



Evolution of Antibacterial Activity of Various Solvents Extracts of *Annona Squamosa* Fruit

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Abstract

This research project was designed to evaluate the antibacterial activity of three different portions (pulp, peel, seeds) of *Annona squamosa* fruit using three different extraction solvents (water, ethanol, and acetone). The experiment performed by using agar well diffusion method against Gram-positive human pathogenic bacteria, *Staphylococcus aureus* and Gram-negative bacteria, *Klebsiella pneumoniae*, and *Escherichia coli* and each organism showed different patterns of inhibition zones. Antibacterial activity of various solvent extracts of the pulp showed noticeable inhibitory activity against almost all the tested pathogens except *K. pneumoniae* who was resistant to water extract of pulp. Although, different solvents extracts of peel were found to be efficient in inhibiting the test pathogens, ethanolic extract of peel exhibited the best antibacterial activity against all the test pathogens in this study. Maximum inhibition activity was found with the peel ethanolic extract against *E. coli* and *K. pneumoniae*, followed by *S. aureus* at crud extract concentration of 50 mg ml⁻¹, which is comparable to the inhibition zones of the standard antibiotic (*Tetracycline* and *Ceftriaxone* 100mg ml⁻¹) used in this study. The water extract of peel also exhibited fairly good antibacterial activity against *E. coli* approximately similar to the ethanolic extract. Conversely, *S. aureus* and *K. pneumoniae* showed complete resistance against water extract of peel. Regarding seeds bactericidal abilities, the water and acetone extracts of seeds showed remarkable inhibitory action against *K. pneumoniae* followed by the water extract against *E. coli*. None of the test pathogens showed inhibition of growth response to seed ethanolic extract.

In conclusion, antibacterial ability of different portions of *A. squamosa* fruit extracts against different types of bacteria used in this experiment signified their remarkable potential for exploration and using effective antibacterial agents from natural resources to inhibit the growth of different types of pathogenic bacteria.

Keywords: *Annona squamosa*, antibacterial activity, fruit, pulp, peel, seeds, extracts, solvents.

دراسة الفعالية المضادة للبكتيريا لمستخلصات فاكهة تفاح القشطة *Annona squamosa* باستخدام مختلف أنواع المذيبات

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الخلاصة

تم تصميم هذا المشروع البحثي لتقييم الفعالية التثبيطية ضد الاحياء المجهرية لمستخلصات ثلاثة أجزاء مختلفة (لب، قشر، بذور) من فاكهة تفاح القشطة *Annona squamosa* وذلك باستخدام ثلاثة انواع

مختلفة من مذبيبات الاستخلاص (الماء والإيثانول والأسيتون) . نفذت التجربة باستخدام طريقة نشر آجار ضد البكتيريا لمرضية الموجبة لصبغة الجرام (المكورات العنقودية الذهبية *Staphylococcus aureus*) والبكتيريا سالبة لصبغة الجرام (كليبسيلا الرئوية *klebsiella pneumoniae* ، والإشريكية القولونية *Escherichia coli*). أظهر كل كائن حي أنماطا مختلفة من مناطق التثبيط لمستخلصات الاجزاء المختلفة من الفاكهة. أظهرت مستخلصات اللب نشاطا تثبيطيا ملحوظا لجميع الانواع البكتيرية المستخدمة في التجربة باستثناء بكتيريا الكليبسيلا الرئوية *K. pneumoniae* الذي كان قد اظهر مقاومة ملحوظة ضد المستخلص المائي لللب الفاكهة.

على الرغم من أن مستخلصات القشرة وباستخدام المذبيبات المختلفة اظهرت فعالية جيدة في تثبيط جميع انواع السلالات البكتيرية ، الا ان المستخلص الكحولي (إيثانول) للقشرة اظهر أفضل فعالية تثبيطية ضد جميع الجراثيم المرضية المستخدمة في هذه الدراسة. أقصى نشاط تثبيطي مع مستخلص الكحولي لقشرة الفاكهة عند تركيز 50 ملغم/ مل كان ضد بكتيريا *E. coli* و *K. pneumoniae* معا بنفس المستوى تقريبا ، يليها و بمستوى اقل نسبيا ضد *Staphylococcus aureus* ، وهو كان مماثلا لحجم مناطق التثبيط البكتيري باستخدام المضادات الحيوية القياسية (Tetracycline and Ceftriaxone 100 ملغم / مل) المستخدمة في هذه الدراسة. كما أظهر المستخلص المائي للقشرة نشاطا تثبيطيا ملحوظا ضد بكتيريا *E. coli* و عند مستوى مماثل تقريبا للمستخلص الكحولي للقشرة . وعلى العكس من ذلك، أظهرت *Staphylococcus aureus* و *K. pneumoniae* مقاومة كاملة للمستخلص المائي للقشرة. أما فيما يتعلق بالقدرات التثبيطية للبدور ضد البكتيريا ، فقد أظهرت المستخلصات المائية والأسيتون للبدور قدرات تثبيطية ملحوظة ضد بكتيريا *K. pneumoniae* يليها قدرة تثبيطية لكن بمستوى اقل للمستخلص المائي ضد نمو بكتيريا *E. coli* . لم تظهر أي من مسببات المرضية المستخدمة في التجربة اي استجابة تثبيطية ضد للمستخلصات الكحولية للبدور .

الكفاءة التثبيطية لمستخلصات الاجزاء المختلفة لفاكهة تفاح القشطة *A. squamosa* ضد نمو مختلف انواع الجراثيم المستخدمة في هذه التجربة يدل على امكانية استكشاف واستخدام المضادات البكتيرية الفعالة من مصادر طبيعية لتثبيط قدرة نمو انواع مختلفة من الميكروبات المسببة للأمراض .

Introduction

Bacterial resistance to antibiotics became a considerable clinical problem as bacteria is able to develop resistance to antimicrobial agents, which is inconstant in different geographic regions and it has been connected with general population consumption of antibiotics [1]. Moreover, antibiotics resistance serious threat to global public health, which decreases the efficacy of these remedies and compromises patient.

Medicinal plants and herbs have been considered one of the important type of medicines from the start of human development. Moreover, number of medicinal plants used in indigenous medicine have been tested as alternative medicines because of their bactericidal properties. For that reason, many research focused on the potential application of natural antimicrobial compounds in food, cosmetic and pharmaceutical manufacturing which has obtained considerable attention. In the recent years, apart from medicinal plants, fruits, which are a rich source of bioactive compounds have become popular subjects for such investigations [2].

Annonaceae is one of the biggest families, which containing about 130 genera over 2000 species are *Annona*, with 150 species genera, and the species of *Annona squamosa* is a small evergreen tree reaching 6-8 meters. It is also known as ' graviola', 100 species of *Annona*, only 5 species, namely the custard apple, cherimoya, soursop, bullock's heart and atemoya are of major commercial importance [3]. The plant originate in deciduous forests and cultivated because of its fruit in most South-East Asian countries such as Malaysia, Indonesia and the Philippines. The genus name *Annona* is from the Latin word 'anon' meaning 'yearly produce' mentioning to the fruit production habits of the various species in this genus [4].

A. squamosa plant is employed for a number of medicinal purposes and it has been the object of much research due to its multiple uses and well-known bactericidal effect. Literatures of many

research works demonstrate that various parts of plants such as the fruits, leaves, the barks, roots and even the seeds are therapeutically helpful [5]. The plant is traditionally used for the treatment of dysentery, worm infestation, epilepsy, cardiac problems, malignant tumors, ruinting, ulcer, hemorrhage, constipation, dysuria, fever. The plant extracts has been showed remarkable antimicrobial and cytotoxic activities [6]. The root is considered as an effective therapy for hepatic problems [7]. The dried unripe fruit powder is used to destroy vermin while the ripe fruits of this plant are applied to malignant tumors to hasten suppuration [2]. The seed extracts have been reported by several researchers for their antimicrobial activity [8]. A paste of seed powder has been also used for destroying worm in the wound of cattles [9]. Ethanol and methanol extract of the plant leaves have insecticidal activity against *Aedes albopictus* and *Culex quinquefasciatus* [10]. The potential effect of this plant on different microorganisms have been found to be due to the presence of certain substances such as alkaloids, glycosides, tannins, volatile oils, gums, steroids, saponins, flavonoids, phlobatannins [11]. This may be elucidate its diversity of curing diseases and well tolerated therapies compared to the conventional medicines.

During *A. squamosa* plant extracts production for medical applications, various factors need to be considered, such as the best solvent, part of the plant that has to be used, and degree of fruit ripening. Solvent normally used for bioactive compounds extraction for various applications are water, ethanol, methanol, chloroform, ether, hexane and acetone.

There have been many studies on the different parts of *A. squamosus* plant as an antibacterial agent [4, 6, 11-17]. However, there is no clear study to determine the best part of fruit as antibacterial agent related to the type of solvent used for extraction. There is no previous local studies revealed the relationship between the kind of solvent used for extraction and different portions of *A. squamosa* fruit on the inhibition activity of the tested microorganisms.

Therefore, the aim of present study is to evaluate antibacterial potential of different extraction solvents (water, ethanol and acetone) of different *A. squamosa* fruit portions independently.

Materials and Methods

Plant material

Throughout the study, a fresh sample of *A. squamosa* fruits were obtained from local fruit shops, washed thoroughly under tap water, separated i.e. between pulps, peels, and seeds to be dried with tray dryer at temperature of 55°C constantly for 72 h. Different parts of dried fruit were chopped separately with blender to produce powder samples. The powder samples was packed and stored at temperature of 20°C in the dark storage room before extraction was done.

Preparation of Crude Extracts

The solvents were used for the present study included distilled water, ethanol, and acetone. 10 g of each fruit parts powder was extracted with 200 ml of each solvent using Soxhlet extractor. The extracts were concentrated separately under vacuum, and the resulting dried extracts were weighed and used for the study [18]. Each of the dried extracts of the fruit parts was stored in closed vial at 4°C. Working stocks were prepared by dissolving each fruit peel extract in appropriate amount of respective solvent in order to obtain a final concentration of 50 mg ml⁻¹.

Agar well Diffusion Method

Three bacterial cultures of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*, which were obtained from clinical microbiology laboratories of the hospitals and private clinics in Kirkuk province. The agar cup method was used to study the antibacterial activity of the extracts as described by Padhi, *et al.* [19]. Mueller-Hinton agar (MHA) was used as bacteriological medium and MHA plates were prepared. Bacterial suspension adjusted to 0.5 McFarland standard (1.5×10⁸ CFU/ml) was used by sterile swab. 0.1% inoculums suspension of bacteria was swabbed uniformly and the inoculums were allowed to dry for 5 min. The extracts were diluted in appropriate amount of respective solvent. A total of 6 mm diameter wells were punched into these seeded agar plates using a sterile cork-borer. 0.1 ml (100 µl) of each extracts (from stock solution 50 mg ml⁻¹) was then introduced into each well using a micropipette, and 100 µl of standard antibiotic (Tetracycline and Ceftriaxone 100 mg ml⁻¹) were used as a positive control. The plates thus prepared were left for diffusion of extracts into media for one hour in the refrigerator and then incubated at 37°C for 24 h. After the incubation period, formation of zones around the wells confirms the antibacterial activity of the respective extracts. The experiment was carried out in triplicates to get rid of any error. The antimicrobial activities were calculated by measuring the diameter of inhibition zone in millimetres

around the periphery of the discs. The same procedure was followed for each isolate of bacteria and extract. The mean \pm SD of the inhibition zone was taken for evaluating the antibacterial activity of the extracts.

Results and Discussion

The problem of antimicrobial resistance knows no boundaries. Dangerous antibiotic resistant bacteria have been observed with increasing level over the past several decades owing to unselective use of commercial antimicrobial drugs. This situation has imposed a search for a new natural source of antimicrobials from plants.

A. squamosa is one of multipurpose tree with edible fruits and it has enormous medicinal and industrial value. All parts of plants individually or in combination show antimicrobial properties against different microorganisms [4]. Choosing an appropriate solvent is very crucial for the selective extraction of fractions with high antimicrobial activity from natural sources. Therefore, in this study the antibacterial activities of the different parts of *A. squamosa* fruit have been evaluated against bacteria using different types of extraction solvents.

Figure-1. shows the antimicrobial activity of various solvent extracts from pulp of *A. squamosa* fruit. Approximately, different extracts from pulp demonstrated effective inhibitory results for all bacterial isolates tested in this study except *K. pneumoniae*, which exhibited resistance to the water extract. The antimicrobial activities of fruit pulp extracts showed that highest zone of inhibition was by ethanol and water extracts followed by acetone extract. The inhibition zone of *S. aureus*, *E. coli*, and *K. pneumoniae* were (25 mm, 21mm, 21mm) respectively for ethanol extract, while (21 mm, 25mm) were for *S. aureus* and *E. coli* against water extract. However, resistant isolate against pulp extract in water was *K. pneumoniae* and this may belong to the bacteria has a polysaccharide capsule, which makes the treatment of *K. pneumoniae* particularly difficult, since, the cell capsule provides the bacteria increased resistance to most antibiotics.

Acetone extract of pulp was found to exert low to moderate antibacterial activity against *S. aureus*, *E. coli*, and *K. pneumoniae* (16 mm, 16mm, and 14mm) compared to a standard antibacterial agent (fig 1). The results of pulp indicated that the extracts showed antibacterial activities towards the gram-positive and negative bacteria, but with variability in inhibition level correlated to the bacterial isolates [20].

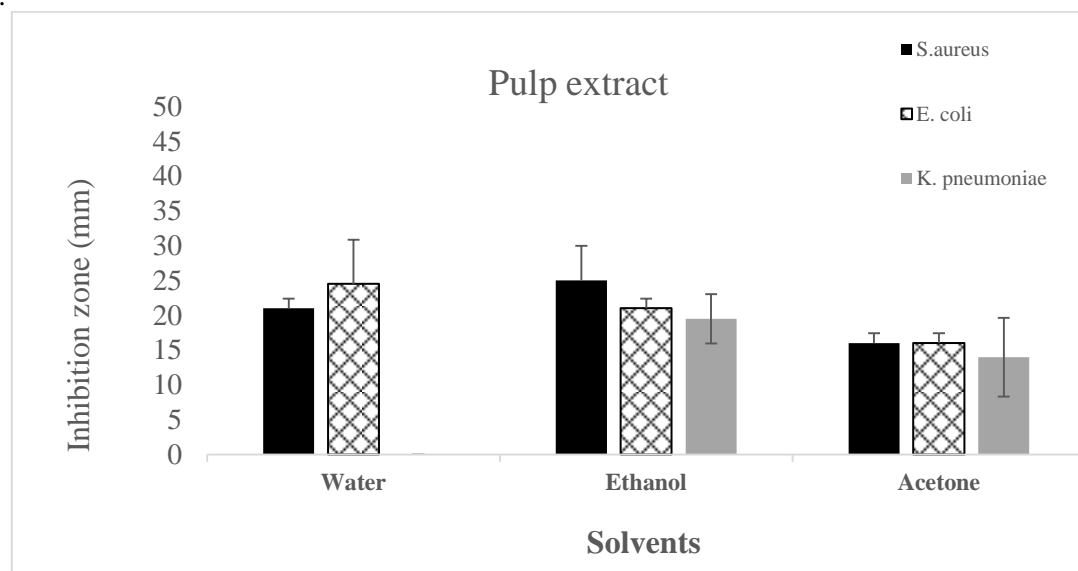


Figure1- Antibacterial activity of pulp extract of *Annona squamosa* fruit by agar diffusion method.

A. squamosa peel extracts antibacterial activity results showed the best antibacterial activities against the tested pathogenic bacteria in this study Figure-2. Highest antibacterial activity was seen in the ethanol extract and the zones of inhibition ranged between 34-38 mm, with largest inhibition diameters seen against *E. coli* and *K. pneumoniae*, which is consistent with the report of Abdulsalami *et al.* [21]. Interestingly the inhibition zone of *E. coli* (38 mm) and *K. pneumoniae* (37 mm) with ethanolic extract of peel is comparable or even greater than the inhibition zones observed of the standard antibiotics (Tetracycline and Ceftriaxone)(33-37 mm respectively) used in this study as a

positive control Figure- 2. Although the concentration of the plant crude extracts was (50 mg ml^{-1}) less than standard antibiotics concentration (100 mg ml^{-1}) used in the experiment Figure-2, superior antibacterial activity was shown with the ethanolic extract of peel against all the tested microorganisms. Similarly, water extract was found to be highly inhibited to *E. coli* (33 mm) when the results were compared with Tetracycline and Ceftriaxone Figure-2. The potential sensitivity of *E. coli* to the peel water extract may be due to the presence of saponins in the peel. Previous study of Pawaskar and Sasangan [22] reported the presence of big amount of saponins in the water extract than in the ethanolic extract of *A. squamosa*. Recent results agree with previous studies (Arabski *et al.* [23], Hassan *et al.* [24]) who documented effectiveness of saponins against *E.coli*. The result of antimicrobial activity of peel extracts in ethanol and water implies that these extracts may contain compounds with therapeutic potential comparable to the antibiotics.

Furthermore, it is seen that the acetone extract of peel showed low to moderate antimicrobial activity against two of the tested bacteria (*S. aureus* and *K. pneumoniae*) with the highest zone of inhibition was against *S. aureus* (16 mm) Figure-2. In contrast with the acetone-based extracts of *A. squamosa* peel used by Roy and Lingampeta [15] who showed inhibition zone diameter of $> 20 \text{ mm}$ against *E. coli*, the current study extract failed to inhibit the growth of the same bacteria isolate. Such a difference in the result between these two studies may be explained by the variances in the *E.coli* isolates who have been used in these two studies. Since *E.coli* isolate show differences in genetic variability by geographic variation, difference in host population characteristics, or differences in sampling method, or a combination of these three factors [25]. Moreover, the method or source of materials for the extraction process were used in the present study are completely different from the previous study, which may produce the differences in the results between these two studies.

The highest inhibition zone for Gram-negative and positive bacteria with ethanolic extract of peel in this study could mean that the ethanol extract has ability to dissolve more active components responsible for the antibacterial activity of the plant. According to Abdulsalami *et al.* [21], *Annona muricata* extracts resulted in higher yield observed for the ethanolic extract compared to the aqueous extract and with various secondary metabolites (phytochemicals) such as flavonoids, tannins, acetogenins, saponins, alkaloids. This could mean that active previous components who are responsible for the antibacterial activity of the plant were dissolved by ethanol more than water and acetone.

The extraction of different bioactive compounds from plants was seen to be more efficient in polar solvents (ethanol, methanol and water) than the nonpolar solvents [26]. Particularly, ethanolic peel extract of the fruit followed by water showed the best inhibition zone in this study. Hence, it can be reported that ethanol and water were the best solvents for extracting the bioactive component from *A. squamosa* fruit than acetone.

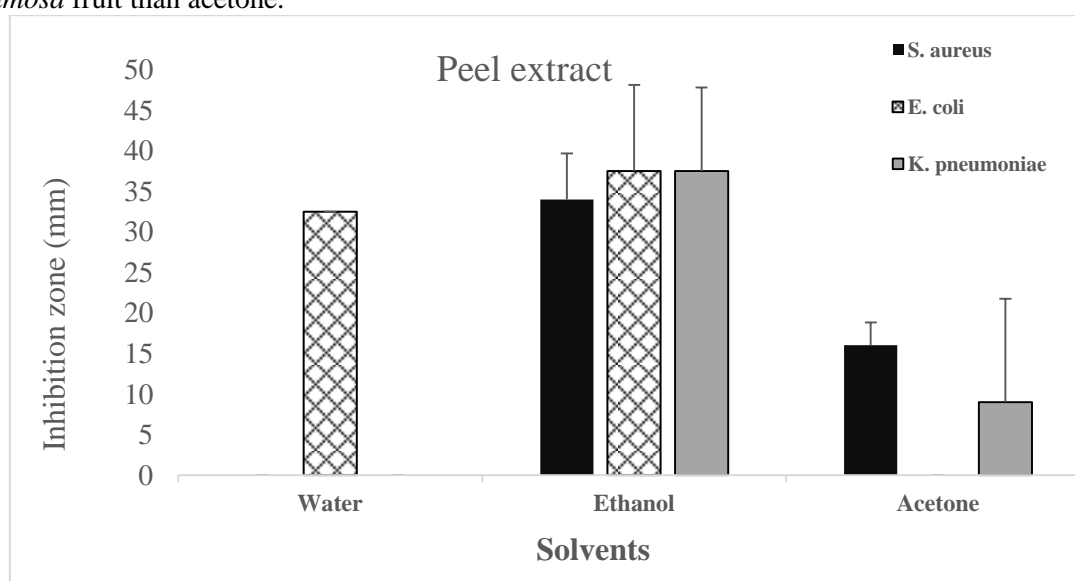


Figure 2- Antibacterial activity of peel extract of *Annona squamosa* fruit by agar diffusion method

Large diameters of inhibition zones ranging between 28- 33 mm were observed with the water extracts of *A. squamosa* seeds, thereby suggesting extremely high antimicrobial activity Figure- 3. The highest zone of inhibition (33 mm) was observed against *K. pneumoniae*, which is comparable to the inhibition zone of the standard antibiotic Tetracycline (33mm). It can be observed from the above results that the *K. pneumoniae* is highly sensitive to the seed extracts; probably the mechanism of inhibition of this pathogenic bacterium by the seed extracts is the same as antibiotics (Tetracycline and Ceftriaxone). Furthermore, acetone extract of seeds also showed highest inhibition zone (28 mm) against *K. pneumoniae*, while the lowest inhibition zone was against *S. aureus* (20 mm). Conversely, *E.coli* did not show any susceptibility to acetone extract of seeds (fig 3). Acetone extract of seeds revealed to be more efficient as it showed the highest antibacterial activity against the tested pathogens compared with acetone extracts of pulp and peel Figures-(1, 2 and3).

Bioactivity of water and acetone extracts of seeds against *K. pneumoniae* ensure that the most of the potent active substances of *A. squamosa* seeds were soluble in water and acetone that can be considered as possible medicine to treat bacterial infections such as *K. pneumoniae*. The phytochemical studies of *A. squamosa* seed extracts determine that water extract of seed contain high level of phenols and flavonoids which are well known as an active chemical substance for antibacterial activity that can be used to prevent bacterial infection [27, 28].

On the other hand, the ethanolic extract of seeds showed no appreciable antibacterial effect against all tested microorganisms Figure-3. The absence of bactericidal effects in our ethanol-based *A. squamosa* seeds extract matches the results published by Lima *et al.*[29], who reported *S. aureus* and *E. coli* to be resistant to all *A. squamosa* extracts based on ethanol.

The present investigation is the first local study to report the promising antimicrobial potential of different solvents extracts of different portions of *A. squamosa* fruit and it focuses on the probability of using these extracts that represent a huge portion of the discarded part of these fruits such as peel and seeds as sources of new and low cost natural antimicrobials agents.

In the recent study, the antimicrobial activity observed is seen to be similarly effective against both, Gram-negative as well as gram-positive pathogenic bacteria that indicate the broad-spectrum nature of the antimicrobial compounds responsible for the activity and therefore could be possible source for chemical classes of antibiotic substances that could serve as selective agents for infectious disease.

The results indicate that although most of the various solvents extracts demonstrated antimicrobial activity against the tested pathogenic bacteria, ethanol was proved the ideal choice of solvent for extraction of antimicrobial compounds from the waste peels.

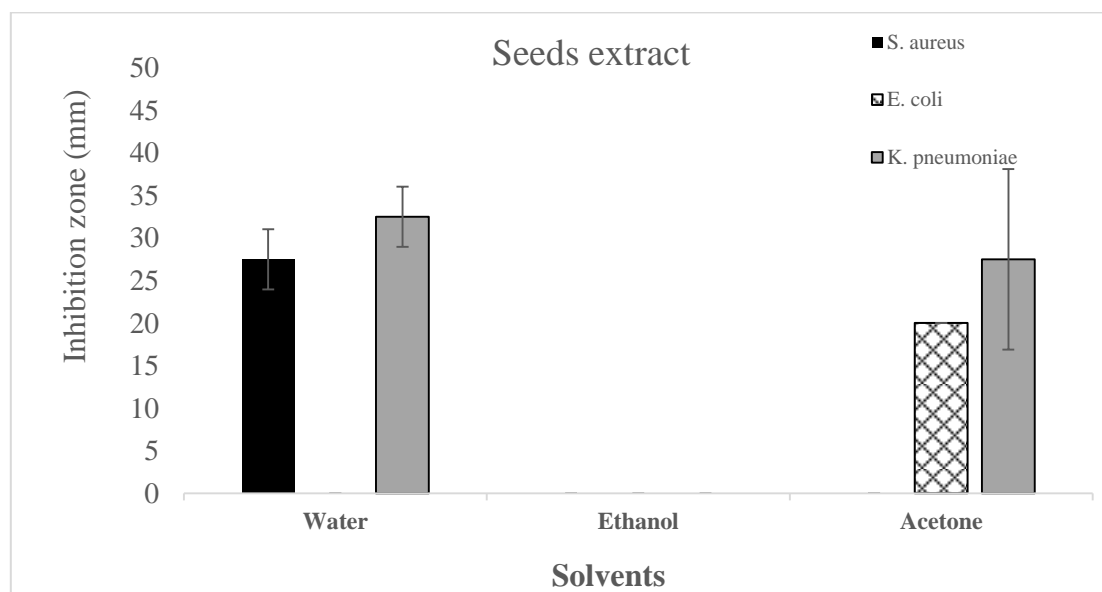


Figure 3-Antibacterial activity of seed extract of *Annona squamosa* fruit by agar diffusion method.

Conclusion

The results of the present study have provided promising evidence of utilizing the crude extracts of different portions of *A. squamosa* fruit as source for natural antibacterial. Based on this study, the differences in extraction solvent and fruit parts influence antibacterial activity of *A. squamosa* fruit. Among three different portions of *A. squamosa* fruit, peel showed the highest antimicrobial activity than pulp and seeds. The best solvent for peel extraction was ethanol against Gram-positive and Gram-negative bacteria, while the best solvent for peel extraction against *E. coli* bacteria was water. Although different solvents extracts of pulp and seeds presented moderate antimicrobial activities against Gram-positive and Gram-negative bacteria, water extract of seeds displayed comparable antimicrobial activity to standard antibiotic tetracycline against *K. pneumoniae*. These promissory results form a primary platform for antimicrobial study may open the possibility of finding new clinically effective antibacterial compounds.

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