

Original Research

Antimicrobial potency of red and yellow varieties of grapefruit (Citrus Paradisi Macfad) grown in Jordan

Malek A. Zihlif, Fatima Afifi, Ahmad Sharab, Paayam Maroof, Ala' Abu-Odeh, Ahmad R. Alsayed

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Abstract

Introduction: Worldwide, grapefruit is known to have medicinal values with antimicrobial, anti-inflammatory, antioxidant, and astringent propensities. Only a few researchers studied its antibacterial effects underlining the efficacy of the flavonoids extracted from the grapefruit peels against gram-positive and negative bacteria. **Aims:** The purpose of this work was to evaluate the antibacterial activity of the grapefruit water and ethanol extracts and their furanocoumarins against three common infectious bacteria and their multi-drug resistant clinical isolates. **Methods:** Antimicrobial activity of 4 different grapefruit extracts (Red-Ethanol, Yellow-Ethanol, Red-n-Hexane, Yellow-n-Hexane) from 2 different varieties (Red & Yellow) along with 4 of its active materials (naringin X, Bergaptol, Bergapten, Bergamottin) have been evaluated *in vitro* against 7 different bacteria that are frequently incriminated in human infection four of which were ATCC (*Salmonella paratyphi*, *E.coli*, *Staphylococcus aureus*, *Pseudomonas*) and three clinical bacteria (*Pseudomonas aeruginosa* drugs sensitive, *Klebsiellas pneumonia* multi-drug resistant, *Pseudomonas aeruginosa* multi-drug resistant) by assessing the minimum inhibitory concentration (MIC), using the microdilution method to test the microorganisms. **Results:** Most of the grapefruit's tested extracts showed great activity level, including the active materials (Furanocoumarins) of the grapefruit, which showed a strong level of antimicrobial activity against the tested microorganisms, especially against the gram-negatives. **Conclusions:** The results obtained from this study confirm that the grapefruit extracts had a significant antimicrobial effect on the tested microorganisms, especially the Red-Ethanol extract, which had more activity against gram-negative bacteria, including *E.coli*, *Pseudomonas* as well as *Pseudomonas aeruginosa* drugs sensitive.

Keywords: grapefruit; antimicrobial; microorganisms; natural sources; Jordan

INTRODUCTION

Grapefruit is a subtropical plant that is grown at sea level where there is enough moisture and can also be grown worldwide at different times of the year, depending on the species and its variety. Grapefruit is a member of the genus Citrus (family Rutaceae), and its scientific name is Citrus paradise Macfad. The grapefruit was found first in Barbados Island and is regarded as the result of a natural cross-breeding between the orange and the pomelo. It is now planted in Mediterranean countries such as Spain, Turkey, Palestine, Jordan, Morocco, South Africa, South America, and many other countries.¹

The fruit is round to slightly pear-shaped and 4 to 6 inches in diameter. The pale-yellowish or pink pulp of the grapefruit has a thin membrane with somewhat bitter walls and very juicy acid to sweet-acid flavor when fully ripe (Morton, n.d.). Grapefruit comes in two varieties white and red (Pink). The red color is due to the high amount of carotenoids that is fifty times that of the white one.¹ According to previous studies, grapefruit had an effect in lowering blood pressure, lipid levels, cardiovascular disease prevention, and lowering the complications of diabetic ketoacidosis.^{2,3} This table fruit and its constituents are considered active agents in detoxification, cellular regeneration, maintenance of heart health, rheumatoid arthritis, and cancer prevention. Furthermore, grapefruit juice is also a great source of vitamin C, folic acid, phenolic acids, potassium, iron, calcium, limonoids, terpenes, monoterpenes, and d-glucaric acid.^{4,5} The red (pink) variety is a rich source of beta-carotene and lycopene that can be converted to vitamin A by the body. The flavonoid fraction of the red variety has the greatest concentration of naringin that the human body is able to metabolize to naringenin, an important antimutagenic agent.¹

Worldwide grapefruit is known to have medicinal values with antimicrobial, antifungal, anti-inflammatory, antioxidant, antiviral, and astringent propensities. The fruit and its seeds, peels, juice extracts, and essential oils are recommended for building up resistance to common colds and wound infections.^{1,6}

Only a few researchers studied its antibacterial effects underlining the efficacy of the flavonoids, such as naringin that was extracted from the grapefruit peels against both gram-

Malek A. ZIHLIF*. Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan. m.zihlif@ju.edu.jo

Fatima AFIFI. Department of Pharmaceutical Sciences, The University of Jordan, Amman, Jordan.

Ahmad SHARAB. Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan.

Paayam MAROOF. Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan.

Ala' ABU-ODEH. Pharmaceutical Chemistry and Pharmacognosy Department, Faculty of Pharmacy, Applied Science Private University, Amman 11937, Jordan. a_abuodeh@asu.edu.jo

Ahmad R. ALSAYED. Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science Private University, Amman 11937, Jordan. a_alsayed@asu.edu.jo



positive and negative bacteria.^{6,7} Other researchers reported the effectiveness of the essential oils extracted from the grapefruit peels against different strains of microorganisms, such as (*Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsellia pneumonia*, *Proteus vulgaris*, *Pseudococcus spp.*, *Salmonella typhimurium*, *Serratia marcescens*, *Shigella flexneri*, *Staphylococcus aureus*, *S. epidermidis*).⁸⁻¹⁰ On the other hand, grapefruit seed extracts were effective against a wide range of gram-negative bacteria.^{11,12} It was also reported in 2008 that grapefruit juice and its furcoumarins are effective against the bacterial autoinducer signaling that are the main cause of pathogenicity in bacteria as well inhibited biofilm formation in *E. coli*, *S. typhimurium* and *Pseudomonas aeruginosa*.¹³ The juice extract was also effective in inhibiting survival and adherence of *S. entericaserovar Typhimurium PT193* at low PH to intestinal epithelial cells.⁷ Other studies revealed the role of grapefruit in the preservation of vegetables, fruits, peanuts, beef, chicken meat¹² and hypromellose gel¹⁴ about the juice extraction of grapefruit mostly dealt with the preservation of vegetables and fruits, peanuts, beef, chicken meat¹² and hypromellose gel¹⁴ and prevention of growth of pathogenic bacteria by using grapefruit juice extracts.⁷ One of the world's greatest challenges in health care that we are facing today, according to the Worlds Health Organization (WHO) in 2014, is the continuous growth of resistant microorganisms to the available antimicrobial drugs. Meanwhile, most t plant-based antimicrobials are usually devoid of many side effects of synthetic antimicrobial drugs. Thus, searching for new antimicrobial compounds that are extracted from natural sources is still an ongoing process.¹⁵

This work aims to evaluate the antibacterial activity of the grapefruit extracts and their furanocoumarins against three common infectious bacteria and their multi-drug resistant clinical isolates with the hope of increasing the existing pool of the currently available antibiotics from the plant kingdom.

MATERIALS AND METHODS

Collection and identification of plant materials

Each 4kg of Yellow and Red varieties of Fresh grapefruit was purchased from the local market in Amman, Jordan, during early winter. The fruits were identified by Prof. B. Abuirmaleh, School of Agriculture, The University of Jordan.

Preparation of fruit juice concentrates

Fresh juice extraction of grapefruit, both the red and yellow varieties of grapefruit were rinsed thoroughly with water to remove their peel and the white tissue covering the fruit. The fruits were cut into smaller pieces and weighed. Each variety was placed in a jar and was blended separately. After obtaining the fresh juice, they were filtered by (Whatmann, No 4) with avoidance of sunlight exposure to prevent the loss of effective ingredients. The filtered juices were then lyophilized (by freeze-drying) for 24 hours, and the obtained powders from them were preserved at 4°C until subsequent use. The extracted powder of each of the grapefruit varieties was dissolved in

distilled water (to a final concentration equal to 100 mg/ml).

Ethanol extraction of grapefruit

Both the red and yellow varieties of grapefruit were rinsed thoroughly with water with the removal of their peel and the white tissue covering the fruit, then they were cut into smaller pieces and weighed. A 1:1 concentration of 70% Ethanol was mixed with each variety of Red and White and then blended separately to obtain a homogenous mixture for each one of them. The red variety was 541.83g and was mixed with 540ml 70% Ethanol, while the yellow variety was 563.92g and was mixed with 564ml 70% Ethanol. After that, they were placed in a shaking water bath for 3 continuous hours at room temperature. Left for 24 hours and was filtered by filter paper (Whatmann, No 4). Direct exposure to sunlight was avoided to prevent the loss of effective ingredients. Then, ethanol was evaporated using a rotary evaporator (Buchi, Swizerland) and was lyophilized (by freeze-drying), and the obtained powders from the 1:1 concentration 70% Ethanol extracts were preserved at (4°C until subsequent use). The extracted powder of each grapefruit variety was dissolved in (5%) Dimethyl sulfoxide (DMSO) to obtain the final desired concentration 2.2.3. n-Hexane extraction of grapefruit, both the red and yellow varieties of grapefruit were rinsed thoroughly by water with the removal of their peel and the white tissue that was covering the fruit, then they were cut into smaller pieces and weighed. A 1:1 concentration of n-Hexane was mixed with each variety Red and White and then blended separately to obtain a homogenous mixture for each one of them. The red variety was 464.81g and was mixed with 462 ml of N-Hexane, while the yellow variety was 677.39g and was mixed with 675 ml of n-Hexane. After that, they were placed on a shaking water bath for 3 continuous hours at room temperature. Left for 24 hours and was filtered (by Whatmann, No 4) with avoidance of sunlight exposure to prevent the loss of effective ingredients. The two filtered solutions were then placed in two different rotary evaporators with a vacuum system to separate the solvent (n-Hexane) from the extracts. The result was a sticky, viscous material for each red and yellow varieties, finally dissolved in (5%) Dimethyl sulfoxide (DMSO) to obtain the final desired concentration.

Assessment of antimicrobial activity of each ingredient

The antimicrobial activity was assessed based on the minimum inhibitory concentration (MIC), using the microdilution method to test a total of seven different microorganisms (Table 1); four of which were ATCC (American Type Culture Collection) and the remaining three were clinical bacteria that were obtained from Jordan University Hospital, Amman, Jordan.

Microbial inoculums

Microbial cultures were grown in the bacteriological Mueller-Hinton Broth (MHB) at 37 °C for 24 hours adjusted at 2x10⁶ colony-forming units (CFU mL⁻¹).

Antimicrobial activity screening tests

The antimicrobial activity of the tested organism towards various plant extracts (four different extracts from both yellow



and red varieties of grapefruit) was evaluated using the Agar-well diffusion method, as described by Perez et al. (1990).¹⁶ Additionally, the three active compounds present in the extracts were identified. A uniform inoculum suspension was applied to a solidified 20ml Mueller-Hinton Agar (MHA) medium for the bacteria and left to dry for a duration of 5 minutes. The seeded agar was punctured with sterile cork borers, creating holes with a diameter of 6 mm. A volume of 100µl of liquor, containing plant extracts at a concentration of 1000 µg ml⁻¹, was introduced into each well of the seeded medium. The samples were left undisturbed on the laboratory bench for a duration of 1 hour to ensure proper diffusion. Subsequently, the samples were incubated at a temperature of 37°C for a period of 24 hours. The measurement of the inhibition zones was conducted in millimeters (mm). Positive controls were conducted using 100µL of Gentamicin, following the same procedure and in parallel with the treatment. The experiments were conducted in triplicate.

Determination of minimum inhibitory concentration

The Minimum Inhibitory Concentrations (MIC) were determined for the grapefruit extracts and their active constituents, which exhibited the most extensive range of antimicrobial activity against the microorganisms tested. The method employed for determining MIC values involved the utilization of techniques described in references 14 and 15. The MIC was determined as the lowest concentration of the sample that exhibited inhibitory effects on visible growth. The examination of all samples was conducted in triplicate.

Statistical analysis

The mean values were presented as the mean ± standard deviation (SD) and were subjected to statistical analysis using one-way ANOVA (Tukey's Studentized range) with the aid of SPSS 24.0 for Windows. Statistical significance was determined at a significance level of P<0.05.

RESULTS

The antimicrobial activity of the grapefruit extracts and their active materials were assessed in Table 2. The Red Ethanol variety revealed the highest significant antimicrobial activity against all the tested microorganisms with inhibition zone starting from 9.5 to 22 mm. Although Red n- Hexane belongs to the same variety, that has been extracted through a different solvent showed a much weaker activity to the same microorganisms based on the measured inhibitor zone. The Yellow Ethanol extract showed high activity against most tested microorganisms, with the highest inhibition zone being recorded against Salmonella paratyphi. However, it displayed negative results against Klebsiellas pneumonia multi-drug resistant. As for the Yellow n- Hexane, like the red n- Hexane?? had a much weaker activity compared to the Yellow Ethanol of the same variety. However, it was surprisingly highly active against Pseudomonas aeruginosa drugs sensitive microorganisms and showed negative activity against the Klebsiellas pneumonia multi-drug resistant just like the Yellow Ethanol.

Table 1. Tested microorganisms in this study

ATCC (American Type Culture Collection)				Clinical Bacteria		
<i>Staphylococcus aureus</i>	<i>E.coli</i>	<i>Salmonella paratyphi</i>	<i>Pseudomonas</i>	<i>Klebsiellas pneumonia</i> multi-drug resistant	<i>Pseudomonas aeruginosa</i> drugs sensitive	<i>Pseudomonas aeruginosa</i> multi-drug resistant

Table 2. Antimicrobial activity (diameter of inhibition zone) of 4 grapefruit extracts with their 3 active materials against seven different microorganisms

Grapefruit extracts	Solvents	<i>S. paratyphi</i>	<i>E.coli</i>	<i>S.aureus</i>	<i>P. aeruginosa</i>	<i>p. aeruginosa</i> drugs sensitive	<i>K. pneumonia</i> multi-drug resistant	<i>P. aeruginosa</i> multi-drug resistant
Yellow	Ethanol	29.0 ± 1	12.5 ± 0.5	19.7 ± 3.8	14.5 ± 0.5	12.3± 1.5	-ve	10.5 ± 0.5
Yellow	n -Hexane	9.0 ± 1.0	10.3 ± 1.0	11.8 ± 1.6	12.8 ± 0.76	21 ± 1	-ve	13 ± 1.7
Red	Ethanol	22.0 ± 2.0	9.5 ± 0.8	18.0 ± 3.45	13.7 ± 1.16	20.3 ± 1.8	16.3 ± 1.5	15.0 ± 2.6
Red	n -Hexane	-ve	10.0 ± 0.8	11.5 ± 1.5	10.2 ± 1.3	15.6 ± 1.2	14.0 ± 2.7	13.0 ± 1.0
Bergaptol		-ve	15.0 ± 2.0	15.0 ± 2.0	20.4 ± 1.5	15.8 ± 1.9	-ve	19.6± 3.2
Bergapten		-ve	13.7 ± 2.1	13.3 ± 1.2	19.7 ± 1.5	17.5 ± 1.3	-ve	15.7 ± 3.2
Bergamottin		-ve	19.0 ± 2.0	14.4 ± 1.5	19.0 ± 3.0	16.5 ± 1.3	13.4 ± 1.5	21.7 ± 3.2
Positive control Gentamycin		7.8 ± 1.6	14.0 ± 1.0	16.2 ± 2.0	14.4 ± 1.5	21.4 ± 1.5	17.5 ± 1.5	12.8 ± 1.8

Inhibition zone diameters are expressed as Mean ± ST. Diameter of Inhibition zone in (mm).



Table 2 also illustrates that the three active materials, Bergaptol, Bergapten, and Bergamottin, showed no activity against *Salmonella paratyphi* while both Bergaptol and Bergapten had no activity against *Klebsiellas pneumonia multi-drug resistant*. However, Bergamottin showed an effect on inhibition zone of 13.3 mm. The highest activity of Bergaptol was revealed against *Pseudomonas aeruginosa* 20.33 ± 1.53 mm and *Pseudomonas aeruginosa multi-drug resistant* 19.67 ± 3.22 mm. While the highest activity for Bergapten was against *Pseudomonas aeruginosa*, 19.67 ± 1.53 mm, and for Bergamottin was against *E.coli*, with a recorded inhibition zone of 19 ± 2 mm. The antimicrobial activity of grapefruit extracts was also expressed in Minimal Inhibition Concentration (MIC) against tested Microorganisms shown in Table 3. Among the most active grapefruit extract were Red Ethanol showing an MIC of only 8.3 µg/m against both *E.coli*, and *Pseudomonas* and 10 µg/m against *Staphylococcus aureus*, while the Red n-Hexane showed equal MIC of 12.5 against *Salmonella paratyphi*, *E.coli*, *Staphylococcus aureus* and *Pseudomonas* and weaker MIC against the rest of the microorganisms. The Yellow Ethanol extract showed an MIC of 10µg/ml against *Pseudomonas* and MIC of 12.5µg/ml against *E.coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* drugs sensitive. And the Yellow n-Hexane MIC against *Pseudomonas* was 10µg/ml and 12.5µg/ml against *E.coli* and *Staphylococcus aureus*.

As for the active materials of grapefruit, it showed much weaker MIC than the extracts. The most effective for Bergaptol was against *E.coli* with an MIC of 15.62µg/ml, while for Bergapten was 15.62µg/ml against both *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* drugs sensitive and negative against

Staphylococcus aureus (table 4). Bergamottin showed an MIC of 31.25µg/ml against *E.coli*, *Pseudomonas aeruginosa*, and *Pseudomonas aeruginosa* drugs sensitive and proved to be the weakest MIC effect among all the tested ingredients for grapefruit (both extracts and active materials of grapefruit) against the tested microorganisms see Table 4.

DISCUSSION

The initial emergence and widespread distribution of antimicrobial-resistant microorganisms have resulted in the diminished effectiveness of numerous antibiotics currently available.¹⁷ Consequently, there has been a significant emphasis on exploring novel antimicrobial compounds derived from natural sources. Antimicrobial activity of 4 different grapefruit extracts (Red-Ethanol, Yellow-Ethanol, Red-n-Hexane, Yellow-n-Hexane) from 2 different varieties (Red & Yellow) along with 4 of its active materials (Naringin, Bergaptol, Bergapten, Bergamottin) have been evaluated in vitro against 7 different bacteria that are frequently incriminated in human infection four of which were ATCC (*Salmonella paratyphi*, *E.coli*, *Staphylococcus aureus*, *Pseudomonas*) and three clinical bacteria (*Pseudomonas aeruginosa* drugs sensitive, *Klebsiellas pneumonia multi-drug resistant*, *Pseudomonas aeruginosa multi-drug resistant*) by assessing the minimum inhibitory concentration (MIC), using the microdilution method to test the microorganisms. Most of the tested extracts of the grapefruit showed a great level of activity, including the active materials (Furanocoumarins) of the grapefruit, which showed a strong level of antimicrobial activity against the tested Microorganisms,

Table 3. Minimal Inhibition Concentration (MIC) of the grapefruit extracts

Plant extract	Solvent	<i>S. paratyphi</i>	<i>E.coli</i>	<i>S. aureus</i>	<i>Pseudomonas</i>	<i>P. aeruginosa</i> drugs sensitive	<i>K. pneumonia multi-drug resistant</i>	<i>P.aeruginosa multi-drug resistant</i>
Red	Ethanol	12.5	8.3	10	8.3	12.5	50	25
Red	n- Hexane	12.5	12.5	12.5	12.5	25	50	33.3
Yellow	Ethanol	16.6	12.5	12.5	10	12.5	100 (-ve)	50
Yellow	n- Hexane	16.6	12.5	12.5	10	25	100(-ve)	33.3
Gentamicin	-----	25	15.62	125	50	25	7.81	500

MIC in µg/ml

Table 4. Minimal Inhibition Concentration (MIC) of grapefruit active materials

Plant active materials	<i>S. paratyphi</i>	<i>E.coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>P.aeruginosa</i> drugs sensitive	<i>K. pneumonia multi-drug resistant</i>	<i>P.aeruginosa multi-drug resistant</i>
X	62.5	15.62	31.25	7.8	7.8	250	125
Bergaptol	62.5	15.62	62.5	31.25	62.5	250	500
Bergapten	62.5	31.25	-ve	15.62	15.62	1000	250
Bergamottin	125	31.25	-ve	31.25	31.25	1000	500

MIC in µg/ml, X: naringin



especially against the gram-negatives. Starting with the grapefruit juice extracts, the Red variety revealed a stronger antimicrobial activity against most bacterial strains compared to the Yellow variety extracts that we have obtained, consistent with some other obtained studies that were done before.¹² The obtained results also indicated that Red- Ethanol extracts had a much better antibacterial effect against gram-negative organisms than the Gram-positive ones, proving that: Ethanol is the best effective solvent with more consistent extraction of antimicrobial substances from the grapefruit extract compared to n-Hexane in which similar findings were also reported previously.^{18,12} Then exploring the activity of the extracts against the gram-negative bacteria showed interesting results, which included a strong activity against *E.coli*, *Pseudomonas* as well as *Pseudomonas aeruginosa* drugs sensitive with Minimal Inhibition Concentrations (MIC) of 8.3 µg/ml, 8.3 µg/ml, 12.5 µg/ml, respectively and comparing it with the MIC of the used positive control of this study Gentamicin which was 15.62 µg/ml, 50 µg/ml, 25 µg/ml. The findings of the present investigations unequivocally demonstrate that there is variation in the antibacterial activity observed across different varieties of grapefruit extracts and their respective active constituents. Hence, the antimicrobial efficacy of the extracts is contingent upon the specific variety, the solvent employed, and the microorganism being tested and digging deeper in the hope of finding the exact active material that is responsible for this activity, a gas chromatography-mass spectrometer sensor (GC-MSS) was used to determine the highest ingredients that make up the Red- Ethanol variety which was found to be Bergaptol, Bergapten, and Bergamottin. As for active materials, Bergaptol, Bergapten and Bergamottin had higher activity than the tested positive control Gentamicin, including their activity against the tested clinical organisms.

Going one step further in the hope of finding an explanation for the high activity of the extracts that were tested at the beginning of this study, the synergistic effect of the active materials of the grapefruit has also been tested, which, unfortunately, the combination of more than one active material showed equal or even weaker activity than the active materials by themselves. The findings of this study provide the initial documentation

of the antimicrobial properties of grapefruit juice extracts and their active constituents. Additionally, the combination of these active constituents demonstrates a synergistic effect in combating the tested microorganisms, indicating a wide-ranging efficacy against the tested microorganisms. Additional phytochemical investigations are encouraged to purify and characterize the bioactive constituents of this particular botanical species and examine the efficacy of the naringin compound against various other microorganisms.

Limitations: There may be some possible limitations in this study, including the inability to provide the other minor ingredients that are found in the grapefruit juice extract, that might have been the main cause of the high antibacterial activity if they were evaluated along with the major ingredients that were tested, whether by themselves or as a combination.

CONCLUSION

The results obtained from this study confirm that the grapefruit extracts had a significant antimicrobial effect on the tested microorganisms, especially the Red-Ethanol extract, which had more activity against gram-negative bacteria, including the *E.coli*, *Pseudomonas* as well as *Pseudomonas aeruginosa* drugs sensitive. Interestingly, naringin proved to have strong antibacterial activity against *Pseudomonas aeruginosa* drugs sensitive and *Pseudomonas aeruginosa* multi-drug resistant.

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CONFLICTS OF INTERESTS

The authors declare that there is no conflicts of interest.

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