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Impact of Alcoholic and Aqueous Extracts of *Annona Muricata on* the Growth of *Staphylococcus aureus and Escherichia coli* Isolates

Zainab M. Alzubaidy[1], Dhary A. Almashhadany[2*], Sherko M. Abdulrahman[3], and Sawsan M. Sorchee[4]

[1]Department of Biology, College of Science, Diyala University, [2]Department of Medical Laboratory Science, College of Science, Knowledge University, Erbil 44001, Iraq, [3]Department of Biology, College of Education/Shaqlawa, Salahaddin University, Kurdistan Region/Iraq, [4]Salahaddin Univ/Coll.Education- Kurdistan Region/Iraq

Abstract

Annona muricata (a.k.a. Soursop or Graviola) is fruit plant native to warm and humid regions in Africa and Asia. *A. muricata* is rich of carbohydrates, vitamins, antioxidants, and important minerals such as ZX. This study aimed at evaluation of aqueous and alcoholic extracts of the peels and pulp of *A. muricata* for their antibacterial activities against *Escherichia coli* and Staphylococcus aureus using the standard well diffusion assay. The results of aqueous extract of the peels, revealed 16 mm inhibition zone for E. coli and S. aureus. The inhibition zone of aqueous extract of the core part was 18.2 mm for E. coli and 17 mm for S. aureus. The alcoholic extract of peels showed 16 mm of inhibition zone of S. aureus compared to 17 mm E. coli. Moreover, the alcoholic extract of the fruit pulp revealed 19.2 mm inhibition growth zone of E. coli and 17.5 mm against of S. aureus. In conclusion, the aqueous and alcoholic extract of peel and pulp contain inhibitory activity compounds against different clinical bacterial species and the inhibitory effect of the alcoholic extract was more evident than that of the aqueous extract due to the solubility of many active compounds in the alcohol.

Keywords: *Annona muricata*, Aqueous extract, Antibacterial activity, ethanol extract, *Staphylococcus aureus*, E.coli ,Well diffusion

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*Corresponding Author: Dhary A. Almashhadany E-mail: dhary.alewy@knu.edu.iq

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Introduction

Humans have been using certain plants as medications or supplements for thousands of years. With the advancements in sciences over the past century, chemical contents of huge number of plants have been studied and evaluated for their bioactivities. Indeed, phytochemicals have been the stockpile of the majority of pharmaceutical industry (Moghadamtousi et al., 2013). *Annona muricata* (*Annonaceae* family) is not an exception. It is an evergreen fruit tree mostly found in tropical and subtropical regions. The fruits of *A. muricata* are used for different industrial purposes such as preparation of syrups, candies, beverages, ice creams and shakes. *A.* communities in Africa and South America extensive*muricata* has a well-known history of traditional use. Indeed, A wide array of ethnomedicinal properties of *A. muricata* contributed to its popularity in indigenous ly use this plant in their folk medicine (Moghadamtousi et al., 2015). Through in vitro and in vivo evaluations, extracts from *A. muricata* exhibit therapeutic activities including anticancer, anti-inflammatory, antiulcer, anthelmintic, antibacterial, antioxidant, anticonvulsant, antiarthritic, antiparasitic, hepatoprotective, antidiabetic and antimalarial (Mutakin et al., 2022). More than 150 compounds have been characterized from seeds, fruits, flowers, stem, barks, leaves and roots (Newman & Cragg, 2020).

In terms of antimicrobial activities, alcohol extracts of *A. muricata* were the most effective extracts against different bacterial species such as *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Bacillus* and *E. coli* (Vijayameena et al., 2013). The ethanol leaf extract showed maximum activity against *Pseudomonas* and *Staphylococcus*, representative models of gram-negative and gram-positive bacterial pathogen. Similarly, bark alcohol extract showed a maximum antibacterial activity against *E. coli* (Vijayameena et al., 2013). However, other studies had found that aqueous and ethanolic extract to show comparable antibacterial activities. For instance, methanolic and aqueous extract of the leaves of *A. muricata* showed inhibitory effects against different bacterial reference strains including *S. aureus* ATCC29213, *E. coli* ATCC8739, *Proteus vulgaris* ATCC13315, *Streptococcus pyogenes* ATCC8668, *Bacillus subtilis* ATCC12432, *Salmonella typhimurium* ATCC23564, *Klebsiella pneumoniae* NCIM No.2719 and *Enterobacter aerogenes* NCIM No. 2340. *B. subtilis* and *S. aureus* were the most susceptible gram positive bacteria while *K. pneumoniae* and *P. vulgaris* were the most susceptible gram-negative species (Pathak et al., 2010).

In addition to the antibacterial activities, *A. muricata* has also antiviral properties. In fact, 1 mg/ml of ethanol extract was reported to be the minimum inhibitory concentration for Herpes simplex virus-1 (HSV-1) (Padma et al., 1998). Additionally, three antiviral compounds were isolated from *A. muricata*, namely annonacin, annonacin A and annomuricin A (Jaramillo-Flores & Hernandez-Sanchez, 2000; Mutakin et al., 2022).

Regarding the other antimicrobial activities of *A. muricata*, alcoholic extracts were also found to inhibit the growth of *Leishmania braziliensis* and *L. panamensis* promastigotes when tested *in-vitro*, where ethyl acetate extract was found to be more

active than the reference substance used in the experiment (Gajalakshmi et al., 2012). Moreover, an *in-vitro* evaluation of aqueous leaf extract against *Haemonchus contortus*, showed 89.08% and 84.91% toxicity against larvae and eggs, respectively. A promising anthelmintic activity was also found against the adult worms via the immobilization test were mobility was significantly reduced within 6 to 8 hours of exposure to the extract (Ferreira et al., 2013). Lastly, two strains of *Plasmodium falciparum*: the Nigerian chloroquine-sensitive strain and FcM29-Cameroon (chloroquine-resistant strain) were found to be susceptible to leaf extract from *A. muricata*. The IC_{50} values for the strains were 16 and 8 µg/mL after 72 hours, respectively (Ménan et al., 2006). Another study also showed a promising antiplasmodial effect where the leaf extract, at 20 µg/mL, showed a 67% inhibition against an asynchronous F32 strain of *P. falciparum* (Titanji et al., 2004). Another study on different extracts of *A. muricata* leaves and stems confirmed the reported cytotoxic effects against the chloroquine-sensitive (F32) and resistant (W2) *P. falciparum* (Ferreira et al., 2013). These findings substantiated the traditional use of *A. muricata* as an antimalarial agent.

It is well known that bioactive compounds concentrations in plants are affected, to different degrees, by the geographical location and growth conditions (Al-Juhaimi et al., 2021; Giangrieco et al., 2016; Urbonaviciene et al., 2022). Therefore, this study aimed at evaluating the antibacterial activity of aqueous and methanolic extracts from *A. muricata* against two representative bacteria: *Staphylococcus aureus* and *Escherichia coli*.

materials and methods

Collection and processing of samples

A. muricata fruit samples were randomly collected from the local market and stored at 4°C until processing. The fruits were washed with a clean water and then peeled to remove the fresh pulp. The pulp was then cut into small pieces and placed in a hot air oven to dry at 50 °C for a week. The dried pulp was then milled into a powder using an electric grater to obtain a fine powder, which was kept in a sterile and closed glass vial at 4°C until further investigations (Gavamukulya et al., 2014).

Preparation of extracts

Aqueous extracts

The aqueous extract of fruit was prepared according to a published protocol (Raybaudi-Massilia et al.; 2015). Briefly, 10 g of fruit mash was added to 100 ml of distilled water and thoroughly mixed for 30 minutes at 28 °C. The homogenized solution was filtered using filter paper (Whatman No.1). The final filtrated solution was concentrated using a rotary evaporator and gradually dried in an electric oven for 30 min at 40 ° C. Dry powder was stored in a sterile container and kept at 4 ° C until further analysis.

Alcoholic extracts

In a volumetric flask, 10 g of pulp powder were added to 100 ml ethyl alcohol (70%) and mixed thoroughly. The mixture was placed in a shaker incubator at 35° C for 24 h, after which the extract was filtered using filter paper (Whatman No.1). The extract was concentrated using a rotary evaporator, then the filter was dried in an electric oven at 40 °C until the complete evaporation of the solvent. The dry extract powder was kept in a sterile

and closed glass vial at 4°C until antimicrobial testing.

Bacterial isolation and phenotypic identification

Isolates of *E. coli* and *S. aureus* were obtained from clinical specimens collected in Baqubah Teaching Hospital. Specimens were collected and transported to the microbiology laboratory in the department of Biology. For isolation, specimens were plated on blood and MacConkey agar plates to detected hemolysis by *S. aureus* and pink growth of *E. coli*, respectively. The suspected colonies were identified by their morphological features and biochemical tests according to (Cheesbrough, 2006).

Molecular identification by PCR

Both species were identified by detection of the 16S rRNA gene of using the primers pairs in table 1. For confirmation of *E. coli*, each 20 µl of the reaction mixture consists of 3 µl genomic DNA, 10 µl PCR master mixtures (Promega, USA), 1 µl of each of the two primers with the final volume adjusted to 20 µl with 5 µl of nuclease-free water. Amplification was done by initial denaturation at 95° C for 5 minutes, followed by secondary denaturation at 94° C for 45 sec, annealing temperature of primers was carried out at 55° C for 45 sec and extension at 72° C for 1 minutes. The final extension was conducted at 72° C for 5 minutes.

For confirmation of *S. aureus*, the extraction of DNA was performed via QIAamp DNA mini kit according to the instructions of the manufacturer (QIAGEN, Hilden, Germany). Preparation of uniplex PCR Master Mix was performed according to Emerald Amp GT PCR master mix (Takara, Shiga, Japan). The volume of PCR mixture was 25 µl that contained 12.5 µl of Master mixture, 5 µl of template1 µl of each primer, and 5.5 ul PCR grade deionized water. Amplification was done by initial denaturation at 95° C for 5 minutes, followed by denaturation at 94° C for 45 sec, annealing temperature of primers was 55° C for 45 s. and extension at 72°C for 1 minutes. The final extension was conducted at 72°C for 5 minutes. The total reaction was performed at 30 cycles. The amplified PCR products were resolved by electrophoresis in 2% agarose gel at 100v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator.

Table 1. PCR primers for detection of *S. aureus* and *E.coli*.

	Primer	Ref.
S. aureus	F: 5'-CCTATAAGACTGGGATAACTTCGGG-3' R: 5-'CTTTGAGTTTCAACCTTGCGGTCG-3'	(Mason et al., 2001)
E. coli	F: 5'-GACCTCGGTTTAGTTCACAGA-3' R: 5'-CACACGCTGACGCTGACCA-3'	(Schippa et al., 2010)

Antimicrobial inhibition testing

Extract re-suspension was done using sterile distilled water by dissolving 1 gm of dry extract in 2 ml of sterile distilled water to obtain a concentration of 500 mg/ml. The suspension was sterilizing by Millipore filter paper with a diameter of 0.22 µm. This concentration was considered as a stock solution from which

the downstream concentrations were prepared; 25, 50, 75, and 100 mg/ml.

The Agar well diffusion method was used according to Obeidat and colleagues (Obeidat et al., 2012). Briefly, a bacterial suspension was prepared from an overnight growth and its turbidity was matched with the standard MacFarland solution (equivalent to 1.5 x 10^8 CFU/ml). Using a sterile swab dipped into the suspension with no excess load, the surface of Mueller-Hinton agar plates were inoculated by triple streaking to make an even lawn of bacteria. The inoculated plates were left to dry for a while before 5-mm holes were made using sterilized a cork borer. From each concentration, 0.5 ml was delivered into a hole in addition to a negative control hole containing 0.5 ml of sterile distilled water. Each test was done in triplicates and plates were incubated aerobically at 37 °C for 24 h. Inhibition zones diameters around wells were measured in mm.

3.Results

3.1. Molecular identification carried out by PCR

The results illustrated in Figure 1 confirmed that the morphological and biochemical tests identified *S. aureus* and *E. coli* from clinical specimens were consistent with the molecular identification.

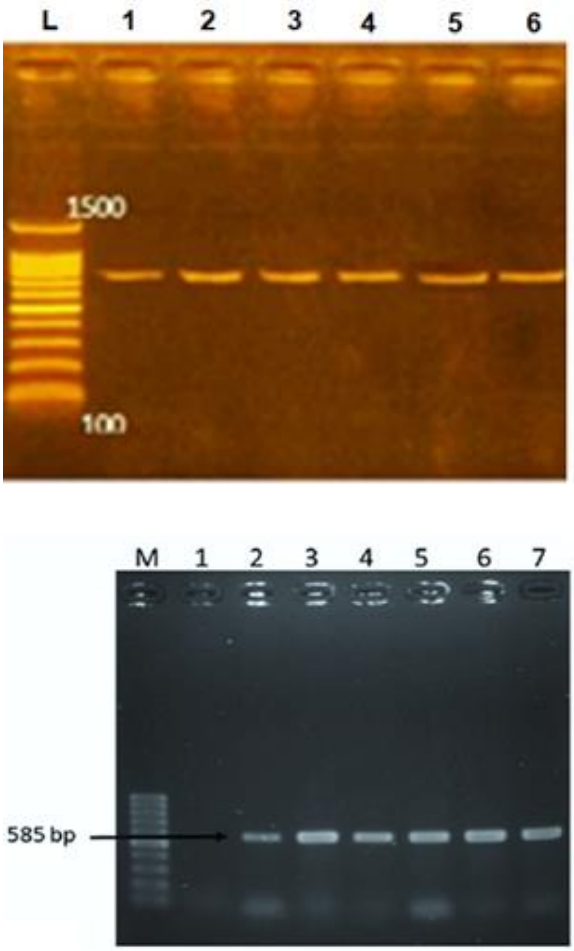

Figure 1. Agarose gels of conventional PCR of 16Sr RNA gene in *S. aureus* (upper panel) and *E. coli* (lower panel). In the upper panel, Lane L: molecular weight marker (100 – 1500 bp). Lanes 1-6: positive samples with amplicon size of 791 bp. Similarly, in the lower panel, Lane M: 100 bp DNA Marker, 1: Negative control, 2: Positive control and 3-7 are representative *E. coli* isolates.

3.2. The inhibition zone diameters of *S. aureus* and *E. coli*

Regarding the inhibition zone diameters, our results clarified that this zone range from 16-17 mm for *S. aureus* and from 16-19 for *E. coli* , by agar well diffusion assay(Table 1 and Figure 2).

Table 1. Inhibition zone diameter (in mm) of A. muricata extracts

Extract	*S. aureus*	*E. coli*
Aqueous skin extract	16	16
Aqueous core extract	17	18.2
Ethanol skin extract	16.2	17
Ethanol core extract	17.5	19

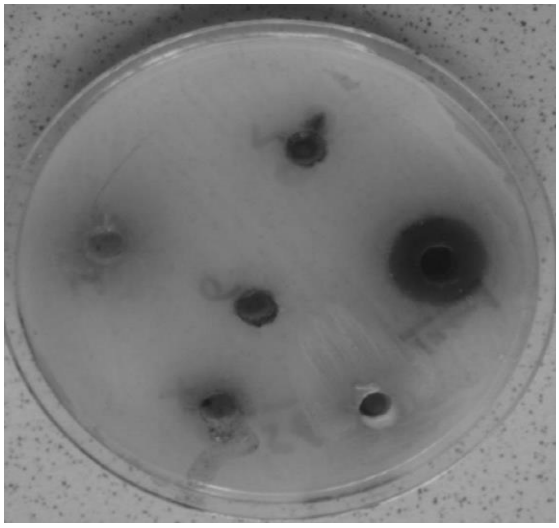

Figure 2. Agar diffusion plates showing the activities of *Annona muricata* extracts (100 mg/ml) against *S. aureus* (upper panel) and *E. coli* (lower panel).

4.Discussion

Morphological and biochemical tests identified *S. aureus* and *E. coli* from clinical specimens were consistent with the molecular identification carried out by PCR (Figure 1). These two species have been the model organisms to study antibacterial activities and resistance development. Antimicrobial resistance mechanisms against one of the highest challenges to contemporary medicine worldwide due to the dissemination of antibiotic resistance determinants via plasmids and transposable elements between microbial communities, leading to limited efficacy of currently available antimicrobial drugs. Over-usage of antimicrobial agents in hospitals and the community, the resistance problem is expected to continue to transform even intrinsically susceptible phenotypes into resistant species (Nagel et al., 2016).

The inhibition zone diameters range from 16-17 mm for *S. aureus* and from 16-19 for *E. coli* (Table 1) by agar well diffusion assay (Figure 2). Generally, alcoholic extracts showed higher efficacy in comparison to the aqueous extracts. These results are consistent with previous reports that found the zones of inhibition were 17 mm, 15 mm, 12.5 mm at a concentration of 200 mg/ml, 100 mg/ml, and 50 mg/ml respectively for *E. coli* and 17 mm, 15 mm, 13.5 mm for *S. aureus*. However, the ethanol extract of the plant leaves was found to exert slightly lower inhibition in comparison to the aqueous extract (Jemikalajah et al., 2021). In contrast, ethanol and aqueous leaf extracts of *A. muricata* in this work have some degree of efficacy against the test organisms, *E. coli* and *S. aureus*, which confirmed bioactivity of the extract as

earlier reported (Radji et al., 2015; Solomon-Wisdom et al., 2014; Vijayameena et al., 2013).

It was reported that 81% of the ethanol extracts inhibited the growth of *P. aeruginosa* while 36% inhibited *E. coli* with a minimum inhibitory concentration range between 0.008 - 256 mg/ml (Jemikalajah et al., 2021). The difference in efficiency of the ethanol and aqueous extract is actually as a result of the difference in polarity of solvents (Meto et al., 2020). During the extraction process, solvent polarity influences solubility of the main active phytochemicals, which leads to differences in their biological activities (Kossouoh et al., 2007).

Medicinally important plants continue to be a cornerstone source for biologically active and safe chemicals (Manandhar et al., 2019). They may not only increase the efficiency of treatments by synergy, but also minimize the possibility of developing resistant phenotypes (Wagner & Ulrich-Merzenich, 2009). Moreover, plants are not merely collections of random chemical compounds, but their components may act synergistically on multiple targets. Over the last decades, *A. muricata* has been a plant of interest for medicinal chemists and its phytochemicals have been analyzed for their biological activities including their anti-microbial activity (Chowdaiah et al., 2019). Furthermore, other studies investigated the synergy between the ethanol extract of *A. muricata* and four antimicrobial agents (gentamicin, kanamycin, amikacin and neomycin) and reported that, in all cases, there was a reduction in MIC when compared to antimicrobial agents acting alone within the 75.0 - 99.9% range (Bento et al., 2013).

The antibacterial activity of *A. muricata* was attributed to its content of alkaloids, steroids, saponins, and falvonoids. Alkaloids mechanism of action is believed to be mediated by its basic functional groups when they establish chemical interactions with the bacterial cell wall. These compounds are believed to interact with the amino acids in the bacterial peptidoglycan layers. This reaction thus leads to changes in the stability of cell wall and bacterial DNA is released after damage and lysis of the cell walls (Pujiyanto et al., 2018). The same process also occurs with the flavonoids. The biological activity in such processes is exerted by damaging the cell walls lipids and short peptides of bacterial cell envelope leading to cell burst (Cushnie & Lamb, 2005). On the other hand, the mechanism of action of steroids as antibacterial agents is actually by disruption of bacterial cell membranes (Jannah et al., 2017). Lastly, saponins causes increase in the permeability of cell membranes and thus inhibit bacterial growth and render cells unstable which leads to cell lysis (Verstraeten et al., 2020).

Conclusion

This study reveals that *A. muricata* possesses significant antibacterial activity against the tested organisms. Further research should be carried out on the plant to determine the concentration needed to archive similar result in-vivo.

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