Evaluation of antibacterial activity of crude extracts of *Annona senegalensis* (wild custard apple) against *Staphylococcus aureus* and *Pseudomonas aeuginosa*, and formulation of a topical gel

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Abstract

Annona senegalensis is a plant well known as the wild custard apple and has been widely reported for its various medicinal uses. This study investigates the potential of *Annona senegalensis* extracts as antibacterial agents against common pathogenic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The leaves of *Annona senegalensis* were collected, dried, and ground into fine powder. The ground leaves were extracted using a soxhlet apparatus, and the solvents were methanol, ethyl acetate, and chloroform. A rotary evaporator was used to concentrate the extracts. Phytochemical analysis was carried out to investigate the phytochemicals present in different extracts of *Annona senegalensis*. The antibacterial activity of *Annona senegalensis* against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was performed using the agar disc diffusion method. Ciprofloxacin was used as a positive control at different concentrations, and DMSO as a negative control. The topical gel was then formulated using *Annona senegalensis* leaf extracts, glycerine, and xanthan gum.

The results show that *Annona senegalensis* leaf extracts of ethyl acetate, chloroform, and methanol contain saponins, flavonoids, tannins, glycosides, anthraquinones, terpenoids, steroids, phenol, and alkaloids. However, the ethyl acetate extract of *Annona senegalensis* exhibited the highest antibacterial activity compared to other extracts. *Staphylococcus aureus* was found to be the most susceptible.

This study indicated that *Annona senegalensis* can effectively treat bacterial infections like wound infections associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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Introduction

Annona senegalensis, also known as the wild custard apple, has widely been reported to have antibacterial activity, particularly against *Staphylococcus aureus*.[1] It has also shown efficacy against *Escherichia coli* and *Bacillus subtilis* using the solvent method.[2] Ethanolic and aqueous leaf extracts of *Annona senegalensis* showed antibacterial activity against the bacterial strains *Staphylococcus aureus*, *Shigella flexneri*, *Salmonella paratyphi*, *Pseudomonas aeruginosa*, and clinical isolates *E. coli*, *S. dysenteriae*, and *S. enteriditis*.[3] Thus, the current study focuses on plants for medicinal purposes.

This is because, in developing nations, 90% of the population relies on plants for medication.[4] Vinblastine, vincristine, etoposide, teniposide, and taxanes are among the medications in clinical trials for cancer derived from natural sources.[5] In Zimbabwe, traditional medicine is one of the oldest medicinal systems available to the bulk of the population, and some people still cannot afford or access hospitals and clinics. Many people employ herbal medicines, and it is believed that more than 80% of the world's population relies primarily on traditional herbal therapy.[6] Extensive scientific research on plants is required to integrate traditional medicines into modern treatment. The phytochemical constituents, pharmacological effects, and toxicological standards of the selected plants must be established for dose recommendations.[7] *Annona senegalensis*' bark is used in Eswatini to heal open wounds;[8] thus, this study considered qualitative analysis of phytochemicals in this plant[9] and their effects on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Hospitals

and communities have reported the rise of *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains resistant to antibiotics.[10-12] Therefore, there is a need to formulate new treatments that can be used as an alternative.

Thus, this study aims to evaluate the antibacterial activity of *Annona senegalensis* and develop a topical gel for treatment against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Materials and Methods

Sample collection and preparation

Healthy and mature leaves of *Annona senegalensis,* as shown in Figure 1, were collected between June and September 2023 from Marondera, Zimbabwe. The leaves were washed thoroughly with distilled water to remove dirt. The leaves were dried in a well-ventilated area away from direct sunlight. The dried leaves were ground into a fine powder using a blender.

Extraction of crude extracts using soxhlet apparatus

A measure of 20 g of powdered *Annona Senegalensis* leaves was weighed using a balance. This sample was placed in a thimble and put in the extraction chamber of the Soxhlet apparatus. Round-bottomed flasks measuring 250 ml were each filled with methanol, chloroform, or ethyl acetate. The Soxhlet apparatus was assembled, and the solvents in the flasks were heated in the round bottom flasks, allowing them to vaporize and rise into the condenser. As the vapor condensed, it dripped into the extraction chamber, extracting the desired compounds from the plant material. The extraction process continued for 6 hours to ensure efficient extraction.

Filtration and concentration

The extract was collected in the round-bottom flask, filtered using a filter, and filtrate collected in a clean glass container. The filtrate was concentrated using a rotary evaporator until a semi-solid residue was obtained. The formula calculated the yield (R) of the extract:

R (%) = mass of the extract/mass of plant product used x100

Phytochemical screening

Phytochemical analysis assays were carried out on ethyl acetate, chloroform, and methanol extracts from the Annona *senegalensis* powdered specimens using standard procedures to identify the constituents with some modifications.

Test for alkaloids

To 1 ml of extract, three drops of Wagner's reagent were added. A reddish-brown precipitate indicated a positive test.

Test for tannins

The test conducted was the Braymer's test, and to 0.5 ml of extract, 7 ml of water was added, followed by the two drops of ferric chloride. A blue color for gallic tannins and a green and black color for catecholic tannins indicated a positive test.

Test for phenolics

The lead acetate test was conducted, and a white precipitate indicated a positive test when 3 ml of lead acetate was added to 1 ml of extract.

Test for saponins

A foam test was conducted, and a persistent foam test was indicated as positive when 1 ml of extract was added to 3 ml of water.

Test for glycosides

The sodium nitroprusside test was conducted; a blue color indicated a positive test when a few drops of NaOH were added and shaken and added to 1 ml of extract, followed by sodium nitroprusside.

Test for flavonoids

An alkaline test was performed. A few drops of 10% NaOH were added to 2 ml of extract, and an intense yellow, which became colorless in addition to HCl, indicated a positive test.

Test for terpenoids

A volume of 1 ml extract was placed into the test tube, and 0.4 ml of chloroform was added. A volume of 0.6 ml of concentrated H□SO□ was poured gently into the tube at an inverted position. The appearance of a reddish-brown color indicated the presence of terpenoids.

Test for steroids

A volume of 1 mL of extract was dissolved in 2 mL of chloroform, and a few drops of concentrated H_2SO_4 were carefully added to form a lower layer. The formation of a reddish color in the lower layer indicated the presence of steroids.

Test for anthraquinones

A mixture of 0.5 mL extract and 5 mL of chloroform was shaken for 5 minutes and filtered. The filtrate was then shaken with an equal 30% ammonia solution. A pink-violet or red color indicated a positive test.

Antibacterial activity determination

Media preparation

All media (nutrient agar, Mueller-Hinton agar, and nutrient broth) was prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. For quality control purposes, all culture media were checked for sterility by incubation overnight at 37°C and stored at 2°C to 10°C until use.

Figure 1. *Annona senegalensis* leaves.

Quadrant streak method

Using the streaking method, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were cultured in nutrient agar petri dishes. The agar plates were sealed with parafilm to prevent contamination. These were incubated at 37°C for approximately 18-24 hours to allow growth of the cells.

Pseudomonas aeruginosa and Staphylococcus aureus nutrient broth cultures

Nutrient broth of volume 20 mL was poured into two different 50 mL falcon tubes labeled *P. aeruginosa* or *S. aureus - pure* cultures. *S. aureus* and *P. aeruginosa,* grown in the streak plates, were collected using a sterile inoculating loop to add a small amount of bacterial culture to the broth. The cells were incubated at 37°C for 24 hours. Changes in turbidity indicated the presence of the two bacteria in the broth. The cultured cells were stored at 4°C for antimicrobial sensitivity testing assays.

Disc diffusion for sensitivity tests using extracts

After the Mueller-Hinton Agar (MHA) had cooled, *Pseudomonas aeruginosa* and *Staphylococcus aureus* diluted cells were added to (MHA). The MHA mixture was poured into sterile plates under aseptic conditions. After the media had solidified, filter paper discs containing the test compound at a desired concentration were placed on the agar surface. Extract concentrations were 12.5, 25, 50, and 100 µg/ml. The plates were left overnight for incubation and observed the following day.

Formulation of topical gel

Aloe vera measuring 5 g, 5 g extract of ethyl acetate or methanol extract, 1 g xanthan gum, 10 g glycerine, 10 g of potassium sorbate, and 69 mL of water was used as the ingredients. In a separate beaker, 10 g of xanthan gum was mixed with a small amount of water to create a smooth paste. Glycerine was added to the xanthan gum paste and mixed well. Gradually, water was added to the xanthan gum mixture, and stirring continued. Ethyl acetate extract and *Aloe vera* mixture were added to the xanthan gum mixture. Potassium sorbate was then added. The mixture was then placed in a homogenizer for 5 minutes for uniform distribution. The mixture was poured into a clean container and let sit for about 30 minutes to thicken. A pipette was used to withdraw the gel and transfer it into a clean, sterile tube. For the formulation of topical gel with methanol or ethyl acetate extract only, the ingredients were 10 g of the extract, 1 g of xanthan gum, 10 g of glycerine, and 69 ml of water. The same procedure was followed without the addition of potassium sorbate.

Statistical analysis

Statistical analysis of the results was carried out using One-Way Analysis of Variance (ANOVA) followed by multiple comparisons with Dunnett's test to determine which mean values were significantly different from the control values. All computations were done using Graph Pad Prism software (Version 9.5.0; Graph Pad Software Inc., San Diego, USA). Significant differences were considered at $p<0.05$, and all the results were expressed as the mean ±standard deviation of the mean.

Results

Extraction and percentage yield

The yield of the dried extracts of *Annona senegalensis* after extraction with different solvents is shown in Table 1. The different solvents are methanol, ethyl acetate, and chloroform. The percentage yield was calculated as the mass of dried extract divided by the mass of the original sample and multiplied by 100. Ethyl acetate was the best solvent as it extracted the highest yield.

Qualitative analysis of phytochemical constituents

The phytochemical identification in each extract was done using different methods, and the observations are shown in Table 2. In solvent extracts to which a phytochemical was positively identified, the color or precipitate of the result was found to be different relative to each solvent extract. Methanol extract shows the highest number of phytochemicals, followed by ethyl acetate and chloroform extract.

Antibacterial sensitivity testing of extracts

The antibacterial activity of *A. senegalensis* crude extracts against *S. aureus* was evaluated using agar disc diffusion assay, and results are shown in Figure 2. The bacterial cells were exposed to varying concentrations (12.5, 25, 50, and 100 µg/ml), and the inhibition zones of different extracts at varying concentrations

Table 1. The yield of dried extracts from *Annona senegalensis* leaves after extraction with different solvents.

Solvent	Yield/%
Methanol	3.03
Ethyl acetate	16.1
Chloroform	10

Table 2. Phytochemical analysis of *Annona senegalensis* extracts of different solvents.

Phytochemical	Chloroform	Ethyl acetate	Methanol
Phenolics	-	+	-
Flavonoids	+	-	+
Tannins	-	+	+
Glycosides	+	+	+
Alkaloids	-	-	+
Steroids	-	+	+
Terpenoids	+	+	-
Anthraquinones	+	-	+
Saponins	+	+	+

+ indicates the presence of phytochemical; - indicates the absence of phytochemical

were measured in millimeters. For all the concentrations tested, the ethyl acetate extract was the most potent compared to the other extracts. However, as expected, the positive control, ciprofloxacin, proved more potent than the ethyl acetate against *S. aureus*. The antibacterial activity of *A. senegalensis* crude extracts against *P. aeruginosa* was evaluated using agar disc diffusion assay, and the results are shown in Figure 3. The bacterial cells were exposed to varying concentrations (12.5, 25, 50, and 100 µg/ml), and the inhibition zones of different extracts at varying concentrations were measured in millimeters. For all the concentrations tested, the antibacterial effect of all the extracts showed the same trend as the effect against *S. aureus*, with a slight decrease in zones of inhibitions. Like against *S. aureus*, the ethyl acetate extract was shown to be the most potent compared to the other extracts, with the positive control, ciprofloxacin being more potent than the ethyl acetate.

Topical gel formulation

Four different topical gels were formulated, and each gel consisted of ethyl acetate extract, ethyl acetate and *Aloe vera*, methanol extract, and methanol and *Aloe vera*, as shown in Figure 4.

Discussion

According to the results, the higher the extract concentration, the greater the zone of inhibition on the bacterial strains. For both bacteria *S. aureus* and *P. aeruginosa*, ethyl acetate exhibited the most significant zone of inhibition at a concentration of 100 µg/ml, followed by methanol extract and chloroform extract. This study also showed that *S. aureus* was more susceptible to all the extracts at all tested concentrations than *P. aeruginosa*. *Staphylococcus aureus* is a Gram-positive bacterium with a thick peptidoglycan cell wall, while *P. aeruginosa* is a Gram-negative bacterium with an outer membrane and a thinner peptidoglycan layer. The differences in cell wall composition and permeability can affect the ability of antibacterial compounds to penetrate and disrupt the bacterial cell, making *S. aureus* more susceptible to the extract compared to *P. aeruginosa.*[13] *Staphylococcus aureus* has been found to develop resistance mechanisms, such as the production of β-lactamases, which can degrade and inactivate certain antibiotics. However, the extract from *Annona senegalensis* leaves might contain bioactive compounds with alternative mechanisms of action that are effective against *S. aureus*, even in the presence of β-lactamases.[14] On the other hand, *P. aeruginosa* has a higher intrinsic resistance and can acquire various resistance mechanisms, including the production of β-lactamases, efflux pumps, and alterations in cell wall permeability. These resistance mechanisms in *P. aeruginosa* may contribute to its reduced susceptibility to the extract.[15]

The polarity of the solvent can influence the types of phytochemicals extracted from plant material.[16] Compounds with moderate polarity may have more potent antibacterial properties, which could explain why ethyl acetate extracts showed more excellent antibacterial activity. Some antibacterial compounds may also be more stable in ethyl acetate than methanol or chloroform. Solvents can cause degradation or denaturation of certain compounds, potentially reducing their antibacterial efficacy. This degradation could reduce their antibacterial activity, leading to the absence of inhibition at lower concentrations.[17] Ethyl acetate has a relatively high evaporation rate, which can concentrate the extracts more effectively during evaporation, potentially leading to higher concentrations of antibacterial agents in the residue.[18] The specific interactions between the test compounds and the components present in ethyl acetate could have contributed to the observed inhibition at lower concentrations.[18]

A topical gel was formulated better to distribute the active compounds onto the target site, leading to a larger zone of inhibition.[19] The topical gel formulation is designed to optimize active compounds' delivery, stability, and bioavailability. It may contain

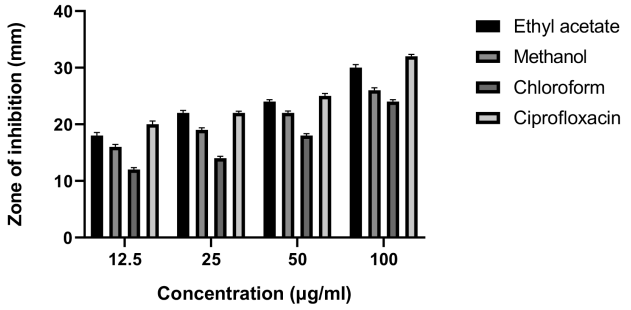

Figure 2. Effect of different concentrations of different extracts on *S. aureus*. The agar disc diffusion assay was conducted in triplicates. Values are for mean ± standard deviation, for n=4.

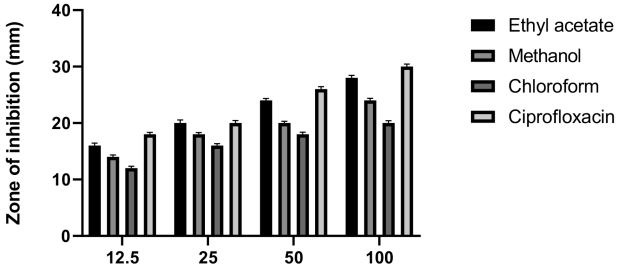

Figure 3. Effect of different concentrations of different extracts against *P. aeruginosa*. The disc diffusion assay was conducted in triplicates. Values are for mean ± standard deviation, for n=4.

Figure 4. Different topical gels with extract, in sterilised containers.

specific excipients or additives that enhance the antibacterial agents' solubility, permeability, and release. *Aloe vera* has been reported to possess inherent antibacterial properties such that when combined with other antibacterial agents present in the gel formulation, *aloe vera* can exhibit synergistic effects. The interaction between the active compounds from the extracts and *Aloe vera* may enhance the overall antibacterial activity, resulting in a larger zone of inhibition compared to the extracts alone. *Aloe vera* contains various bioactive compounds that can contribute to its antibacterial activity. These compounds can work in synergy with the active compounds in the extracts, providing a broader spectrum of antibacterial action.[20] Including *Aloe vera* in the formulation of a topical gel can provide added skincare benefits and improve the overall efficacy and tolerability of the gel.[21]

Potassium sorbate is a preservative commonly used in cosmetic and personal care products, including topical gels. It helps prevent the growth of microorganisms, such as bacteria and fungi, in the gel formulation.[22] By inhibiting microbial growth, potassium sorbate helps maintain the stability and shelf life of the gel. In topical gel formulations, xanthan gum is a thickening and stabilizing agent. It increases the viscosity of the gel, giving it a desirable consistency and texture. Xanthan gum also enhances the spreadability and adherence of the gel to the skin, improving its overall application properties.[23] Glycerine, or glycerol, is a humectant commonly used in skincare products, including topical gels. It attracts moisture from the environment and helps retain it in the skin, keeping it hydrated. Glycerine also provides a smooth and moisturizing feel to the gel, enhancing its sensory properties and making it more pleasant to use.

Conclusions

Annona senegalensis leaf extracts may be used to treat bacterial infections caused by *S. aureus* and *P. aeruginosa,* as they showed inhibitory activities against these species. *Annona senegalensis* extracts may be easily used in the form of a topical gel.

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