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Coumarins from Red Delicious Apple Seeds: Extraction, Phytochemical Analysis, and Evaluation as Antimicrobial Agents

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ABSTRACT
The evolution of resistance to many valid antimicrobial agents and the demand for exploring novel agents to deal with this emerge are well-highlighted. In this report, the seeds of Red Delicious apples were extracted by four solvents; n-hexane, chloroform, methanol and water. This was carried out using three methods, which are kinetic maceration, ultrasound- and microwaves- expedited extraction techniques. These methods were performed in three modes including non-serial, serial rising- and falling- arranged in polarity. Phytochemical study indicated the presence of coumarins in the methanol and chloroform extracts obtained from the applied methods and modes. Four novel furanocoumarins were isolated and their chemical structures characterized by corresponding their spectroscopic data with those detected in literature. Two in vitro antimicrobial studies were verified for the isolated products via a broth dilution method; the antibacterial activity versus the following standard strains: *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Haemophilus influenzae* and *Escherichia coli* utilizing Ciprofloxacin as a reference, and the antifungal activity versus the following standard strains: *Candida albicans* and *Aspergillus niger* using Nystatin as a reference. The results indicated that the isolated furanocoumarins have a promising antimicrobial activity against the test microorganisms with superiority attributed to compound **R1**. Also, the isolated products showed encouraging bactericidal and fungicidal activities according to the values of MBC/MIC and MFC/MIC ratios. It is concluded that these novel furanocoumarins may provide a convenient scaffold for the development of new antimicrobial agents.

Keywords: Apple, Phytochemical study, Furanocoumarins, Antibacterial, Antifungal.

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INTRODUCTION
The harmful effects of pathogens on human health have increased primarily due to the multiple drug resistances developed by these microorganisms (1). Currently, the confrontation between the valid antimicrobial agents and infectious diseases is biased toward the first side, but this is not for a long time. Expansion in the antimicrobials abuse and escalation of pathogenic defense mechanisms may change the equation in the favor the second side (2). To handle with this consequence, there is a developing concern for exploring novel antimicrobial agents especially those isolated from natural sources (3).

Nature can be considered as an inexhaustible source of products which are characterized by their diversity in the chemical structures and biological activities. This diversification may serve better in the drug design and development (4). Nowadays, there is a growing global trend to employ natural products in the management of many human health problems. This is principally due to their long history of safety and acceptability (5).

The apple cultivar named Red Delicious is the most frequently devoured apple phenotype and is qualified by its charming red color, crispy flesh, and high nutritional profits (6). In addition to the highest amounts of antioxidants and dietary fibers among the other apple phenotypes, this cultivar is high in vitamins (C, A, B6) and minerals (Iron, Potassium, Calcium), and low in calories and sugars (7). Therefore, consuming of this apple cultivar is highly recommended in many slimming programs (8).

Generally, the seeds of apple contain a belittle amount (0.6 mg/g of dry seeds) of cyanogenic glycoside named amygdalin. This compound with a misnomer vitamin B17 can cause some deleterious effects upon ingestion; however, such effects may progress into toxicity when an excessively high quantity of seeds has been digested (9).

Coumarins are a class of heterocyclic phytochemicals which isolated primitively from plants. These secondary metabolites have been broadly investigated for their pharmacological properties such as: anti-inflammatory, anticoagulant, antimicrobial, analgesic, antitumor, antioxidant, antidiabetic, and antithrombotic activities (10). In the plant realm, coumarins can be detected in different plant sectors including roots, leaves, flowers, and fruits (11); besides they can be found in the seeds of some plants (12).

As reported by our survey fulfilled in September 2019, there are many reports concerned with the phytochemistry and pharmacologically-related properties of Red Delicious apple. These two main subjects were studied on the following fruit parts: peel (13), pomace (14), and fruit flesh (15). The endeavor to find any report dealing with the chemical content of Red Delicious apple seeds was reached to closed end. This motivated the work team to launch this report.

The aim of the present work is to separate coumarins from Red Delicious apple seeds and examine their in vitro antimicrobial activity. This target was fulfilled by attaining the following objectives: (I) Extracting the seeds with four solvents; n-hexane, chloroform, methanol and water. Extraction was carried out by three different methods, which are kinetic maceration, ultrasound- and microwaves-expedited extractions. For each method, three modes were

followed including non-serial, serial rising- and falling-ordered in polarity. (II) Submitting the resultant extracts to qualitative phytochemical assay. (III) Separating the coumarins from the elected extract. (IV) Characterizing the chemical structures of the separated coumarins, and finally (V) Testing their antimicrobial activity against selected standard bacteria and fungi using a broth dilution method.

MATERIALS AND METHODS

Chemicals, solvents, and microbiological cultures used in this work were obtained from Tokyo Chemical Industry and Sigma-Aldrich. Silica gel (100-200 mesh size) was acquired from Sisco Research Laboratories Pvt. Ltd. Standard bacterial and fungal strains were obtained from Microbiologics®. The fruit was acquired from a resident market and botanically described by professionals from the College of Agriculture and Forestry/University of Mosul. The λ_{max} and IR spectra of the separated coumarins were documented via Varian UV/ Visible and Bruker-Alpha ATR spectrophotometers. Utilizing DMSO-d_6 as a solvent, ^{1}H NMR and ^{13}C NMR spectra of the separated products were scanned on Bruker Analytische Messtechnik GmbH (300 MHz).

Preparation of plant materials

Each single apple of the obtained batch (145 kg) was rinsed carefully with faucet water, followed by distilled water and divided into four slices by steel blade. The gained seeds were dried in shade at room temperature for two weeks, crushed by a home blender, and sieved producing a smooth powder (257 g). This was preserved in well-sealed containers and stored in the refrigerator for the following step (16).

Extraction

The powdered seeds were extracted with the following solvents: n-hexane, chloroform, methanol and water. Three methods were employed; kinetic maceration (KM), ultrasound-expedited extraction (UEE), and microwaves-expedited extraction (MEE). For each method, extraction was carried out in three modes including non-serial, serial rising- and falling- ordered in polarity. In the serial modes, the powder was extracted by the first solvent in the order. The extract mixture was filtered and the filtrate submitted to phytochemical assay while the residue was extracted with solvent next to the first in the applied order. These sequences were also employed for the third and the fourth solvents in the same fashion (9).

Kinetic maceration

The powdered seeds (2 g) were kinetically macerated in 20 ml extracting solvent at 30°C for 3 days using a shaker water bath (SWBR17 SHEL LAB shaking water bath, USA) . The mixture was filtered and the filtrate stored in refrigerator until be passed into the phytochemical assay (17).

Ultrasound-expedited extraction

The powdered seeds (2 g) were sonicated in 20 ml extracting solvent at 30°C for 30 min using an ultrasonic water bath (40 kHz, 350 W, Power sonic410, Korea). The mixture was filtered and the filtrate refrigerated for the use in a subsequent phytochemical assay (18).

Microwave-expedited extraction

The powdered seeds (2 g) were irradiated in 20 ml extracting solvent at 100 W for 5 min using a domestic microwave oven (Moulinex - MW Steam 23L, MW531070, France). The mixture was filtered and the filtrate kept in refrigerator until be used in the phytochemical assay (19).

Qualitative phytochemical assay

From the formerly defined extraction methods and modes, the resultant thirty six extracts were assessed for the presence of the following phytochemicals: fixed oils, anthraquinones, coumarins, glycosides, saponins, proteins, amino acids, anthocyanins, betacyanins, steroids, phenols, emodins, alkaloids, carbohydrates, terpenoids, tannins, and flavonoids. This assessment was performed according to the devilishly admitted methods reported by Harborne (20). Tables showed the results of this assay are attached in a Supplementary file.

Separation and isolation (9)

Seed powder (200 g) was extracted by chloroform (2 L) using UEE operated in a non-serial mode and the resultant extract was evaporated to dryness using a rotary evaporated under reduced pressure. The mixture of raw products (8.112 g) was suspended in 82 ml of 1M NaOH, stirred at 50°C for 50 min, and filtered. The deep yellowish filtrate was soured by dropwise addition of 1M HCl in an ice bath; the addition was stopped as the solution color vanished. The mixture was preserved in a refrigerator for one day to complete the precipitation process, filtered and then weighted (2.776 g).

To identify the number of isolated coumarins, a solution of small amount of resultant powder in 2 ml chloroform was used to make spots on TLC plates. The applied spots were eluted by a mobile phase consists of chloroform: ethyl acetate (4:1) and the separated points were settled via UV light (366 nm). The results of triple trials marked the presence of four products.

The isolation of these products was fulfilled via gravitational column chromatography utilizing mixtures of ether: ethyl acetate in a gradient ratio of 9:1 to 1:9 as mobile phases, and silica gel as a stationary phase. Four products named E1-E4 were detected, each one disclosed as a single spot in TLC, in different mobile phases.

Physical properties and spectral characterization of compounds E1-E4

(E)-12-(2'-Chlorovinyl)bergapten (E1): Yellow powder; Mobile phase (8:2); Weight 1.106 g; 36.84% Yield; mp 186-189°C; R_f 0.81; UV (EtOH) λ_{max} 357 nm; IR ν_{max} 3086, 3056, 2916, 1735, 1671, 1627, 1589, 1553, 1244, 1049, 756 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.15 (1H, d, J= 9 Hz, H-4), 7.10 (1H, s, H-8), 7.00 (1H, s, H-11), 6.75 (1H, d, J= 15 Hz, H-1'), 6.55 (1H, d, J= 15 Hz, H-2'), 6.28 (1H, d, J= 9 Hz, H-3), 4.25 (3H, s, H-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 160.8 (C, C-2), 158.7 (C, C-7), 157.1 (C, C-12), 154.4 (C,

C-5), 152.0 (C, C-9), 143.7 (CH, C-4), 130.2 (CH, C-1'), 126.4 (CH, C-2'), 115.5 (CH, C-3), 114.6 (C, C-6), 113.8 (C, C-10), 110.2 (CH, C-11), 99.1 (CH, C-8), 64.2 (CH_3, C-13) ppm.

12-(1',1'-dihydroxyethyl)bergapten (E2): Yellow powder; Mobile phase (4:6); Weight 0.105 g; 3.78% Yield; mp 211-214°C; R_f 0.63; UV (EtOH) λ_{max} 321 nm; IR ν_{max} 3383, 3085, 3048, 2906, 1736, 1628, 1590, 1555, 1246, 1048 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.15 (1H, d, J= 9 Hz, H-4), 7.14 (1H, s, H-8), 7.02 (1H, s, H-11), 6.27 (1H, d, J= 9 Hz, H-3), 4.24 (3H, s, H-13), 3.66 (2H, s, OH-1'), 1.85 (3H, s, H-2') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 160.8 (C, C-2), 159.4 (C, C-12), 154.4 (C, C-5), 154.0 (C, C-7), 152.0 (C, C-9), 143.8 (CH, C-4), 115.5 (CH, C-3), 113.8 (C, C-10), 111.9 (C, C-6), 107.3 (C, C-1'), 103.3 (CH, C-11), 99.1 (CH, C-8), 64.2 (CH_3, C-13), 31.7 (CH_3, C-2') ppm.

12-(2'-chloropropan-2'-yl)-8-hydroxybergapten (E3): Yellow powder; Mobile phase (5:5); Weight 0.942 g; 33.93% Yield; mp 224-227°C; R_f 0.66; UV (EtOH) λ_{max} 333 nm; IR ν_{max} 3403, 3060, 2893, 1735, 1631, 1590, 1555, 1249, 1051 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 9.81 (1H, s, OH-8), 8.09 (1H, d, J= 9 Hz, H-4), 6.69 (1H, s, H-11), 6.23 (1H, d, J= 9 Hz, H-3), 4.26 (3H, s, H-13), 2.01 (6H, s, H-1', H-3') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 160.8 (C, C-2), 159.4 (C, C-12), 147.0 (C, C-5), 143.8 (CH, C-4), 141.2 (C, C-7), 139.2 (C, C-9), 127.9 (C, C-8), 115.5 (CH, C-3), 114.9 (C, C-10), 113.3 (C, C-6), 103.3 (CH, C-11), 64.2 (CH_3, C-13), 62.6 (C, C-2'), 30.9 (CH_3, C-1', C-3') ppm.

12-Hydroxy-11-chloromethylbergapten (E4): Yellow powder; Mobile phase (7:3); Weight 0.617 g; 22.22% Yield; mp 195-198°C; R_f 0.75; UV (EtOH) λ_{max} 342 nm; IR ν_{max} 3208, 3056, 2916, 2852, 1735, 1637, 1589, 1553, 1244, 1049 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.07 (1H, d, J= 9 Hz, H-4), 7.82 (1H, s, OH-12), 7.08 (1H, s, H-8), 6.26 (1H, d, J= 9 Hz, H-3), 5.02 (2H, s, H-1'), 4.20 (3H, s, H-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 160.8 (C, C-2), 155.3 (C, C-7), 154.2 (C, C-5), 152.6 (C, C-9), 143.8 (CH, C-4), 130.1 (C, C-12), 118.9 (CH, C-11), 115.4 (CH, C-3), 113.4 (C, C-10), 112.6 (C, C-6), 99.1 (CH, C-8), 64.2 (CH_3, C-13), 29.6 (CH_2, C-1') ppm.

Antimicrobial studies

Assay of antibacterial activity

The antibacterial activity of the isolated products was investigated versus standard pathogenic bacterial strains including *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603), *Haemophilus influenzae* (ATCC 49247), and *Escherichia coli* (ATCC 25922). This investigation was performed via a well-documented broth dilution method using Mueller-Hinton broth as a nutrient medium, Ciprofloxacin as a golden reference, and DMSO as a negative control. Briefly, a stock solution of 1 mg of the isolated coumarin in 1 ml DMSO was prepared. From this solution, the double-fold diluted concentrations starting from 512 and ending to 0.25 μg/ml were achieved by using distilled water as a diluent. To a labeled test tube, nutrient medium (3 ml), inoculum of 0.5 McFarland standard (0.2 ml), and elected concentration of the isolated coumarins (1 ml) were added sequentially. The test tubes were incubated at 37°C for 24 hr and the bacterial growth was detected visually. Depending on the concentration which revealed no bacterial evolution, the experiment was repeated using a second dilution prepared on the basis of 4, 1, 0.5 or 0.05. Subsequently, the minimum inhibitory concentration (MIC) for each test bacterial strain was detected (21). The minimum bactericidal concentration (MBC) was determined by further incubating 0.5 ml of the second diluted concentrations with 3 ml of nutrient medium for 24 hr at 37°C (22). Eventually, this method was triplicated to optimize the results.

Assay of antifungal activity

Two fungal strains were used to investigate the antifungal activity of the isolated coumarins including *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16888). The method of this assay is similar to that of testing the antibacterial activity except the nutrient medium was Sabouraud dextrose broth, the golden reference was Nystatin, and the incubation was performed for 48 hr at 30°C (23).

RESULTS AND DISCUSSION

The availability and practical safety of a vast number of natural products advocate for their utilization by several ancient and urban civilizations to take the advantages of their medicinal properties. In a phytochemistry, there are many obstacles facing the researchers in this field; the most important are the potential isolation and structural identification of the phytoconstituents. These obstacles are becoming more complicated via the attempt to link the presence of certain functional groups in the chemical structures of the isolated products with the diverse biological activities (24).

Phytoconstituents screening assay

The crushed seeds of Red Delicious apple were extracted by four solvents including n-hexane, chloroform methanol and water. Three methods were engaged in this extraction, which are KM, UEE, and MEE. Separately, each method was performed in three modes including non-serial, serial rising- and falling- arranged in polarity. The corollary of this process was thirty six extracts, which were screened for the existence of selected primary and secondary phytoconstituents. The data presented in the Tables included in a Supplementary file revealed that coumarins were detected in the methanol and chloroform extracts which resulted from the employed extraction methods and modes. Frequently, coumarins could be extracted by different solvents; this is mostly depended on the nature of functional groups attached to the coumarin nucleus and on the applied volume of the extracting solvent (25).

Isolation and separation

The characteristic chemical feature of coumarin is the presence of lactone, which is readily hydrolyzed when attacked via a strong nucleophile like sodium hydroxide into water-soluble derivatives of *cis*-cinnamic acid. The original coumarins are reformed once these derivatives treated with strong acid (26). In the current work, this chemical motif was employed as a method for the isolation of natural coumarins. The chloroform extract from non-serial UEE was elected for separating its coumarin contents. This was built on the vacancy of certain phytoconstituents in this extract including fixed oils, tannins, alkaloids, and flavonoids; where their presence may overlap with the method of separation (27).

Qualification of the chemical structures

By analyzing the spectroscopic data of the isolated coumarins and corresponding them with those detected in literature, it was concluded that the isolated products have a furanocoumarin nucleus substituted at position 5 with methoxy group. Since this skeleton is referenced as bergapten (28–31), the isolated products E1-E4 can be recognized as derivatives of this furanocoumarin. The chemical structures of these novel furanocoumarins, as displayed in Figure 1, were qualified based on the aforementioned chemical guide and on the interpretation of their FTIR, ^{1}H-NMR and ^{13}C-NMR spectra.

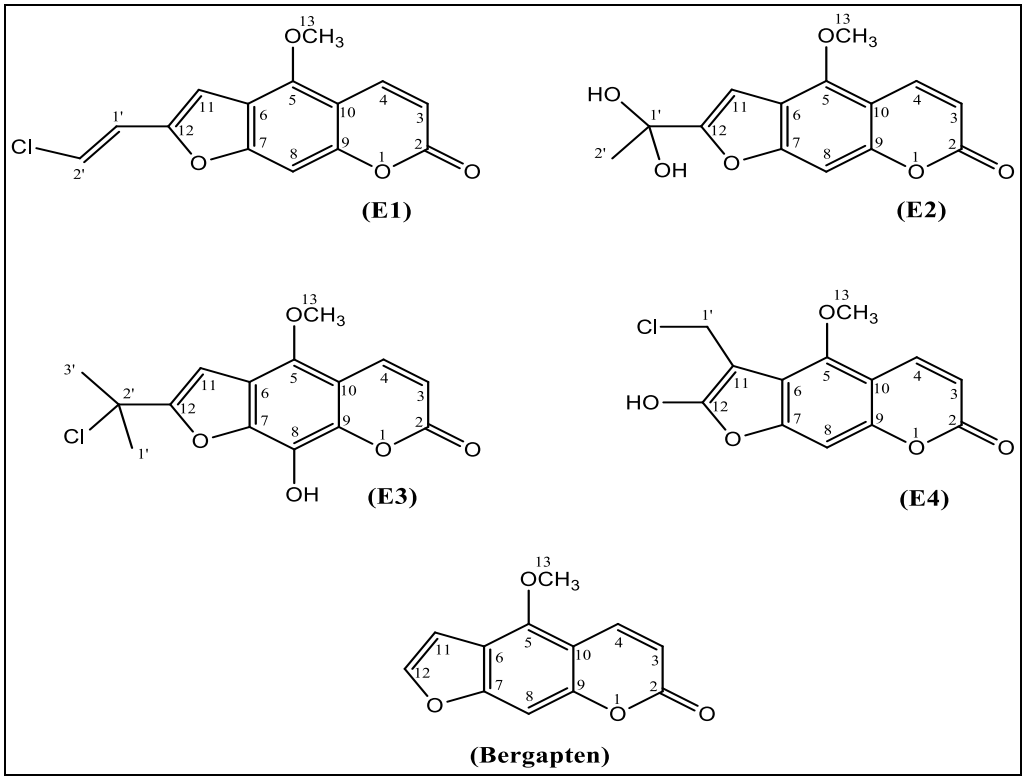

Figure 1. The chemical structures of bergapten and of the isolated furanocoumarins.

Antimicrobial activity

During urban history, there are ongoing battles between humans and pathogenic microorganisms which occasionally result in crises due to the failure of current therapies to adequately counteract the resistant pathogens. Since the potential antimicrobials are still acting as a strategic weapon in this war, the search for effective agents demands an extensive investigation to find new antimicrobials especially those separated from the natural products (32).

In this context, there are several research papers reported the antimicrobial activity of many natural and synthetic furanocoumarins (31). However, the correlation between their structural characteristics and the antimicrobial activity has been insufficiently highlighted in literature. Based on the available data, furanocoumarins with improved lipophilicity may show a bitter antimicrobial activity than those with hydrophilic substituents (33–35); this is complied with the results shown in Table 1.

Table 1: Results acquired from testing the antimicrobial activity of references and isolated furanocoumarins utilizing a broth dilution method.

Microorganisms	Test name	R	R1	R2	R3	R4
Pseudomonas aeruginosa ATCC 27853	MIC	0.75	2.15	7.50	4.50	5.00
	MBC	0.85	4.50	20.00	12.00	14.00

Klebsiella pneumonia ATCC 700603	MIC	0.40	2.05	7.00	6.00	7.50
	MBC	0.45	3.15	24.00	15.00	14.00
Haemophilus influenzae ATCC 49247	MIC	0.60	2.25	9.00	5.50	7.50
	MBC	0.65	5.00	20.00	15.00	16.00
Escherichia coli ATCC 25922	MIC	0.85	2.45	9.00	6.50	6.00
	MBC	0.95	5.00	32.00	14.00	16.00
Candida albicans ATCC 10231	MIC	4.00	9.00	24.00	12.00	13.00
	MFC	6.00	11.00	64.00	24.00	24.00
Aspergillus niger ATCC 16888	MIC	8.00	13.00	32.00	15.00	20.00
	MFC	12.00	14.00	72.00	28.00	32.00

MIC, MBC, and MFC (minimum fungicidal concentration) are reported in µg/ml, R symbol refers to the reference compound which is either Ciprofloxacin for bacteria or Nystatin for fungi.

Based on the data displayed in Table 1, compound R1 revealed the best antimicrobial activity against the test bacteria and fungi while compound R2 has the least activity; compounds R3 and R4 are in between. Also, this Table indicated that the isolated furanocoumarins are less active as antibacterial agents than Ciprofloxacin and less active as antifungal agents than Nystatin in spite of they showed encouraging results.

According to the values of MBC/MIC and MFC/MIC ratios included in Table 2, it is concluded that Ciprofloxacin has a bactericidal activity versus the test bacteria (36), Nystatin has a fungicidal activity versus the test fungi (37), and the isolated furanocoumarins may have bactericidal and fungicidal activities against the test microorganisms. This is because the values of aforementioned ratios are less than 4 for the tested products and references (38).

Table 2: Values of MBC/MIC and MFC/MIC ratios for the tested products and references.

Microorganisms	Ratio type	R	R1	R2	R3	R4
Pseudomonas aeruginosa ATCC 27853	MBC/MIC	1.13	2.09	2.67	2.67	2.80
Klebsiella pneumonia ATCC 700603	MBC/MIC	1.13	1.54	3.43	2.50	1.87
Haemophilus influenzae ATCC 49247	MBC/MIC	1.08	2.22	2.22	2.73	2.13
Escherichia coli ATCC 25922	MBC/MIC	1.12	2.04	3.56	2.15	2.76
Candida albicans ATCC 10231	MFC/MIC	1.50	1.22	2.67	2.00	1.85
Aspergillus niger ATCC 16888	MFC/MIC	1.50	1.08	2.25	1.65	1.60

R symbol refers to the reference compound which is either Ciprofloxacin for bacteria or Nystatin for fungi.

CONCLUSION

This work demonstrated the success in the isolation and structural elucidation of four novel furanocoumarins from Red Delicious apple seeds. The isolated products showed hopeful antibacterial and antifungal activities versus the test bacteria and fungi. Also, the results acquired from this study reported that the isolated novel furanocoumarins have bactericidal and fungicidal activities versus the test microorganisms and these products may consider as an optimistic scaffold for the development of new antimicrobial agents.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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