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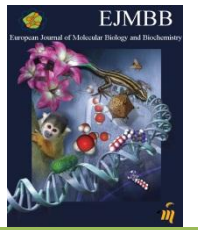

ANTIBACTERIAL ACTIVITY OF *ANNONA SQUAMOSA* L. AGAINST *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PYOGENES*

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ABSTRACT

Evaluated the antibacterial effect of aqueous and ethanolic extract of the leaf, bark of *Annona squamosa* L. against *Staphylococcus aureus* and *Streptococcus pyogenes* was analyzed in this study. All the selected strains were previously characterized. Aqueous and ethanolic extract of the leaf, bark of *Annona squamosa* L. were prepared with help of soxhlet unit. Further, evaluated the antimicrobial activity of these extract were analyzed against *S. aureus* and *S. pyogenes*. Aqueous and ethanolic extract of the leaf and bark of *Annona squamosa* leaf showed significant antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*. The 15 µg/ml aqueous extract of leaf of the *Annona squamosa* showed 1.66, 24.02% antimicrobial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* respectively as compared to the control. Similarly, 15 µg/ml ethanolic extracts of *Annona squamosa* leaf showed more inhibition zones (23.16 and 21.34 %, respectively) against *Staphylococcus aureus* and *Streptococcus pyogenes*. The 15µg/ml aqueous extract of bark of the *A. squamosa* showed 51.88, 78.77% more inhibition zone against *Staphylococcus aureus* and *S. pyogenes* respectively whereas 15 µg/ml ethanolic extract of bark of the *A. squamosa* exhibited 59.02, 99.45% more inhibition zone against *Staphylococcus aureus* and *S. pyogenes* respectively as compared to 15µg/ml norflox (control drug). Aqueous and Ethanolic extract of bark of *Annona squamosa* showed 5µg/ml MIC and 10µg/ml MBC against *Staphylococcus aureus* and *Streptococcus pyogenes*. Ethanolic extract of leaf of *Annona squamosa* showed 5µg/ml MIC but aqueous extract of leaf of *Annona squamosa* showed 10µg/ml MIC.

INTRODUCTION

Medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis [1]. Now present scenario, medicinal plants are a source of great medicinal value all over the world [2]. Many plants have medicinal value and recently and there are more than 35,000 plants species being used in medicinal purpose [3].

The respiratory tract infection are divided into the upper respiratory tract (nasal passages) prior to dissemination to the lower respiratory tract (airways and lungs). Respiratory infections are common in both hospital and community. The reports suggested that acute respiratory infections remain one of the most important causes of death in both adults and children [4]. Usually, the human upper respiratory tract is the reservoir of a diverse community of commensals and potential pathogens such as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* [5,6] which occasionally turn into pathogens causing infectious diseases. This respiratory infection effects on major population of world wide.

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The bacterial cells divide rapidly and evolve resistance to most treatments rather quickly and converted into resistance [7]. Continuous use of drug makes the micro-organisms into multi drug resistant (MDR). The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the spectra of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies [8]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions [9]. The use of antibiotics is not safe so scientists are more focus on alternative. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases [10]. Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [11]. The root of *Annona squamosa* is considered as a drastic purgative [12]. Further, a paste of seed powder has been applied to the head to kill lice. It is also used for destroying worm in the wound of cattles [13].

Annona squamosa Linn. also known as Sitaphala, Custard-Apple, belongs to the family Annonaceae. *Annonaceae* is commonly found in India, Thailand & originates from the West Indies & South America [14]. *Annona squamosa* Linn. is a large evergreen, straggling shrub or small tree, 7 m in height, introduced into India, found wild and cultivated in various parts, up to an altitude of 900m. Bark thin, grey; leaves oblonglanceolate or elliptic, pellucid-dotted, peculiarly scented, 5.0–15.0 cm × 1.9- 3.8 cm; flower 1-4, greenish, fleshy, drooping, extra-axillary, more on the leaf shoot than on the older wood [15].

The leaves of *Annona squamosa* are used as a vermicide, for treating cancerous tumors and are applied to abscesses, insect bites, other skin complaints and scrapings of root-bark are used for toothache, and powdered seeds are used to kill head-lice [16].

Various phytochemical analysis of *Annona squamosa* has been carried out. This plant is reported to contain glycoside, alkaloids, saponins, flavonoids, tannins, carbohydrates, proteins, phenolic compounds, phytosterols, amino acid. The various chemical constituents isolated from leaves, stems and roots of the plant including anonaine, aporphine, coryeline, isocorydine, norcorydine, glaucine **[**17, 18].

All the literature suggests that plant are good sources of medicine and it has antimicrobial activity. The medicinal plants are better alternative of antibiotics. Therefore, this study was carried out to evaluate antibacterial activity of aqueous and ethanolic extract of leaf as well as bark of the *Annona squamosa* L. against *Staphylococcus aureus* and *Streptococcus pyogenes*.

MATERIALS AND METHODS

Selection of strains: In present study, we used characterized *Staphylococcus aureus* and *Streptococcus pyogenes* strains [19].

Preparation of aqueous extraction: Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extract was done at 95^{o}C for 24 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth and then stored at 4 ºC.

Preparation of ethanol extraction: Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extract was done at 45^{o}C for 72 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth and then stored at 4 ºC.

Preparation of different concentration: The extracts were sieved through a fine mesh cloth and sterilized using a membrane filter (0.45-micron sterile filter). This extract was considered as the 100% concentration of the extract [20]. The concentrations such as 15, 20, 25, 30, 35 µg/ml were prepared and norflox 15 µg/ml worked as control drug.

Sterilization of extract: The dried extracts were exposed to ultra violet light (UV rays for 24 h to sterilize [21]. Liquid extracts were sterilized using a membrane filter (0.45-micron sterile filter).

Sterility Test: The sterility was checked by streaking the extracts on nutrient agar plate and incubated at 37° C for 24 h. It was confirmed that there were no artifacts to contaminate the sensitivity testing [21].

Antibacterial Activity by disc diffusion method and agar well diffusion method:

The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (30 ml) and incubated on a rotary shaker. Then 0.2 ml of inoculum (inoculum size was 10^8 cells/ml as per McFarland standard) was inoculated into the molten Muller Hinton agar media and after proper homogenization it was poured into the Petri plate. For agar disc diffusion method, the test compound (0.1 ml) was introduced on the disc (0.7 cm) and then allowed to dry. Then the disc was impregnated on the seeded agar plate. The plates were incubated at 37 ºC for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain, controls were maintained in which pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter is

shown in the graph. The experiment was done three times and the mean values are presented [22].

Antibacterial Activity by serial dilution in tubes

Dry the extract of medicinal plant. This powder of medicinal plant was dissolved in sterilized Mueller-Hinton broth and sterilized by membrane filter method. Various concentration of medicinal plants such as 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 µg/ml were prepared. The tubes were inoculated with 20 µL of the bacteria suspension per ml of broth, homogenised and incubated at 37ºC for 24 hours. After incubation, 50 µL were taken from each tube and inoculated in a second tube containing 1 mL of sterile Mueller-Hinton broth, homogenised and incubated for another 24 hours at 37ºC.
The **Minimal Inhibitory Concentration (MIC)** was determined as the lowest concentration of medicinal plant for growth was observed in second set of tubes. The **Minimal Bactericidal Concentration (MBC)** was determined as the lowest concentration of medicinal plant for which no growth was observed in the second set of tubes.

RESULTS

The pathogenic samples had been collected from the hospitals at haridwar, Uttarakhand [19]. Aqueous extract of leaf *Annona squamosa* showed significant antibacterial activity. 15µg/ml extract showed 1.66% more antimicrobial activity against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Staphylococcus aureus* was observed in 35µg/ml which was 267% as compared to control. Similarly, 15µg/ml showed 24.02% more antimicrobial activity against *Streptococcus pyogenes* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Streptococcus pyogenes* was observed in 35µg/ml which was 311.73% as compared to control (Table 1a). Ethanolic extract of leaf of *Annona squamosa* showed significant against *Staphylococcus aureus* and *Streptococcus pyogenes*. 15, 20, 25, 30, 35µg/ml extract showed more inhibition zone by 23.16, 58.75, 139.54, 218.64, 318.64% respectively against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). Similarly, 15µg/ml showed 21.34% more antimicrobial activity against *Streptococcus pyogenes* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Streptococcus pyogenes* was observed in 35µg/ml which was 315.169% as compared to control (Table 1b). Aqueous extract of bark of *Annona squamosa* showed significant antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*. 15µg/ml extract showed 51.88% more antimicrobial activity against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Staphylococcus aureus* was observed in 35µg/ml which was 126.778% as compared to control. Similarly, 15µg/ml showed 78.77% more antimicrobial activity against *Streptococcus pyogenes* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Streptococcus pyogenes* was observed in 35µg/ml which was 237.98% as compared to control (Table 2a). Ethanolic extract of bark of *Annona squamosa* showed significant against *Staphylococcus aureus* and *Streptococcus pyogenes*. 15, 35µg/ml extract showed more inhibition zone by 59.02, 145.85% respectively against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). Similarly, 15µg/ml showed 99.45% more antimicrobial activity against *Streptococcus pyogenes* as compared to 15µg/ml norflox (control drug) but highest inhibition zone against *Streptococcus pyogenes* was observed in 35µg/ml which was 239.67% as compared to control (Table 2b). Aqueous and Ethanolic extract of bark of *Annona squamosa* showed 5µg/ml MIC and 10µg/ml MBC against *Staphylococcus aureus* and *Streptococcus pyogenes*. Ethanolic extract of leaf of *Annona squamosa* showed 5µg/ml MIC but aqueous extract of leaf of *Annona squamosa* showed 10µg/ml MIC (Table 3).

Table 1a. Effect of aqueous extract of leaf of *Annona squamosa* against *Staphylococcus aureus* and *Streptococcus pyogenes*

Pathogen	Inhibition Zone (mm)					
	Medicinal Plant					Control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	9	12	18	26	33	9
SAD-2	10	13	17	27	33	9
SAD-3	9	12	17	26	34	9
SAD-4	9	12	18	26	33	9
SAD-5	9	12	18	25	33	9
SAD-6	9	13	18	26	33	9
SAD-7	9	12	18	26	32	9
SAD-8	10	12	18	26	33	9
SAD-9	10	12	18	26	33	9
SAD-10	9	13	18	26	33	9
SAD-11	9	12	18	25	33	9

SAD-12	9	12	18	25	33	9
SAD-13	9	12	17	26	33	9
SAD-14	9	12	18	26	33	9
SAD-15	9	11	18	26	33	9
SAD-16	9	12	18	26	33	9
SAD-17	9	12	18	26	33	9
SAD-18	9	12	19	26	34	9
SAD-19	9	13	18	26	34	9
SAD-20	9	12	18	26	33	9
Average	9.15	12.15	17.9	25.9	33.1	9
SD	0.357071	0.47697	0.43589	0.43589	0.43589	0
SPD-1	11	13	21	28	37	9
SPD-2	11	14	21	28	37	9
SPD-3	11	14	22	28	37	9
SPD-4	12	14	21	28	37	9
SPD-5	11	14	21	28	37	9
SPD-6	11	14	21	28	37	9
SPD-7	11	13	20	27	36	9
SPD-8	10	14	21	28	37	9
SPD-9	11	14	21	28	37	9
SPD-10	11	14	21	28	37	9
SPD-11	11	14	21	28	37	9
SPD-12	12	14	22	28	37	9
SPD-13	12	14	21	28	37	8
SPD-14	11	13	21	28	37	9
SPD-15	11	15	21	29	37	9
SPD-16	11	14	21	28	36	9
SPD-17	11	14	21	28	37	9
SPD-18	11	14	22	28	37	9
SPD-19	11	13	21	29	36	9
SPD-20	11	14	21	28	37	9
Average	11.1	13.85	21.1	28.05	36.85	8.95
SD	0.43589	0.47697	0.43589	0.384057	0.357071	0.217945

SAD = *Staphylococcus aureus*; SPD= *Streptococcus pyogenes*

Table 1b. Effect of ethanolic extract of leaf of *Annona squamosa* against *Staphylococcus aureus* and *Streptococcus pyogenes*

Pathogen	Inhibition Zone (mm)					
	Medicinal Plant					Control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	11	14	21	28	37	8
SAD-2	11	13	21	28	37	9
SAD-3	11	14	22	28	38	9
SAD-4	10	14	21	28	37	9
SAD-5	11	15	21	29	37	9
SAD-6	11	14	21	28	36	8
SAD-7	11	14	22	28	37	9
SAD-8	11	14	21	28	37	9
SAD-9	10	14	21	28	37	9
SAD-10	11	14	21	28	38	9
SAD-11	11	14	21	29	37	9
SAD-12	11	15	21	28	37	9
SAD-13	11	14	21	28	37	8
SAD-14	11	14	21	28	37	9
SAD-15	12	14	22	28	37	9

SAD-16	11	14	21	29	38	9
SAD-17	11	14	21	28	37	9
SAD-18	10	13	21	28	37	9
SAD-19	11	14	21	28	36	9
SAD-20	11	15	22	29	37	9
Average	10.9	14.05	21.2	28.2	37.05	8.85
SD	0.43589	0.497494	0.4	0.4	0.497494	0.357071
SPD-1	11	14	20	31	38	9
SPD-2	11	14	20	30	37	9
SPD-3	11	13	20	30	37	9
SPD-4	11	14	20	30	37	9
SPD-5	11	14	20	30	37	8
SPD-6	10	15	20	30	36	9
SPD-7	11	14	20	30	37	9
SPD-8	11	14	20	30	37	9
SPD-9	11	14	21	30	37	9
SPD-10	11	14	21	31	37	9
SPD-11	11	14	20	30	37	9
SPD-12	10	13	20	30	36	9
SPD-13	11	14	20	30	37	9
SPD-14	11	14	20	30	37	9
SPD-15	11	14	20	30	37	9
SPD-16	11	14	20	30	37	9
SPD-17	11	14	20	30	36	9
SPD-18	11	14	22	29	37	9
SPD-19	10	14	20	30	37	8
SPD-20	10	14	20	30	38	9
Average	10.8	13.95	20.2	30.05	36.95	8.9
SD	0.4	0.384057	0.509902	0.384057	0.497494	0.3

SAD = *Staphylococcus aureus*; SPD= *Streptococcus pyogenes*

Table 2a. Effect of aqueous extract of bark of *Annona squamosa* against *Staphylococcus aureus* and *Streptococcus pyogenes*

Pathogen	Inhibition Zone (mm)					
	Medicinal Plant					Control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	18	22	22	25	27	12
SAD-2	18	20	22	24	27	12
SAD-3	18	20	22	24	27	12
SAD-4	19	20	23	25	27	12
SAD-5	18	21	22	24	26	11
SAD-6	18	20	22	24	27	12
SAD-7	18	20	22	24	28	12
SAD-8	18	20	22	24	27	12
SAD-9	18	20	22	24	27	12
SAD-10	18	20	23	25	27	12
SAD-11	18	20	22	24	27	12
SAD-12	18	21	22	24	27	12
SAD-13	18	20	22	24	28	13
SAD-14	18	20	22	24	27	12
SAD-15	18	20	22	24	27	12
SAD-16	19	20	22	24	27	12
SAD-17	18	20	22	24	27	12
SAD-18	18	20	23	25	27	11
SAD-19	18	21	22	24	28	12

SAD-20	19	20	22	24	27	12
Average	18.15	20.25	22.15	24.2	27.1	11.95
SD	0.357071	0.53619	0.357071	0.4	0.43589	0.384057
SPD-1	16	18	22	27	30	8
SPD-2	16	19	22	27	30	9
SPD-3	16	18	22	27	30	9
SPD-4	16	18	22	27	30	9
SPD-5	16	18	22	27	30	9
SPD-6	17	19	23	28	31	10
SPD-7	16	18	22	27	30	9
SPD-8	16	18	22	27	30	9
SPD-9	16	18	22	27	30	9
SPD-10	16	18	22	27	30	9
SPD-11	15	17	22	27	31	8
SPD-12	16	18	22	28	30	9
SPD-13	16	18	24	27	30	9
SPD-14	16	18	22	27	30	9
SPD-15	16	18	22	27	31	9
SPD-16	15	18	22	27	30	9
SPD-17	16	18	22	27	30	9
SPD-18	16	18	24	28	30	9
SPD-19	17	18	22	27	32	9
SPD-20	16	18	22	27	30	9
Average	16	18.05	22.25	27.15	30.25	8.95
SD	0.447214	0.384057	0.622495	0.357071	0.53619	0.384057

SAD = *Staphylococcus aureus*; SPD= *Streptococcus pyogenes*

Table 2b. Effect of ethanolic extract of bark of *Annona squamosa* against *Staphylococcus aureus* and *Streptococcus pyogenes*

Pathogen	Inhibition Zone (mm)					
	Medicinal Plant					Control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	16	19	22	24	25	10
SAD-2	16	19	21	23	25	11
SAD-3	16	19	21	23	26	11
SAD-4	17	20	21	23	25	10
SAD-5	17	19	21	24	25	10
SAD-6	16	19	21	23	25	10
SAD-7	16	19	21	23	25	11
SAD-8	16	20	22	24	25	10
SAD-9	17	19	21	23	25	10
SAD-10	16	19	21	23	26	11
SAD-11	16	19	21	23	25	10
SAD-12	16	19	21	23	25	10
SAD-13	17	20	22	24	25	10
SAD-14	16	19	21	23	25	11
SAD-15	16	19	21	23	25	10
SAD-16	17	19	21	23	25	10
SAD-17	16	19	21	23	26	10
SAD-18	16	19	22	23	25	10
SAD-19	16	19	21	24	25	10
SAD-20	17	20	22	23	26	10
Average	16.3	19.2	21.25	23.25	25.2	10.25
SD	0.458258	0.4	0.433013	0.433013	0.4	0.433013
SPD-1	18	24	26	29	31	9

SPD-2	18	24	25	29	31	10
SPD-3	18	23	26	28	31	10
SPD-4	18	24	26	29	32	9
SPD-5	18	24	26	29	31	9
SPD-6	18	24	26	29	31	9
SPD-7	18	24	25	28	31	9
SPD-8	19	24	26	29	32	9
SPD-9	18	24	26	29	31	9
SPD-10	18	24	26	28	31	9
SPD-11	20	25	27	30	32	10
SPD-12	19	24	26	29	31	9
SPD-13	18	24	26	29	31	9
SPD-14	18	24	26	29	31	9
SPD-15	18	24	26	29	31	9
SPD-16	20	25	27	30	32	10
SPD-17	18	24	26	28	31	9
SPD-18	18	23	27	29	31	9
SPD-19	18	24	26	29	32	9
SPD-20	19	24	25	29	31	9
Average	18.35	24	26	28.9	31.25	9.2
SD	0.653835	0.447214	0.547723	0.538516	0.433013	0.4

SAD = *Staphylococcus aureus*; SPD= *Streptococcus pyogenes*

Table 3. MIC and MBC of *Annona squamosa* against *Staphylococcus* aureus and *streptococcus aureus*

S.No.	Plant part	Pathogen	Antimicrobial activity of plant extract			
			MIC (µg/ml)		MBC (µg/ml)	
			Aqueous	Ethanolic	Aqueous	Ethanolic
1	Leaf	*Staphylococcus aureus*	10	5	20	10
2	Leaf	*Streptococcus pyogenes*	5	10	15	10
3	Bark	*Staphylococcus aureus*	5	5	10	10
4	Bark	*Streptococcus pyogenes*	5	5	10	10

DISCUSSION AND CONCLUSION

All the available literature suggested that isolated strains were *Staphylococcus aureus* and *Streptococcus pyogenes*. Use of antibiotics is not safe for human and makes Multi Drug Resistant pathogens. Therefore, scientists are more focus on alternative medicine. Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [11].

Similarly, our results suggested that aqueous and ethanolic extract of plant significantly inhibited the growth of *Staphylococcus aureus* and *Streptococcus pyogenes*. 15µg/ml concentration of aqueous and ethanolic extract of bark of *Annona squamosa* inhibited the growth of *Streptococcus pyogenes* by 78, 99% more as compared to norflox (15µg/ml). Similarly, few phytochemical studies suggested that *Annona squamosa* has antimicrobial activity [17,18] which supported our finding that *Annona squamosa* has antimicrobial activity. Other reports suggested that leaf and flower of various plants such as *Aristolochia indica*, *Cassia angustifolia*, leaf of *Catharanthus roseus*, *Diospyros melanoxylon*, *Dolichos biflorus*, *Gymnema sylvestre* and *Justicia procumbens* showed antimicrobial activity against certain pathogens [20].

Minimum inhibitory concentration of our selected plants varies from 5 to 10µg/ml. Similarly, other report suggested that MIC values of leaf methanol extract of *A. indica* against *K. pneumonia* (22.6 µg/ml), and flower extract showed against *E. coli* (MIC: 24.2 µg /ml) [20]. Similarly other reports stated that extract of ethnomedicinal plants such as *Selaginella bryopteris* (Amarbatoooti Sanjivini), *Lygodium flexuousm* (Kalijar) *Adiantum philippense* (Kalijhant), *Drypteris eochleata* (Jatashankari), *Tectacria coadunate* (Jatamasi) exhibited antimicrobial activity against one or more of the tested microorganism and also mentioned that extract concentrations spanning from 25 µg/ml to 2 mg/ml or even 40 mg/ml [21]. Further, other report suggested that ethanolic leaf extract of *Pongamia pinnata* and *Lowsonia innermis* possessed a highest inhibitory zone against *Pseudomonas aeroginosa* and *Micrococcus luteus* [22]. We concluded that *Annona squamosa* significantly inhibited the growth of isolated pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes*.

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