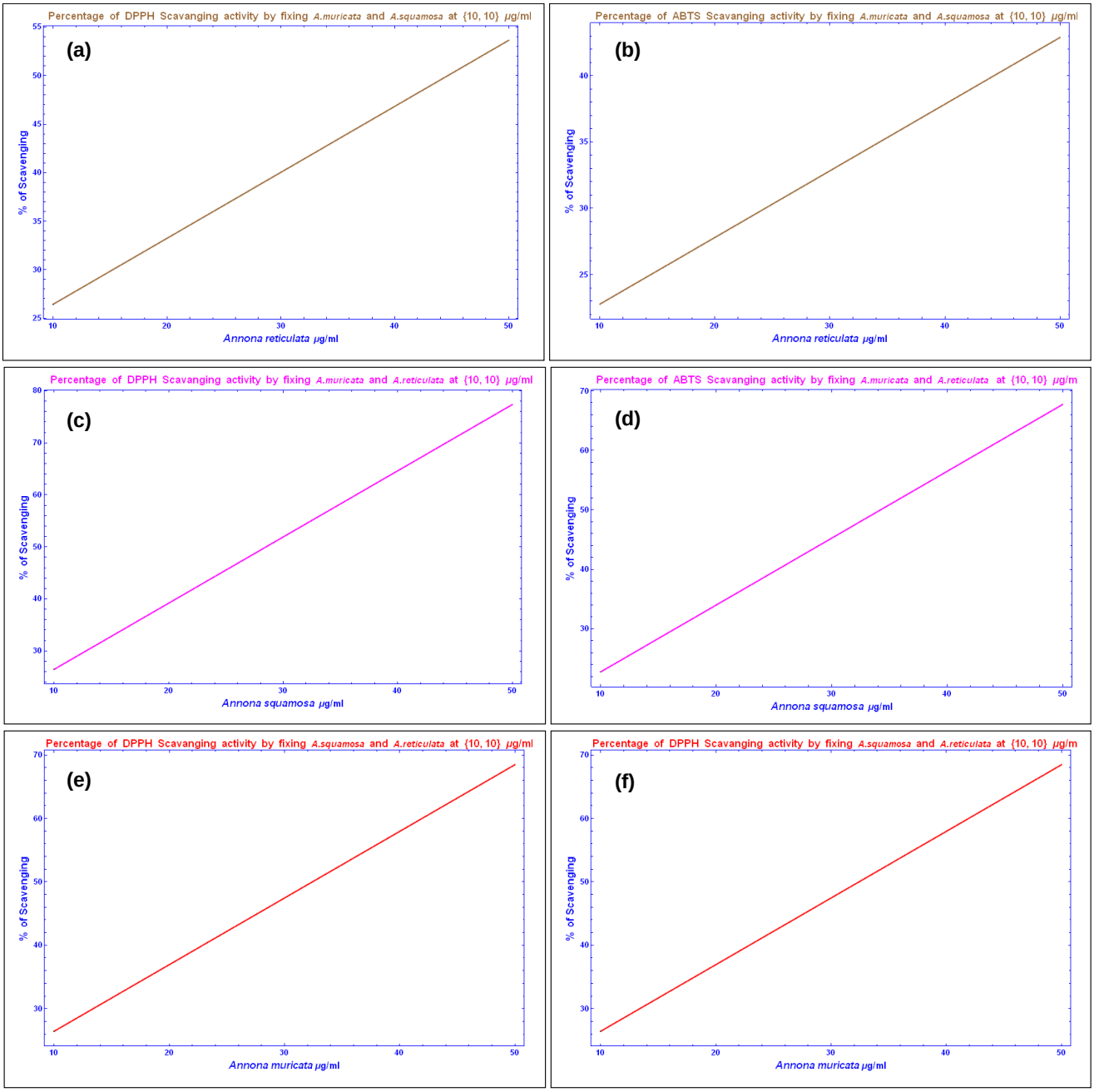
Mathematical Modelling for Antioxidant Activity of Annona reticulata, Annona squamosa and Annona murricata

Using the method of least squares, the effect of the sample extract concentration on antioxidant activity has been analyzed [17]. Figure 2a and 2e illustrates the linear model of the antioxidant activity of A. reticulata (fixing A. squamosa and A. murricata at base level) and A. murricata (fixing A. squamosa and A. reticulata at base level), determined by DPPH method, varying the extracts on the interval [10, 50]μgml^{-1}, respectively. Similarly, Figure 2c represents the linear model of the antioxidant activity determined by DPPH method for A. squamosa wherein, the concentration of A. squamosa varies on the interval [10, 50]μgml^{-1}, fixing the other two extracts at 10μgml^{-1}. Likewise Figures 2b, 2d and 2f depict the antioxidant activities of all the three extracts using $ABTS^{+}$ method.

Figure 2: Linear fit model of A. reticulata, A. squamosa and A. muricata as determined for both DPPH and $ABTS^{+}$ by varying one variable at a time while keeping the other two fixed.



The following are the inferences drawn, based on the least-squares fit:

(i) A. squamosa is found to be more effective than the other two extracts.
(ii) Although A. reticulata is less effective individually compared to the other two extracts, the predictive model suggests that A. reticulata in combination with A. muricata is found to be interactive under ABTS.
(iii) Equal weightage has been given to all the extracts while studying the combinatorial effects. But, the correlation coefficient study shows that the combination of any two at a time is better than considering all the three extracts together.
(iv) If all the three extracts have to be considered, then under DPPH, A. muricata has to be given more weightage. Under ABTS, A. squamosa and A. muricata has to be given more weightage at varied levels.

Scope for future research

While studying the combined effects of all three extracts, it has been realized that by giving different weightages to the individual samples, the antioxidant activity of the combination can be optimized. The research is ongoing.

Correlation Analysis: Based on the correlation coefficient analysis [18], following conclusions can be drawn:

(i) With reference to the DPPH free radical scavenging activity, Table 2 shows that Annona muricata & Annona reticulata are highly correlated and hence is expected to show a higher percentage of scavenging activity as compared with the other two combinations.
(ii) With reference to the ABTS free radical scavenging activity, Table 2 shows that Annona squamosa & Annona reticulata are highly correlated and hence is expected to show a higher percentage of scavenging activity as compared with the other two combinations.
Further, (i) & (ii) are being experimentally validated.
(iii) Similarly, with respect to the DPPH free radical scavenging activity, none of the pair of the extracts interact positively as depicted in Fig. 3a.
(iv) Likewise, with reference to the ABTS free radical scavenging activity, only A. muricata and A. reticulate shows positive interaction, as illustrated in Figure 3b.

Table 2: Correlation Coefficient Analysis of Annona reticulata, Annona squamosa and Annona muricata

TEST	SPECIES PAIR	CORRELATION COEFFICIENT	REMARKS
DPPH	Annona muricata & Annona squamosa	0.9902	
DPPH	**Annona muricata** & Annona reticulate	0.9944	**Highly correlated**
DPPH	Annona squamosa & Annona reticulata	0.9854	
ABTS	Annona muricata & Annona squamosa	0.9595	
ABTS	Annona muricata & Annona reticulata	0.9291	
ABTS	**Annona squamosa** & Annona reticulata	0.9941	**Highly correlated**

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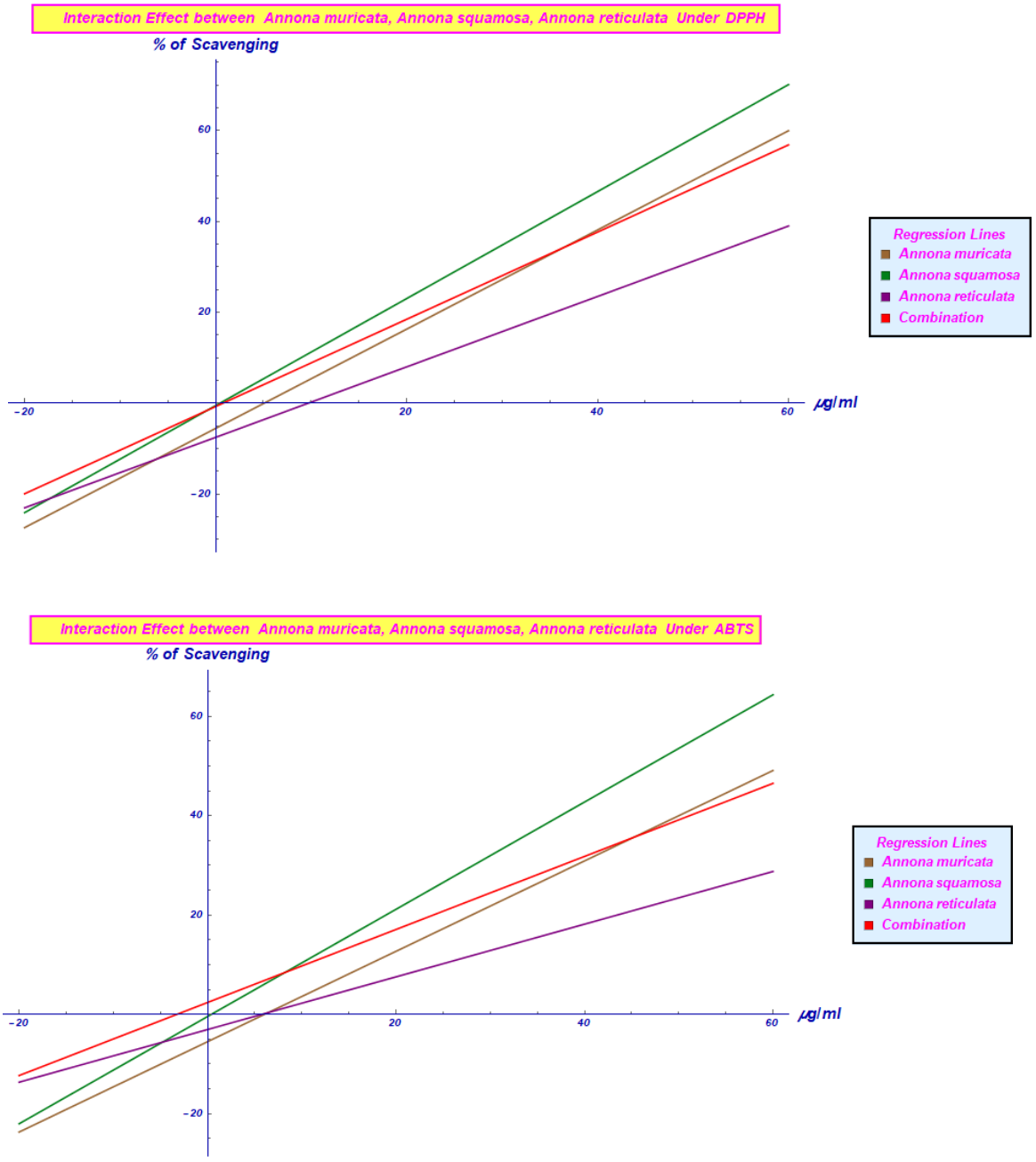
Figure 3: illustrates the interactive effect of the extracts, taken individually and in combination, on the % of scavenging activity under (a) DPPH and (b) ABTS



Antimicrobial Activity of Annona reticulata, Annona squamosa and Annona muricata and its combination

The combination of all three extracts was found to be effective against both Bacillus cereus and Streptococci, while A. reticulata was found to be effective against Escherichia coli and Serratia marcescens (Fig.4). None of the extracts were found to be effective against Enterococcus. Enterococcus was found to be resistant to all the four extracts. Staphylococcus aureus was found to be largely inhibited by A. squamosa. This could be attributed to the fact that A. squamosa contains lot of flavonoids which might have depicted the antimicrobial activity [19, 20]. Similarly inhibition of Aspergillus flavus was greatly influenced by the combination compared to the individual samples (Fig. 5). A. squamosa was found to be effectual against Candida albicans, Aspergillus niger and Cryptococcus. None of the samples were found to be potent against Penicillium purpura.

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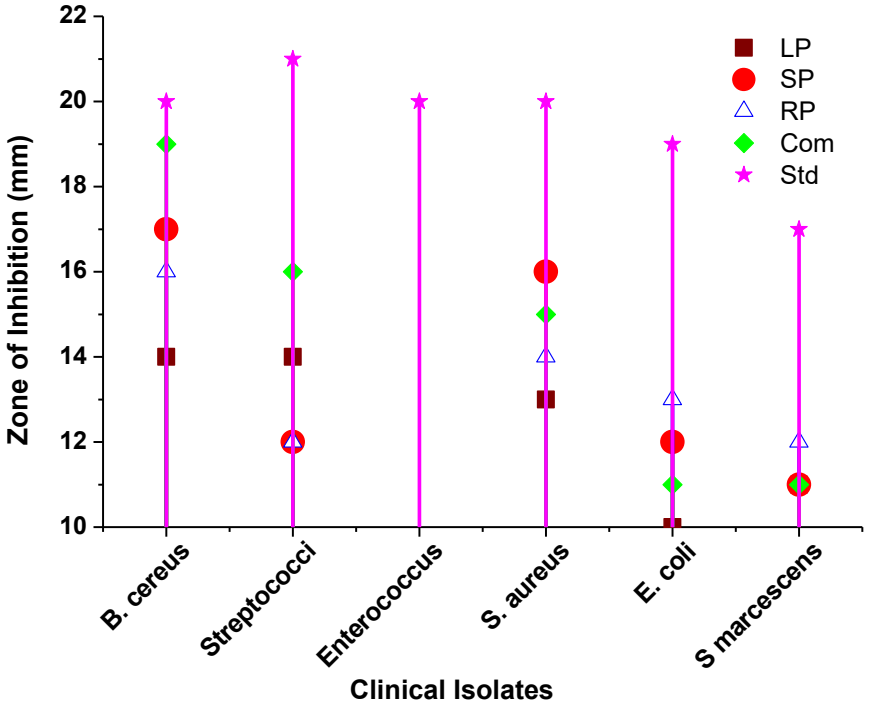
Figure 4: Plot depicting antibacterial activity of A. reticulata, A. squamosa, A. muricata and their combination.



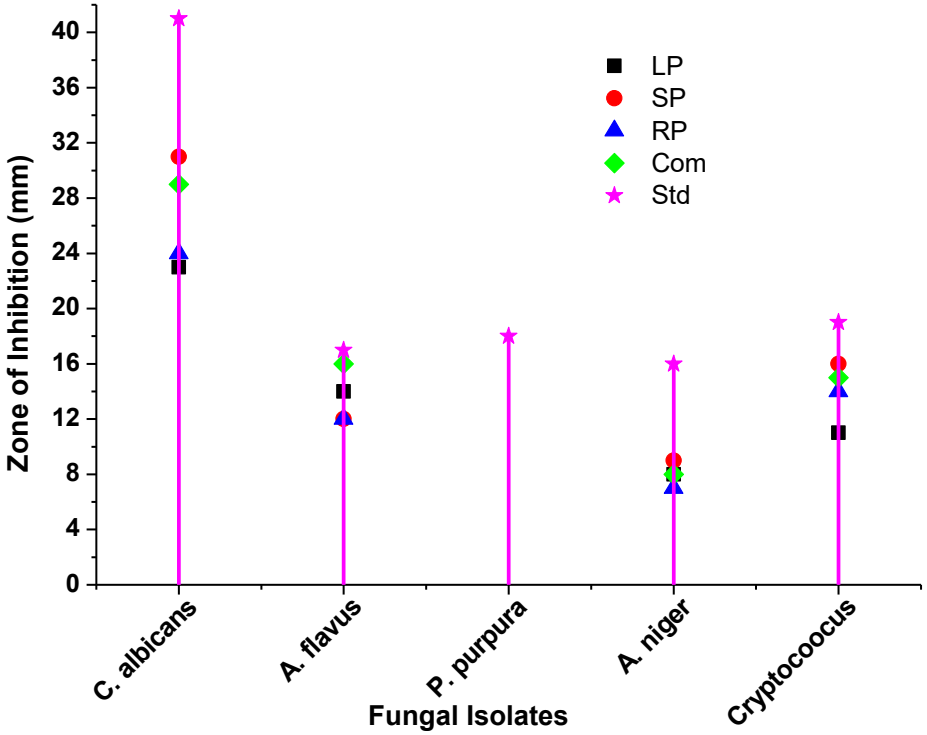


Figure 5: Plot depicting antifungal activity of A. reticulata, A. squamosa, A. muricata and their combination.

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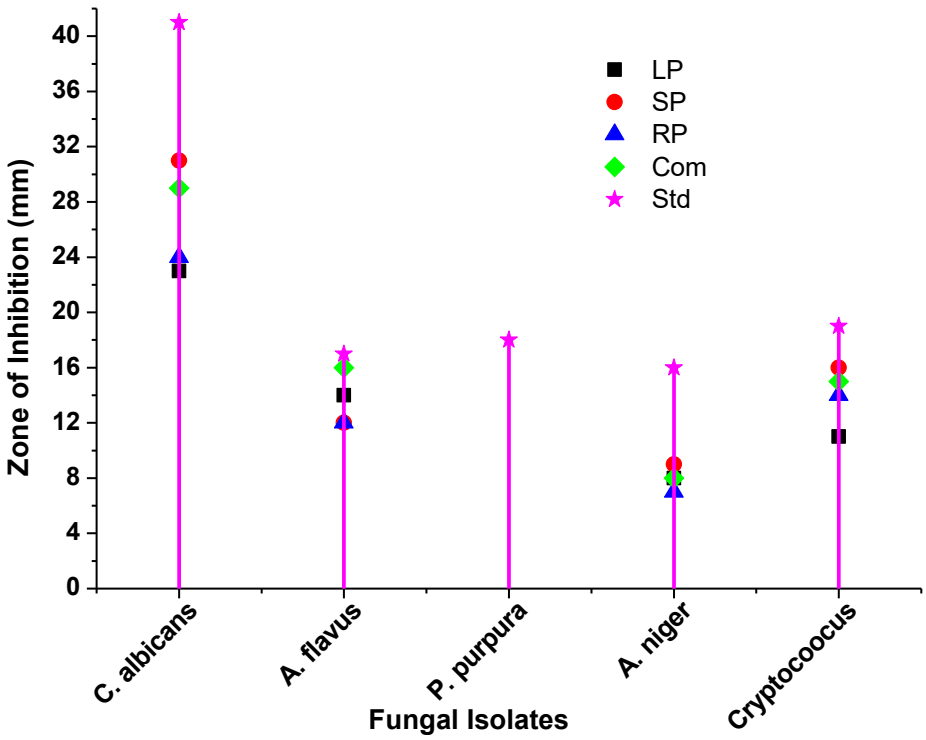

Leaf extracts of A. muricata can be employed in the treatment of various bacterial infectious diseases such as Pneumonia, diarrhea, Urinary Tract infections and also few skin diseases. According to Wisdom et al., [21], A. muricata possess a broad spectrum of activity against a panel of bacteria responsible for the most common bacterial diseases. Flavonoids present in the leaf extracts are potential antibacterial compounds which act upon various bacteria by destroying the cell wall permeability, microsome, lysosomes and bacterial cell as a result of interaction between flavonoids with DNA. Tanins or the polyphenolic compounds present in the extracts are also known to exhibit antimicrobial activity [22, 23] (Pathak, Saraswathy & Savai, 2010; Vijayameena, Subhashini, Loganayagi & Ramesh, 2013). These compounds react with cell membranes, inactivate essential enzymes and alter the metabolism. Various reports on the antimicrobial activity of A. muricata leaf extracts on broad range of pathogens are available in the literature. Recently, Anes et al., [24] has reported the chitosan loaded plant extract of A. muricata as a promising means in treating various infectious diseases. Chitosan loaded A. muricata extracts were found to be very much effective against both Gram positive and Gram negative bacteria. Salman and Senthil Kumar [25] have reported the presence of antibacterial activity of A. squamosa bark against Streptococcus mutans and Streptococcus sobrinus. Similarly, A. reticulata has also exhibited antibacterial activity against both Gram positive and Gram negative strains [26]. A. squamosa illustrated strong antimicrobial activity compared to A. reticulata extracts [27]. The leaf extract of A. squamosa is found to be effective against few respiratory isolates.

Anti-cancerous Activity of Annona muricata and its combination

The anticancer property of both A. muricata extract and the combined sample on A549 cell line is as depicted in Fig. 6. Apoptosis of cancer cell was significantly higher in case of combinational treated sample compared to A. muricata treated cell line. Figure 6a and 6b represents the untreated as well as Camptothecin treated A549 cell lines. Maximum apoptosis was observed at 450μM concentration of both the samples. Paul et al., [28], have reported that HeLa cells treated with 75 μg of a crude leaf extract of A. muricata shows 80% of cell inhibition which was not true in the present study. They have also characterized the crude leaf extract of A. muricata and confirmed the presence of bioactive compounds such as Anonaine, Friedelin, Isolaureline, Annonamine, Anomurine, Kaempferol, Asimilobine, Quercetin, Xylopine.

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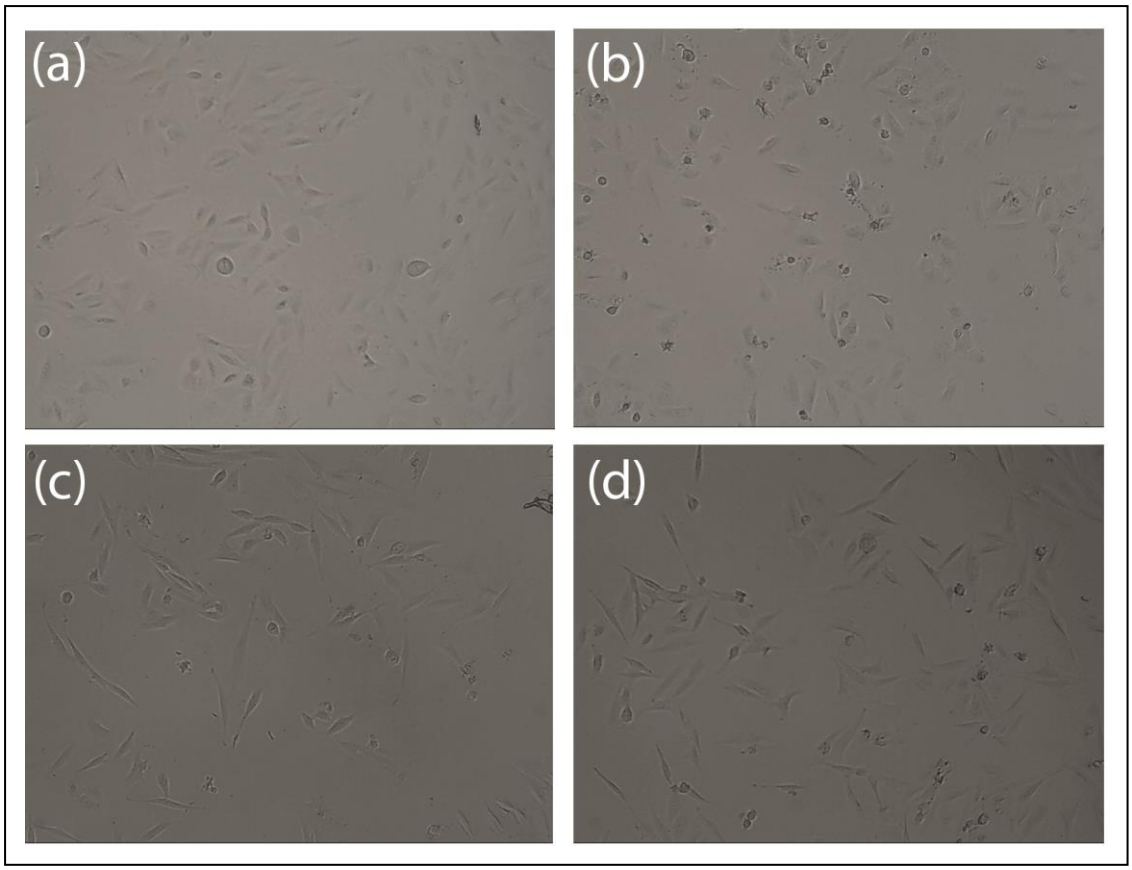

Nature has been a constant source of medicinal plants since time immemorial. Studies on natural products have gained lot of interest by Scientists over decades. Since the beginning of civilization, medicinal plants have been exploited by mankind for various therapeutic applications. Several modern drugs have been isolated from natural sources. Herbal plant based traditional medicine system continues to play a vital role in healthcare. Plants provides a good source of wide variety of compounds such as phenolics compounds, nitrogen compounds, vitamins, terpenoids and certain other secondary metabolites which are rich in bioactivities such as antioxidant, anti-inflammatory, antimicrobial etc.

Annonaceae members are reported to possess numerous medicinal properties. The bioactive compounds such as muricoreacin and murihexocin present in Annonaceous members depicted significant cytotoxicities among six human tumor cell lines with selectivity to the prostate adenocarcinoma and pancreatic carcinoma cell lines [29]. Monotetrahydrofuran acetogenin present in the A. reticulata seeds have illustrated a significant cell death in several cancer cell lines [30]. Root extracts of A. reticulata have depicted strong inhibitory effect against various cell lines namely human lung carcinoma, human chronic myelogenous leukemia bone marrow, human chronic myelogenous leukemia bone marrow and human adenocarcinoma mammary gland which can be due to the presence of acetogenins and alkaloids in the extract.

Seed extract of A. squamosa has depicted anticancerous property against human hepatoma cells both in vitro and in vivo conditions [31]. Bioactive compounds such as squamosa-O^1, squamocin-O^2 and squamostatin isolated from A. squamosa have exhibited cytotoxic activity against several cell lines [32-34]. Aqueous leaf extract of A. muricata on diabetic rats has depicted the hypoglycemic, hypolipidemic and antioxidant effects (Adewole & Ojewole, 2008). Similarly, Adeyemi et al. [35] have also reported the hypoglycemic effects of the methanolic extract of A. muricata on diabetic rats. This can be attributed to the fact that the compounds such as tanins, polyphenols, flavonoids, steroids, saponins present in the leaf extract might have contributed to the hypoglycemic effects in rats.

An in-depth understanding on the medicinal plants by researchers opens a new avenue in discovering and developing novel drugs with no side effects unlike most synthetic drug.

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