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IN VITRO ANTIMICROBIAL ACTIVITY OF THE UNRIPE PERICARP EXTRACTS FROM *Annona reticulata* LINN.

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ABSTRACT:

Hexane, chloroform, ethyl acetate, ethanol and water extracts of unripe pericarp of Annona reticulata Linn. Were assayed for antibacterial and antifungal activities using disc diffusion method. Ethanol proved to be the best when compared with the other solvents. Results of current study was interesting as the inhibition zone observed for crude ethanol extract against *E. coli* (IZ :19.67 mm, AI: 1.283) was even higher than the inhibition zone produced by the standard antibiotic, ampicillin of about 15.33 mm. More detailed pharmacological screening of the crude unripe extracts of this plant should be done for the exploration of effective and natural drug.

Key words: Antibacterial, antifungal, inhibition, screening

INTRODUCTION:

In rural communities the use of local herbs and plants is very common and uses may be for various purposes. There are numerous plants which are still in use since centuries to treat infection caused through microorganisms therefore, act an antimicrobial agent. An antibiotic is a chemical or biological compound that either destroys or inhibits the growth of microorganisms [19]. These antibiotics have saved countless lives and have revolutionized the field of medicine. However, the overuse and misuse of these wonder drugs resulted in appearance of microbial strains developing resistance. The rate of resistance of pathogenic microorganisms to conventionally used antimicrobial agents is increasing with an alarming frequency [21, 25]. In addition to this problem antibiotics are sometimes associated with adverse side effects on the host, which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions [2]. The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to

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antibiotics are continuously increasing. This increase has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agents, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection [8, 11].

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes; as a result, plants are one of the bedrocks for modern medicine to attain new principle [10]. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being [13]. Over the years, the WHO advocated that countries should encourage traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins [30]. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost-effective remedies that are affordable to the population [9]. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19thcentury [32]. Systematic screening of plants may result in the discovery of novel effective antimicrobial compounds [29]. Numerous experiments have been carried out to screen natural products for antimicrobial property [20, 22, 23, 24]. Also, studies have been conducted with the extracts of various plants for the discovery of new antimicrobial compounds.

In the present investigation, hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of the unripe pericarp of *Annona reticulata* Linn. were screened for antimicrobial activity against three Gram positive bacteria, three Gram negative bacteria and three fungi.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains were procured from the culture collection in the Microbiology division of Sree Chitra Thirunal Institute of Medical Sciences and Technology, Thiruvananthapuram. These include *Bacillus subtilis* (MTCC 739), *Enterococcus faecalis* (MTCC 2912), *Staphylococcus aureus* (MTCC96), *Eschericia coli* (MTCC 25922), *Pseudomonas aeruginosa* (MTCC 5210) and *Proteus vulgaris* (MTCC 1771). The fungi used were *Aspergillus fumigatus* (MTCC 277), *Aspergillus niger* (MTCC 2425) and *Penicillium chrysogenum* (MTCC160). The bacterial and fungal stock cultures were incubated for 24 hours at 37°C on Muller Hinton and potato dextrose agar medium (Hi media, Mumbai, India) respectively following refrigeration storage at 4°C.

Preparation of the inoculum

A loopful of inoculum was taken from a pure culture of respective bacteria/fungi inoculated into 10 ml of Muller Hinton broth. The broth suspension was then incubated at 37°C for 3 hours. The growth so obtained was used as inoculum for the sensitivity assay.

Screening of extracts for antimicrobial activity (Disc-diffusion assay)

The disc diffusion method was used to study the antimicrobial activity [4]. Muller-Hinton agar (for bacteria) and Potato Dextrose agar (for fungi) plates were inoculated with freshly grown bacterial culture of approximately 2.56 x 10^3 CFU/ml. About 100 µl of each

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plant extract (1 mg/disc) were loaded in the sterile filter paper discs (6 mm) and placed on the MHA plates. Discs containing 5% DMSO served as negative control. Streptomycin (1mg/disc) for Gram positive bacteria, ampicillin (1mg/disc) for Gram negative bacteria and flucanozole (1mg/disc) for fungi were used as positive controls. The plates were incubated at 37^0C for 18 - 24 hrs.

The Activity Index (AI) for each extract was calculated by using the following formula:

AI = Inhibition zone of the sample/ Inhibition zone of the standard

Determination of minimum inhibition concentration (MIC) of the extract with maximum antimicrobial activity

The MIC of the plant extracts was determined according to the micro broth dilution technique [3]. In this method, the plant extracts were prepared to the highest concentration of 2000µg/ml (stock concentration) in propanol and serially diluted (two-fold) to a working concentration ranging from 2000 µg/ml to 31.25µg/ml using Muller Hinton broth and added in 96 - well microtitre plates. Thereafter, a 100µl inoculum of standard size was added to each well. The positive control was broth with standard drug (gentamycin for bacteria and fluconazole for fungi) while the bacterial and fungal inoculums in broth served as negative control. The microtitre plates were incubated at 37 ± 2°C for 24 hours for bacteria, 27 ± 2°C for 5 -7 days for fungi. After incubation at 37°C, the microtitre wells were observed for turbidity. The turbidity in the wells were interpreted as a visible growth of microorganisms. The least concentration of the extracts that showed no turbidity after incubation was noted as the MIC value.

Determination of MBC/MFC

The minimum bacterial/fungicidal concentration (MBC/MFC) was determined by subculturing 50 µl from each well showing no apparent growth. The least concentration of extract showing no visible growth on subculturing was taken as MBC/MFC.

4.2.8 Determination of total activity

Total activity is the volume at which the test extract can be diluted without losing the ability to kill microorganisms. It is calculated as follows:

TA = Amount of extract from 1g of plant material/ MIC of the extract

RESULTS AND DISCUSSION

In the present study, the highest inhibition zone was recorded for ethanol extract against *E. coli* (IZ :19.67 mm, AI 1.283), even higher than the inhibition zone produced by the standard antibiotic, ampicillin of about 15.33 mm. This was followed by the inhibition zones produced by *E. faecalis* (IZ: 18.67 mm, AI: 1.057), *P. vulgaris* (IZ: 17.67 mm, AI: 1.082), *P. aeruginosa* (IZ: 16.33 mm, AI: 0.766) and *B. subtilis* (IZ: 14.33, AI: 0.632). Of all the tested solvent extracts, ethanol extract was found to be the most effective against all the tested microorganisms, followed by ethyl acetate and water (Table 1, Figures 1, 2 and Plate 1).

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This observation is very significant because of the possibility of developing therapeutic substances that will be active against multi-drug resistant organisms. Literature representing the studies on the antimicrobial activity of selected parts of *Annona reticulata* under study was scanty. The demonstration of antimicrobial activity against both Gram positive and Gram-negative bacteria was an indication that *Annona reticulata* was a potential source of drug with broad spectrum of activity [28]. It was also observed that Gram positive bacteria were more susceptible than Gram negative bacteria. This was supported by the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative bacteria [19]. The Gram-negative bacteria showed less sensitivity to plant extracts possibly as a result of their extra lipopolysaccharide and protein cell wall that provides a permeability barrier to the antibacterial agent [6,1]. Furthermore, the Gram-positive bacteria are more sensitive to the extracts because of the single layer of their cell wall while the double membrane of Gram-negative bacteria make them less sensitive [16].

Although the mechanism of action of these plant constituents is not yet fully known, it is clear that the effectiveness of the extracts largely depends on the type of solvent used. The organic extracts provided a more powerful antimicrobial activity, as compared to the aqueous extracts [7]. These variations in these results might be due to differences in the extraction method, media and solvents used, environmental conditions, metabolic stress on plant, physiological age of plant, seasonality and cultivar type [14,15, 26].

In this study, the mean zone of inhibition produced by the commercial antibiotics ampicillin, vancomycin and flucanozole were larger than those produced by the experimentally tested plant extracts. It may be attributed to the fact that the plant extracts being in crude form contain a smaller concentration of bioactive compounds [33].

All the three extracts exhibited poor or no activity against the three tested fungal strains. Among the three fungi, *A. flavus* produced a maximum inhibition zone of 9.66 mm and 9.67 mm against water and hexane extracts respectively.

The MIC values indicated that the unripe pericarp extracts of *A. reticulata* were more potent against bacteria than fungi. Also, these extracts are more potent against Gram positive bacteria than Gram negative bacteria. The MIC for ethanol extract was the lowest being 1.0 mg/ml compared with other extracts (Table: 2). Determination of MIC is important in diagnostic laboratories, as it helps to confirm the resistance of the microorganism to an antimicrobial agent, and it monitors the activity of new antimicrobial agents. Total activity indicates the volume at which the extract can be diluted with still having the ability to kill the microorganisms. Among the three extracts of unripe pericarp tested, high values of TA were recorded for ethanol extract against all the pathogenic microorganisms, including bacteria and fungi (Tab: 3). The traditional healers usually make use of water primarily as solvent, but the results obtained suggested that ethanol extracts of unripe pericarp were much better, powerful and possess strong antibacterial efficacy. This could be because of the better solubility of the active constituents in organic solvents [31, 17].

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Antimicrobial activity of medicinal plants is also attributed to the various chemical constituents (secondary metabolites) present in it [5, 7, 27]. Thus, the study ascertains the value of plants used in ayurvedha which could be of considerable interest in the development of new drugs.

Table 1: Diameter of the zone of inhibition (mm) of various extracts of unripe pericarp of *Annona reticulata* against selected clinical pathogens

MO	Activity	Hexane	Chloroform	Ethyl acetate	Ethanol	Water	Standard
Bacillus subtilis	IZ	-	-	10.33	14.33	8.66	22.67
	AI	-	-	0.456	0.632	0.382	
Enterococcus faecalis	IZ	-	6.0	8.67	18.67	9.33	17.67
	AI	-	0.34	0.491	1.057	0.528	
Streptococcus aureus	IZ	-	0	0	0	7.33	19.33
	AI	-	-	-	-	0.379	
Eschericia coli	IZ	-	-	-	19.67	13.33	15.33
	AI	-	-	-	1.283	0.87	
Pseudomonas aeuginosa	IZ	-	7.33	7.33	16.33	7.66	21.33
	AI	-	0.344	0.344	0.766	0.359	
Proteus vulgaris	IZ	-	-	10.3	17.67	11.67	16.33
	AI	-	-	0.633	1.082	0.715	
Aspergillus flavus	IZ	9.66	10.0	-	9.67	-	18.33
	AI	0.527	0.546	-	0.528	-	
Aspergillus niger	IZ	5.67	-	-	6.0	-	16.67
	AI	0.34	-	-	0.36	-	
Penicillium chrysogenum	IZ	-	6.33	7.67	8.33	7.33	19.67
	AI	-	0.322	0.39	0.423	0.373	

IZ: Inhibition zone in mm; AI: Activity Index

Fig 1: Antimicrobial activity of various extracts of unripe pericarp of *Annona reticulata* against selected clinical pathogens

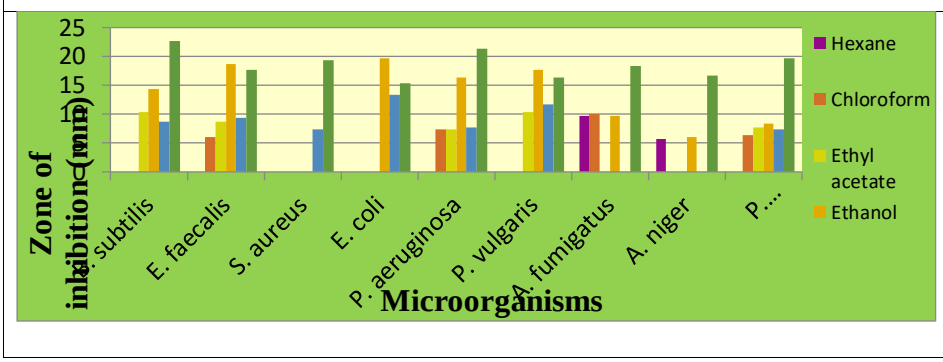

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Plate: 1Antimicrobial activity of unripe pericarp extracts of *Annona reticulata* against selected clinical pathogens

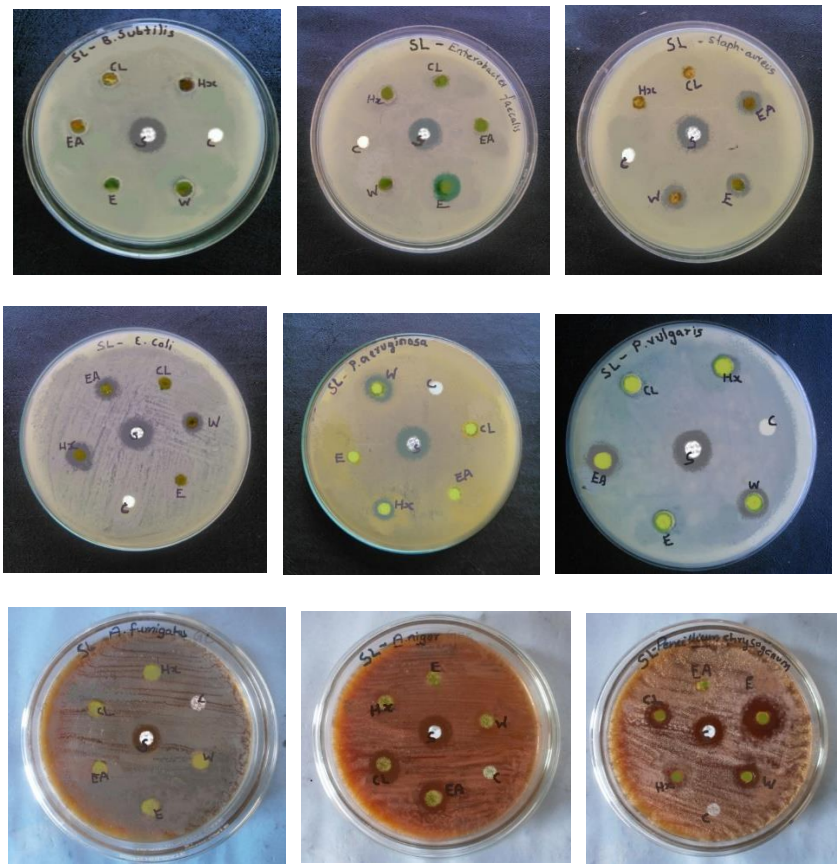
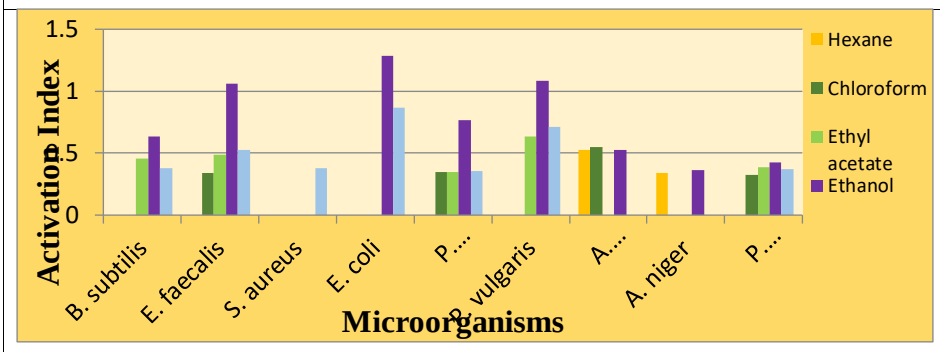


Fig 2: Activity Index of unripe pericarp extracts of *Annona reticulata* against selected clinical pathogens



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Tab: 2 Determination of MIC, MBC/MFC of unripe pericarp extracts of *Annona reticulata*

MO	Activity (mg/ml)	Water	Ethyl acetate	Ethanol
Bacillus subtilis	MIC	2.5	2	2
	MBC	>3	3	3
Enterococcus faecalis	MIC	2.5	2.5	1
	MBC	3	>3	2
Staphylococcus aureus	MIC	3	-	-
	MBC	>3	-	-
Eschericia coli	MIC	1.5	-	1
	MBC	2	-	1.5
Pseudomonas aeruginosa	MIC	3	3	1.5
	MBC	>3	>3	2.5
Proteus vulgaris	MIC	2	2	1.5
	MBC	3	2.5	2
Aspergillus flavus	MIC	-	-	2.5
	MFC	-	-	>3
Aspergillus niger	MIC	-	-	3
	MFC	-	-	>3
Penicillum chrysogenum	MIC	3	3	2.5
	MFC	>3	>3	3

MIC – Minimum inhibitory concentration; MBC – Minimum bactericidal concentration; MFC – Minimum fungicidal concentration

Tab:3 Total activity of unripe pericarp of *Annona reticulata* against selected clinical pathogens

Plant part	Extract	Amount of extract mg/g dried plant part	Test microorganisms	Total activity (ml/g)
		3.0	*B. subtilis*	20
			S. aureus	39.683
			E. faecalis	39.683
			E. coli	10
			P. vulgaris	-
			P. aeruginosa	-
			A. niger	10
			A. fumigatus	10
			P. chrysogenum	-

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Unripe pericarp		3.0	*B. subtilis*	57.6
			S. aureus	228.571
			E. faecalis	114.286
			E. coli	-
			P. vulgaris	-
			P. aeruginosa	-
			A. niger	-
			A. fumigatus	-
			P. chrysogenum	28.8
		3.0	*B. subtilis*	326.984
			S. aureus	20.6
			E. faecalis	163.492
			E. coli	41.2
			P. vulgaris	163.492
			P. aeruginosa	326.984
			A. niger	82.4
			A. fumigatus	163.492
			P. chrysogenum	41.2

CONCLUSION

The result of present study offers a scientific basis for the traditional use of solvent extracts of the selected medicinal plants and ascertains the value of these plants to be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant microbes. A marked inhibitory potential of ethanolic unripe pericarp extracts of *Annona reticulata* Linn. against Gram positive and Gram-negative bacteria and fungi were observed in this study. The antimicrobial potential of extracts could be ascribed to the presence of secondary metabolites detected in the extracts. The unripe pericarp extracts can be the potential candidate for the development of agents active against human pathogenic bacteria and fungi. This finding can form the basis for further studies to prepare and optimized preparation of the herbal extract to further evaluate the effectiveness in more pathogenic bacterial strains and for the efficacy to human health. The results provide justification for the use of these plants in folk medicine to treat various diseases. The inhibitory factor responsible for the antimicrobial activity can further be identified and used as an alternative to currently used drugs against the pathogenic microbes under study. Now a days microbes are increasingly developing resistance against the drugs in use. To combat against these drug resistant microbes, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

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