BIODIVERSITAS
Volume 24, Number 1, January 2023
Pages: 603-608

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240168

Phytochemical analysis of *Annona atemoya* seed extract by HPLC and their ability to inhibit the growth of *Agrobacterium tumefaciens*

NAJWA IBRAHIM KHALEEL AL-BARHAWEE[1,♥], WJDAN SALIM QASIM[2], YOUSRA A. ABDLLA[1]

[1]Department of Biology, College of Education for Pure Sciences, University of Mosul. Mosul, Nineveh, Iraq. ♥email: dr.najwa@uomosul.edu.iq
[2]Medical Laboratory Technique, University of Northern Technical Mosul. Mosul, Nineveh, Iraq

Manuscript received: 14 October 2022. Revision accepted: 26 January 2023.

Abstract. *Al-Barhawee NIK, Qasim WS, Abdlla YA. 2023. Phytochemical analysis of* Annona atemoya *seed extract by HPLC and their ability to inhibit the growth of* Agrobacterium tumefaciens. *Biodiversitas 24: 603-608.* The main objective of this study was to determine the effectiveness of *Annona atemoya* seeds crude alcoholic extract utilizing nine different concentrations (0.0020, 0.0039, 0.0060, 0.0080, and 0.00100 mg/mL). *Agrobacterium tumefaciens* causes crown gall disease in numerous plant crops causing significant economic losses. Medicinal plants contain chemical compounds (a group of secondary metabolites) known to have antibacterial properties. The result showed that the minimum inhibitory concentration was (0.0313 mg/mL) using agar diffusion technique, while it ranged from (0.0156-0.0313 mg/mL) in the minimal microplate inhibitory concentration test. The crude alcoholic extract of *A. atemoya* seeds contains alkaloids, flavonoids, glycosides, tannins, phenols, and proteins, but not saponins, based on the results of qualitative analysis of chemical components. HPLC analysis revealed that the extract contained quercitin, rutin, valine, and camphor which were found in the amount of 33.09, 139.39, 0.061, and 7.43 ppm/mL, respectively. Therefore the present finding suggests that A. atemoya seed extract may be used as a biological control to inhibit crown gall formation in plants. This is the first study of the antimicrobial activity of *A. atemoya* seed alcoholic extract on the growth of plant pathogenic bacteria.

Keywords: *Agrobacterium tumefaciens, Annona atemoy*a, HPLC, phytochemical compounds

INTRODUCTION

Agrobacterium is rod-shaped, motile, aerobic, gram-negative, has a slow generation time (1.5 to several hours) under ideal laboratory conditions, and is resistant to drought. The genus *Agrobacterium* belongs to the family Rhizobiaceae, also includes the genera of atmospheric nitrogen-fixing symbiotic bacteria, three of which are: *A. tumefaciens*, capable of inducing crown gall disease on a wide spectrum of dicotyledonous plants, *A.rhizogenes*, which causes hairy root disease; and *A. radiobacter*, which is non-plant pathogenic (Lacroix and Citovsky 2013; Van Montagu and Zambryski 2013). It is well known that the non-pathogenic biocontrol bacterium *A. radiobacter* uses bioremediation to shield seeds from contamination with this harmful bacteria, and it has been considered an effective and relatively inexpensive way to prevent crown gall formation on sui generis plants (Gupta et al. 2017). Plants are considered the most important sources of biological activity (Gentile 2021). For the past two decades, researchers have been interested in the species of genus *Annona*, mainly due to their unique biological properties. It belongs to the family Annonaceae and includes more than 119 species (Anaya-Esparza et al. 2017). The Annonaceae family includes the commercially significant fruiting plant *Annona atemoya.* Many tropical and subtropical continents, including southern and northern America, Asia, Africa, and Australia, as well as Spain, are home to *A. atemoya* cultivation. *A. atemoya* is a cross between the Cherimoya (*Annona squamosa*) (Li et al. 2019; Kazman et al. 2020). *A. atemoya* is short-branched with low, drooping branches and grows to a height of around 7.5 to 10 m. The leaves of *A. atemoya*, which average 15 cm in length, are alternate, leathery, deciduous, and less hairy than those of the parent cheimola. The triangular, long-stalked, yellow flowers measure roughly 6 cm long and 4-5 cm wide. The fruit has a rough texture and is juicy, light to bluish green, and varies in shape from heart-shaped to round (2-2.5 kg). Its surface is covered with raised, angular areoles. The fruits have a nice flavor, with white flesh and smooth, brown-to-black seeds. The pulp can be easily separated from the white flesh (Kazman et al. 2020; Kupe et al. 2021). There are known uses for several *Annona* species, including Annona muricata, in conventional and alternative medicine. Although there is some limited anecdotal evidence that the leaves of *A. atemoya* are bought from growers to make tea, traditional uses of this hybrid plant are absent. In both in vivo and in vitro experiments, a number of phytochemical components fruit's were isolated from *A. atemoya* which was evaluated for their biological potential. The most extensively researched parts of *A. atemoya* which have chemical and pharmacological qualities are its leaves, fruits, and seeds (Errayes et al. 2020; Nolasco-González et al. 2022). *Annona atemoya* (Atemoya) is a tropical plant famous for its fruits which contain large amounts of phenolic compounds, flavonoids, and alkaloids. Fruits are also known for their biological activity due to some antioxidant properties (Mannino et al. 2020) which were used to treat various conditions in vitro and in vivo (Bhardwaj et al.

2020). Several studies have been conducted to reveal the biological activity of other species of the genus *Annona*, including using the chloroform extract of *Annona squamosa* seeds as an anti-infective (Biba et al. 2013). Its anti-inflammatory activity has also been recorded in the extract of the leaves, roots, and bark stem (Bitar et al. 2019). Its leaves contain about 18 kinds of secondary metabolites of a phenolic or alkaline nature that have antioxidant activities (Mannino et al. 2020), in addition to their use as insecticides, bactericides (Hernández-Fuentes 2016), antimicrobials, and antifungals (León-Fernández et al. 2019). The antibacterial activity of extracts *A. squamosa* leaves was evaluated using six species of human pathogenic bacteria of both gram-negative and gram-positive types, and the results showed moderate antibacterial activity when compared with a standard antibacterial agent, and they confirmed that this activity is positively correlated with its phenolic content (Ali and Mohammed 2020).

Due to the lack of research on how to treat plant pathogenic bacteria, the fact that antibiotics are the only way to treat them and most of them have become resistant to them, and it is now necessary to find natural alternatives. The objective of this study was to discover a natural plant extract that has the ability to inhibit the growth of *Agrobacterium tumefaciens*, which causes crown gall disease in plants.

MATERIALS AND METHODS

Seed collection

Seeds of *Annona atemoya* were purchased at a local market in Baghdad and then identified and confirmed by people from the Ministry of Agriculture in Baghdad, Iraq. All reagents (ethanol, Dragendorff's reagent, NaOH, HCl, acetic acid, $FeCl_3$, Na_2CO_3, $CuSO_4$ and NaOH) used for extraction, isolation, and analysis were of analytical grade and obtained from Sigma Aldrich (Baghdad, Iraq). All of the primary antibiotics used in this study were purchased from Biotechnology, USA.

Microorganisms used

A culture of *A. tumefaciens* obtained by the researcher Sabah Mehdi Hadi /Central Environmental Laboratory/ Baghdad University.

Activation of *A. tumefaciens*

A colony of the bacteria was transferred to 5 mL of liquid LB medium and incubated in a shaking incubator at a speed of 150 rpm and a temperature of 28°C for 24-48 h.

Crown gall formation test on carrot discs

Carrot discs of 0.5 cm thickness were surface sterilized by immersing them in 3 mL of sodium hypochlorite: 1 mL of distilled water solution (volume: volume) for 10 minutes, thereafter washed several times in sterile distilled water, infected with 1 mL of *A. tumefaciens* bacterial culture to an OD 600nm of 0.3, dispersed on a medium surface (1.5% water agar), and incubated for 3-4 weeks with alternating light and dark periods (Ferdous et al. 2021).

Seeds extraction

Annona atemoya seeds were air dried in the shade at a temperature of 25°C for 36 hours and crushed using pestle and mortar to about 70 mesh sizes and then stored in dry containers (Kubmarawa et al. 2012). Rotary evaporator at 35°C was used to obtain concentrated alcoholic extract and soxhlet was used to obtain ethanol-soluble fractions (Vikas et al. 2013). The extract was divided into two parts and preserved at 4°C for further experiments. The first part was used for phytochemical examination, and the second part was used to study its biological effect on the growth of *A. tumefaciens*, which causes crown gall disease in plants.

Determination of antibacterial activity

Agar well diffusion technique

The antibacterial activity of ethanolic extract was determined using agar-well diffusion technique (agar cup method) as described by Balouiri et al. (2016). For this, 0.1 mL (equivalent to $1.5x\ 10^5$ CFU mL /l adjusted to 0.5 McFarland) suspension of *Agrobacterium* was swabbed on the plates of Mueller-Hinton agar media. After that 6 mm diameter wells were made with the help of sterile cork borer and poured 0.1 mL (100 µl) of different concentrations i.e. 0.5, 0.25, 0.125, 0.0625,0.0313, 0.0156, 0.0078, 0.0039, 0.0020 mg/mL of alcoholic extract. The antibiotic cefotaxime at a concentration of 100 mg/mL, and distilled water were used as a positive and negative control, respectively. The plates were incubated at 28°C for 18-24 h. The diameter of inhibition zones was measured in millimetres and compared to the negative and positive control inhibition zones.

Microplate minimal inhibition concentration (MIC) assay

The microplate minimum inhibitory concentration assay was conducted to detect *A. tumefaciens* isolates passed resistance ability to the crude solution of an alcoholic extract of *A. squamosa* seeds by using the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol (Cockerill 2019).

Ten µL alcoholic extract was added to each well of the microplate with different concentrations i.e. 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.0078, 0.0039, and 0,0020 mg/mL. After the absorption spectrum attained 0.15 at 600 nm, 190 µl of Mueller-Hinton broth culture of bacteria was added to bring the volume in these wells to 200 µl. The experiment was performed in triplicates and MIC value was directly determined by the microplate reader (PARAMEDICAL PKL) at a wavelength of 600 nm. The results were analyzed by comparing wavelengths using Excel 2020.

Conventional qualitative chemical testing

The alcoholic seed extract of *A. atemoya* was analyzed for various phytochemical compounds, such as alkaloids, flavonoids, glycosides, saponins, phenol, tannins, and protein.

Alkaloids test: 1 mL of Dragendorff's reagent was added to 2 mL of extract and the formation of a red-orange precipitate indicates the presence of alkaloids.

Flavonoid test: Two to three drops of 10% NaOH were added to 2 mL of the extract. Initially, dark yellow color appeared, but after adding a few drops of dilute HCl, it gradually became colorless, indicating the presence of flavonoids.

Glycosides test: In the test tube a solution for this 0.5 ml, of CH_3COOH (Glacial acetic acid) and 2-3 drops of 0.1% $FeCl_3$, was mixed with 2 mL of the extract. Then add 1 mL of concentrated H_2SO_4 along the walls of tube. The appearance of dark blue color indicates the presence of glycosides.

Saponin test: A drop of Na_2CO_3 solution was added to 5 mL of extract in a test tube. After mixing them vigorously, test tube was kept aside for five minutes. The absence of foam indicates the absence of saponins.

Phenol and tannins test: 2 mL of a 5% $FeCl_3$ solution was added to 1 mL of extract. Appearance of a dark blue color showed the presence of phenols and tannins.

Protein test: Two drops of 3% $CuSO_4$ and a few drops of 10% NaOH were added to 1 mL of extract, indicating the presence of violet or red color showed the positive test for proteins.

High Performance Liquid Chromatography (HPLC)

The HPLC Shimadzu LaSolutions apparatus was used based on the following conditions: Mobile phase, Methanol; Water =70:30(v/v); Detector, at wavelength =280 nm; Flow rate= 1mL/min; Injection volum= 20µl; Column, C18.

Separation was performed using elution duration (0-10 min), the flow rate of 1.0 mL/min, and a column temperature of 20°C. The injection volume was 20 µL, and UV light at wavelength 280 nm was used. The solvents used were obtained from Merck Chemicals ltd. For the detection of phytochemicals content in ethanolic extracts, 10 µg/mL sample was loaded in the HPLC column and the retention time values of the extract were calculated. The compounds were identified by comparison of their retention times with those of the standards.

RESULTS AND DISCUSSION

Formation of crown gall on the carrot disc

It was observed that crown galls formed on carrot disc after one month of infection, and this result confirmed that *A. tumefaciens* (Figure 1) has the ability to cause infection. Most pathogenic strains of *A. tumefaciens* on Ti-plasmid are responsible for stimulating infected cells to form crown galls disease (Ganjeh et al. 2021).

Agar well diffusion technique

The alcoholic extract of *A. atemoya* seeds at different concentrations showed inhibition against bacteria compared to the antibiotic cefotaxime, and the diameter of the inhibition zone ranged from 2-15 mm (Table 1). This result is not the same as the results of (Al-Deen 2017), who reported no inhibitory effect of *Annona* alcoholic seed extract against any of the pathogens *Staphylococcus aureus, Klebsiella pneuomoniae* and *Escherichia coli* using the agar diffusion technique. The agar diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Meshaal et al. 2021).

The results also showed that the minimum inhibitory concentration (MIC) was (0.0313) mg/mL, considering that the MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of microorganisms after the incubation period (Kowalska-Krochmal and Dudek-Wicher 2021). This antibacterial effect can be attributed to several reasons, including the mode of action of antimicrobial plants associated with the presence of phenolic compounds and their effect on cellular membranes. It has been observed that these phytochemicals attack cell walls and membranes, affecting their permeability and release of intracellular components, and they also interfere with membrane functions such as electron transfer, enzyme function, and nutrient absorption, which can thus lead to inhibition of bacterial growth (Othman et al. 2019). Moreover, the inhibitory effect may be due to the alteration of the structure of proteases by some hydrogen bonds that ultimately impede or denature the protein synthesis (Álvarez-Martínez et al. 2021).

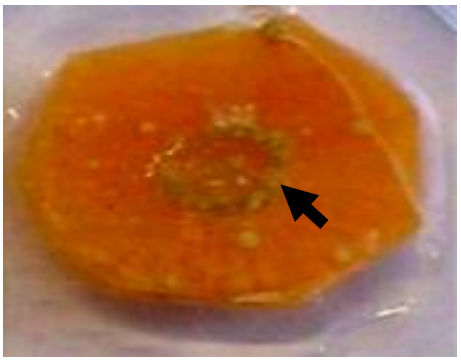

Figure 1. Crown gall formed on carrot disc

Table 1. Antimicrobial activity of *Annona atemoya* seeds alcoholic extract, cefotaxime and distilled water on the growth of plant pathogenic *Agrobacterium tumefaciens*

	Treatment									Cefotaxime (mg/mL)	Distilled water
Alcoholic extract (mg/ mL)	0.0020	0.0039	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	100	-
Inhibition zones (mm)	0	0	0	0	2	5	7	10	13	15	0

Note: Cefotaxime: positive control; Distilled water: negative control

Microplate MIC assay

Microplate assay showed that bacteria were sensitive to the alcoholic extract of seeds at different concentrations. Wells that are still clear after incubation may have low levels of viable microorganisms, which are illegible to the naked eye. As a result, the growth of bacteria is measured in terms of the value displayed in the spectrophotometer (Giuliano et al. 2019). The MIC value for antibacterial activity ranged (0.0156-0.0313 mg/ ml) from alcoholic seed extract after 48 h of incubation (Table 2). This shows that the microplate minimum inhibitory concentration (MIC) assay was about two times more sensitive than agar-well diffusion technique. Al-Hamdoni and Al-Rawi (2020) reported that MIC value of microplate method was more sensitive than agar-well diffusion technique. The antimicrobial properties often are attributed to the hydroxyl group present within the chemical structure of the phenolic secondary metabolites found in various types of fruit like the custard apple (*A. squamosa*) (Al Mamari 2021), this is due to the high affinity of phenolic hydroxyl groups to bind to proteins and thus inhibit the action of microbial enzymes as well as increase the affinity with cytoplasmic membranes, thus enhancing antibacterial activity (Miklasińska-Majdanik et al. 2018). Kumari et al. (2022) noted that the seed extract of *A. squamosa* has an antimicrobial effect on human pathogens and has a promising future in the development of its use as a medical plant in the treatment of pathological conditions caused by various types of pathogenic bacteria.

Phytochemical constituents of the alcoholic extract of *Annona* seeds

Qualitative analysis

The results of qualitative tests revealed that the alcoholic extract of *A.atemoya* seeds contained alkaloids, flavonoids, glycosides, tannins, phenol, and proteins, except for saponins (Table 3). Many studies have confirmed that plants are rich in a variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids (Mapfumari et al. 2022). Shami (2017) reported that alkaloids isolated from *A. squamosa* showed good antibacterial and antioxidant activity, so it could be a new source of antimicrobial agents against pathogenic bacteria. The presence of acetogenin, alkaloids, essential oils, phenolic compounds, cyclic peptides, amino acids, dyes, and vitamins has also been reported from the extract of different *Annona* plant parts (Bhardwaj et al. 2020). In addition, alkaloids and flavonoids are found in the seeds of *A. atemoya* and *A. squamosa* (Al Kazman et al. 2020; Kumar et al. 2021).

HPLC assay

The results of HPLC analysis at wavelength 280 nm showed that the extract contained the following four phytochemical compounds, namely 2.710 quercetin, 3.083 rutin, 3.392 valine, and 3.992 camphor (Figure 2A), based on standard compounds (Figures 2B-E). On the other hand, concentrations of quercetin, rutin, valine, camfor in the crude alcoholic extract were 33.09, 139.39, 0.61, and 7.43 ppm/mL, respectively (Table 4).

Zhu et al. (2020) confirmed the presence of quercetin in *A. squamosa* seed extract. Quercetin is a natural substance from the flavonoid group that is found abundantly in vegetables and fruits. There is a lot of evidence indicating that quercetin has therapeutic potential to prevent and treat various diseases (Maurya 2022). The second chemical compound is rutin, also called vitamin B. It is a low molecular weight flavonoid found abundantly in various vegetables and fruits. Numerous studies have identified many of the pharmacological properties of rutin as well as its antibacterial properties (Pandey et al. 2021). In addition, quantitative analysis using high-performance liquid chromatography revealed that rutin is the most abundant compound in *A. atemoya* leaves generally. Quercetin and rutin are not detected in the fruit of *Annona* plant, but they are present in abundance in the leaves (de Moraes et al. 2020). Valine is one of the groups of essential-branched chain amino acids (Li et al. 2020). Phytochemical analysis of *Annona* extracts revealed the presence of several phytochemicals, including proteins, carbohydrates as well as others (Al Mamari 2021). A study showed the highest protein content was in *A. squamosa* leaves (ASLs) compared to its content in seeds and fruits, as was Proteins and amino acids are also present in high amounts in methanolic and aqueous extracts of ASLs (Kumar et al. 2021). On the other hand, the biuret test revealed the presence of protein and amino acids in the aqueous ASLs extract, while the Millon test revealed their presence in methanolic ASLs extracts. The presence of camphor in the stem and root extracts of *Annona* plant has also been confirmed by Singh et al. (2019). It has many biological properties as anticancer, antimicrobial, antiviral, antipain, and insecticidal (Chen et al. 3013).

Concentration of phytochemicals in alcoholic extract

The concentration of phytochemical compounds in the alcoholic extract of the seeds (Table 5) was calculated based on the concentration of 1000 ppm/mL for standard compounds and based on the following equation: C1:V1=C2:V2.

Table 2. Antibacterial activity of alcoholic extract of *Annona atemoya* seeds on the growth of *Agrobacterium tumefaciens*

Concentration (mg/mL)	0.0	0.0020	0.0039	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5
Crude alcoholic extract	+++	+++	+++	++	+	+	-	-	-	-

Note: +++: good growth ,++: intermediate growth, +: week growth

Table 3. Phytochemical qualitative analysis of alcoholic extract of *Annona atemoya* seeds

Sample	Alkaloids	Flavonoids	Glycosides	Saponins	Tannins and phenol	Protins
Alcoholic extract of seeds	+	+	+	-	+	+

Note: + : Present , - : absent

Table 4. Calibration curve parameters for standard phytochemicals

Standards	Rt	Area	Peak
Quercetin	2.771	4781610	305.850
Rutin	4.252	8474028	320.923
Valine	3.096	47885893	4000.287
Camphor	3.052	24857640	1744.652

Table 5. The concentration of phytochemicals present in the seeds alcoholic extract

Extract	Phytochemicals	ppm/mL
Ethanol	Quercetin	33.07
	Rutin	139.39
	Valine	0.61
	Camphor	7.43

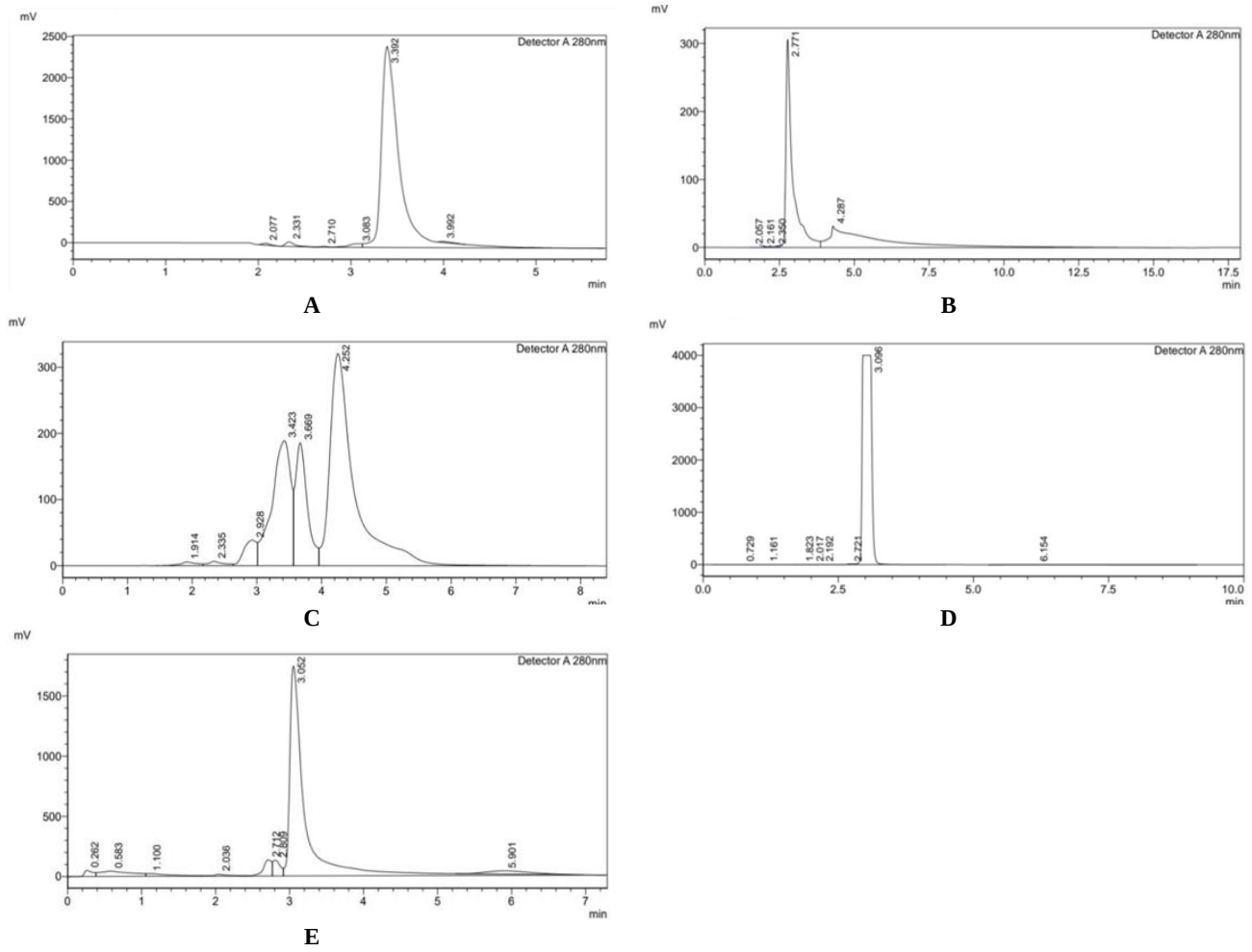

Figure 2. The HPLC chromatogram: A. Phytochemicals present in ethanol extract at 280 nm, B. Quercetin standard, C. Rutin standard, D. Valin standard, E. Camphor standard

In conclusion, the results of the present study revealed that secondary metabolites of *A. atemoya* (Atemoya) seeds play an important role in antibacterial activity against *Agrobacterium tumefaciens*. This may indicate the effectiveness of the solvent used (ethanol) in releasing the active chemical compounds and thus, its effect on inhibiting bacterial growth. Thus, the present research reveals the antibacterial nature of this plant and that it is likely to be a good source for the treatment of the diseases caused by plant-pathogenic bacteria, especially *Agrobacterium*. *A. atemoya* seeds extracts inhibited the growth of plant-pathogenic bacteria in-vitro. Therefore the present study recommends a future study to deal with its effect on bacteria in-vivo. This is the first study of the antimicrobial activity of *A. atemoya* seeds alcoholic extract on the growth of plant pathogenic bacteria.

ACKNOWLEDGEMENTS

The authors are very grateful to the University of Mosul/College of Education for Pure Sciences/ Department of Biology for their provided facilities, which helped to improve the quality of this work.

REFERENCES

Al Mamari HH. 2021.Phenolic Compounds: Classification, Chemistry, and Updated Techniques of Analysis and Synthesis. In Phenolic Compounds-Chemistry, Synthesis, Diversity, Non-Conventional Industrial, Pharmaceutical and Therapeutic Applications. Intech Open, London.

Al-Deen FMN. 2017. Evolution of antibacterial activity of various Solvents Extracts of *Annona squamosa* fruit. Iraqi J Sci 58: 2301-2308. DOI: 10.24996/ ijs.2017.58.4C.5.

Al-Hamdoni SA, Al-Rawi AM. 2020. A comparison between different susceptibility test methods to evaluate the antibacterial activity of *Olibanum* and *Alum* against the "Red Complex" periodontal pathogens. Iraqi J Sci 61: 1913-1925. DOI: 10.24996/ijs.2020.61.8.8.

Ali OT, Mohammed MJ. 2020. Isolation, characterization, and biological activity of some fatty acids and volatile oils from Iraqi *Eucalyptus microtheca* plant. Intl J Pharm Qual Assur 11: 136-140. DOI: 10.25258/ijpqa.11.1.21

Álvarez-Martínez FJ, Barrajón-Catalán E, Herranz-López M, Micol V. 2021. Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. Phytomedicine 90: 153626. DOI: 10.1016/j.phymed.2021.153626.

Anaya-Esparza LMMV, Montalvo-González E, Sánchez-Burgos JA. 2017. Cherimoya (*Annona cherimola* Mill.). Fruit and Vegetable Phytochemicals: Chemistry and Human Health. John Wiley & Sons, Ltd. DOI: 10.1002/9781119158042.ch48.

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal 6: 71-79. DOI: org/10.1016/j.jpha.2015.11.005.

Bhardwaj R, Sagar SNA, Vyas N. 2020. Bioactive compounds of annona. In: Murthy HN, Bapat VA (eds). Bioactive compounds in underutilized fruits and nuts. Springer Nature Switzerlad, Basel, Switzerland. DOI: 10.1007/978-3-030-30182-8_5.

Biba V, Pareek S, Jeba Malarm PW et al. 2013. Antibacterial activity of *Annona squamosa* seed extract. Intl J Pharm Technol 5: 5651-5659.

Bitar RMR Fahmi R, Borjac JM. 2019.*Annona muricata* extract reduces inflammation via inactivation of NALP3 inflammasome. J Nat Remedies 19: 12-23. DOI: 10.18311/jnr/2018/22617.

Chen W, Vermaak I, Viljoen A. 2013. Camphor a fumigant during the black death and a coveted fragrant wood in ancient Egypt and Babylon a review. Molecules 18: 5434-5454. DOI: 10.3390/molecules18055434.

Cockerill FR. 2019. Performance Standards for Antimicrobial Susceptibility Testing. 29th edition. CLSI Supplemented M100.

de Moraes MR, Ryan SM, Godoy HT, Thomas AL, Maia JGS, Richards KM, Tran K, Smith RE. 2020. Phenolic compounds and metals in some edible Annonaceae fruits. Biol Trace Elem Res 197: 676-682. DOI: 10.1007/s12011-019-02005-w.

Errayes AO, Abdussalam-Mohammed W, Darwish MO. 2020. Review of phytochemical and medical applications of *Annona muricata* Fruits. J Chem Rev 2: 70-79. DOI: 10.33945/SAMI/JCR.2020.1.5.

Ferdous ML, Hossain MN, Ali MO, Islam MS, Yasmin S. 2021. Morphological, biochemical and molecular identification of the wild strain of *Agrobacterium tumefaciens* from crown gall infected mango tree. Fundam Appl Agric 6: 43-46. DOI: 10.5455/faa.136134.

Ganjeh A, Rahimian H, Basavand E. 2021. *Agrobacterium tumefaciens* causing crown and stem gall disease of citrus propagation nursery in Iran. J Plant Pathol 103: 355-355. DOI: 10.1007/s42161-020-00679-z.

Gentile C. 2021. Biological activities of plant food components: implications in human health. Foods 10: 456. DOI: 10.3390/foods10020456.

Giuliano CC, Patel R, Kale-Pradhan PB. 2019. A guide to bacterial culture identification and results interpretation. Pharm Therapeut 44: 192-200.

Gupta AK, Sharma G, Khosla AK et al. 2017. *Agrobacterium radiobacter*: A potential biocontrol agent for management of crown gall disease of plants. Microbial Antagonists: Their role in biological control of plant diseases: 461-495.

Hernández-Fuentes LM. 2016. Recursos genéticos de anonáceas (Annonaceae) en México: Situación actual y perspectivas. Agro Productividad 9: 3-8.

Kazman BS, Harnett JE, Hanrahan JR. 2020. The phytochemical constituents and pharmacological activities of *Annona atemoya*: a systematic review. Pharmaceuticals 13: 269. DOI: 10.3390/ph13100269.

Kowalska-Krochmal B, Dudek-Wicher R. 2021. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens 10: 165. DOI: 10.3390/pathogens10020165.

Kubmarawa D, Khan ME, Shuaibu A. 2012. Comparative phytochemical screening and biological evaluation of n-hexane and water extracts of *Acacia tortilis*. Res Pharmaceut Biotech 4: 18-23. DOI: 10.5897/RPB11.029.

Kumar M, Changan S, Tomar M, et al. 2021. Custard apple (*Annona squamosa* L.) leaves: nutritional composition, phytochemical profile, and health-promoting biological activities. Biomolecules 11: 614. DOI: 10.3390/biom11050614.

Kumari N, Prakash S, Kumar M, Radha, Zhang B, Sheri V, Rais N, Chandran D, Dey A, Sarkar T, Dhumal S, Kumar S, Mahato DK, Vishvanathan M, Mohankumar P, Pateiro M, Lorenzo JM. 2022. Seed waste from custard apple (*Annona squamosa* L.): A comprehensive insight on bioactive compounds. Health promoting activity and safety profile. Processes 10: 2119. DOI: 10.3390/pr10102119.

Kupe M, Karatas N, Unal MS, Ercisli S, Baron M, Sochor J. 2021. Nutraceutical and functional properties of peel, pulp, and seed extracts of six 'Köhnü'grape clones. Horticulturae 7: 346. DOI: 10.3390/horticulturae7100346.

Lacroix B, Citovsky V. 2013. Crown gall tumors. Brenner's Enc Genet. DOI: org/10.1016/B978-0-12-374984-0.00360-0.

León-Fernández AE, Martínez-Cárdenas, L. Zepeda-Vallejo LG, Arteaga-Garibay RI, Gutierrez-Martinez P, Montalvo-Gonzalez E. 2019. Antibacterial, antifungal, antioxidant and toxic effect of fractioned extracts from Soursop pulp. Revista Biol Ciencias 6: e400. DOI: org/10.15741/revbio.06.e400.

Li H, Li W, Zhang T, Zhong J, Lu J, Yuan C, Liu K. 2019. Comparative transcriptomic analysis of split and non-split atemoya (*Annona cherimola* Mill.× *Annona squamosa* L.) fruit to identify potential genes involved in the fruit splitting process. Sci Hortic 248: 216-224. DOI: 10.1016/j.scienta.2019.01.017.

Li S, Peng F, Xiao Y, Gong Q, Bao Z, Li Y, Wu X. 2020. Mechanisms of high concentration valine-mediated inhibition of peach tree shoot growth. Front Plant Sci 11: 603067. DOI: 10.3389/fpls.2020.603067.

Mannino GC, Gentile A, Porcu C, Agliassa C, Caradonna F, Bertea CM. 2020. Chemical profile and biological activity of cherimoya (*Annona cherimola* Mill.) and atemoya (*Annona atemoya*) leaves. Molecules 24: 2612. DOI: 10.3390/molecules25112612.

Mapfumari S, Nogbou ND, Musyoki A, Gololo S, Mothibe M, Bassey K. 2022. Phytochemical screening, antioxidant and antibacterial properties of extracts of *Viscum continuum* E. Mey. Ex Sprague, a South African mistletoe. Plants 11: 2094. DOI: 10.3390/plants11162094.

Maurya PK. 2022. Health benefits of quercetin in age-related diseases. Molecules 27: 2498. DOI: 10.3390/molecules27082498.

Meshaal AK, Hetta HF, Yahia R, Abualnaja KM, Mansour AT, Al-Kadmy IMS, Alghamdi S, Dablool AS, Emran TB, Sedky H, Batiha GES, El-Kazzaz W. 2021. In vitro antimicrobial activity of medicinal plant extracts against some bacterial pathogens isolated from raw and processed meat. Life 11: 1178. DOI: org/10.3390/life11111178.

Miklasińska-Majdanik M, Kępa M, Wojtyczka RD, Idzik D, Wasik TJ. 2018. Phenolic compounds diminish antibiotic resistance of *Staphylococcus aureus* clinical strains. Intl J Environ Res Public Health 15: 2321. DOI: org/10.3390/ijerph15102321.

Nolasco-González Y, Chacón-López MA, Ortiz-Basurto RI, et al. 2022. *Annona muricata* leaves as a source of bioactive compounds: Extraction and quantification using ultrasound. Horticulturae 8: 560. DOI: 10.3390/horticulturae8070560.

Othman L, Sleiman A, Abdel-Massih RM.2019. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front Microbiol 10: 911. DOI: 10.3389/fmicb.2019.00911.

Pandey P, Khan F, Qari HA, Oves M. 2021. Rutin (Bioflavonoid) as cell signaling pathway modulator: Prospects in treatment and chemoprevention. Pharmaceuticals 14: 1069. DOI: 10.3390/ph14111069.

Shami AM. 2017. The effect of alkaloidal fraction from *Annona squamosa* L. against pathogenic bacteria with antioxidant activities. Pharm Sci 23: 301-307. DOI: 10.15171/PS.2017.44.

Singh Y, Bhatnagar P, Thakur N. 2019. A review on insight of immense nutraceutical and medicinal potential of custard apple (*Annona squamosa* Linn.). Intl J Chem Stud 7: 1237-1245.

Van Montagu M, Zambryski Zambryski P. 2013. *Agrobacterium* and Ti plasmids. Brenner's Encyclopedia of Enetics. Elsevier Sci 1: 55-57. DOI: 10.1016/B978-0-12-374984-0.01542-4.

Vikas B, Nehru J, Botanic T. 2013. Antibacterial activity of *Annona squamosa* seed extract. Intl J Pharm Technol 5: 5651-5659.

Zhu H, Chen L, Yu J, Cui L, Ali I, Song X, Park JH, Wang D, Wang X. 2020. Flavonoid epimers from custard apple leaves, a rapid screening and separation by HSCCC and their antioxidant and hypoglycaemic activities evaluation. Sci Rep 10: 1-11. DOI: 10.1038/s41598-020-65769-5.