Isolation and diagnosis of phytochemicals extracted from pomegranate husks and study of the biological effect of extract (O-Veratramide) as an antimicrobial activity

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Abstract

Context: The biological efficiency of O-veratramide (2,3-dimethoxybenzamide) isolated from pomegranate extract was studied. Extraction of 400 g of dried pomegranate husks was carried out by Soxhlet, and seven organic compounds were extracted using a mixture of organic solvents. O-veratramide has been deposited and isolated. Several solvents such as methanol, chloroform, ethyl acetate, and hexane have been used. The extraction ratio was higher when using methanol and less in chloroform. **Objective:** The biological efficacy of O-veratramide was investigated against different types of bacteria by taking the highest concentration of this compound and less concentration of the compound. **Materials and Methods:** The purpose of the manuscript was to study the inhibitory activity of the bacteria using MacConkey Agar as an agro-medium. O-veratramide showed excellent bioavailability against different types of Gram-negative and positive bacteria. **Results:** All extracts were analyzed by the GC-Mas technique to identify and distinguish the chemical compounds present in the raw extract in both organic and biological layers. A number of internationally recognized detection methods have been used to detect each type of compound extracted. The spectral analysis of the O-veratramide was studied, and its biological efficacy was studied against Gram-negative and positive bacteria. **Conclusions:** GC-Mas technique revealed the presence of seven compounds in the pomegranate extract. The same technique has demonstrated the possibility of isolating the O-veratramide using different solvents, its potential for estimation, and the study of its biological effect.

Keys words: An antimicrobial activity, phytochemicals O-veratramine, pomegranate husk extract

INTRODUCTION

Benzimidazole compounds and their derivatives are compounds that have long been known as antibacterial agents such as hydroxybenzanilides, which has biological activity and a significant effect on a wide range of bacteria. This can be attributed to the presence of the aromatic ring, which has been widely spread due to its various uses as antagonists for bacteria and viruses as well as infections and pain relievers.[1-6]

O-veratramide is one of the benzimidazole compounds, a substance that is effective against a number of biological activities due to its selective. O-Veratramide (2,3-dimethoxybenzamide), as powder his other chemical name 2,3-dimethoxybenzamide and has molecular formula $C9H11NO3$, molecular weight 181.191 g/mol, has melting point 75–85°C, his chemical is currently classified as antibacterial and a pesticide. It can be classified as a biochemist for its high toxicity, as shown in Figure 1.

Antibacterial activity is usually increased by increasing the number of amine groups in the solution as the NH_2 group
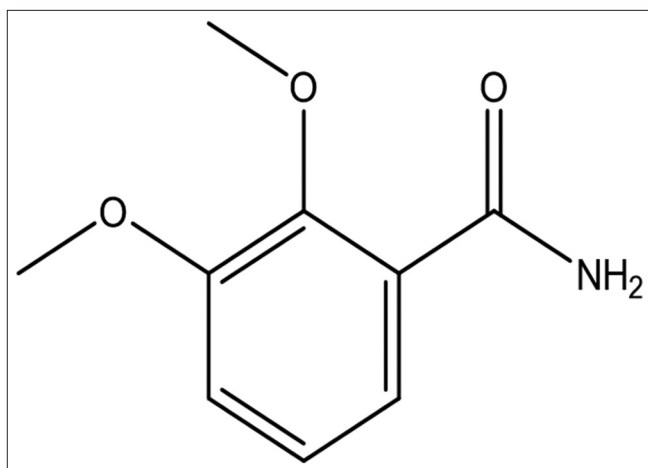
Figure 1: Chemical structure of O-veratramine

enters the chemical composition of O-veratramide. A number of researchers where they mentioned that the presence of amine groups are more effective in the elimination of both positive and negative bacteria.[7-10]

The NH_2 groups are particularly related to the bacterial surface molecules of the negative charge, which disrupt the bacterial membrane leading to its dissolution and thus cell death. On this basis, the amine groups have attracted high antibacterial efficacy as a new class of antibiotics.[11-13]

In 2011, the world Ozkay *et al*. chose a series of 14 compounds of benzimidazole compounds for the purpose of testing their susceptibility to a wide range of bacteria. The results showed a clear effect on the bacteria under examination, especially colon bacteria. This was confirmed by a number of tests of some benzimidazole derivatives used for various types of bacteria such as *Candida albicans*, *Staphylococcus aureus,* and Yeast *Saccharomyces cerevisiae* .[14-18]

In another study by researchers using 2,3-dimethoxybenzamide on a wide range of bacteria, it was observed that it had a inhibitory effect on the formation of whiteboards when added 3 h before the vegetative growth but had a less inhibitory effect after adding the end of the vegetative growth and there are some strains which showed a clear effect regardless of the time to add the compound.[19-23]

Zhang *et al*.[24] said that, for the purpose of obtaining the inhibitory effect against bacteria, the structural properties of benzimidazole should be improved by adding a series of alkyls of different lengths that would increase the efficiency of the compound against the bacteria by binding the gasoline group to another group containing nitrogen. An increase in the double electrons, which the source of nitrogen, is one of the important factors to reach the optimum value to reduce bacterial activity as it is known that the high density of electrons cause a significant loss of activity of bacteria as well as the presence of ammonia in the Benzimidazole structure raises the permeability of the cell membrane and thus the

cell is susceptible to degradation and the permeability of the intracellular components and their death.[25-30]

MATERIALS AND METHODS

All solvents and reagents were of analytical grade unless indicated otherwise, and all experiments were performed with deionized water (18.2 Ω-cm) resistivity at 25°C.[31]

Chemical and Biological Materials

- Hexane for high-performance liquid chromatography (HPLC) grade, BDH Chem. Ltd.
- Ethyl acetate, BDH Chem. Ltd.
- Ethanol and methanol, BDH Chem. Ltd.
- Chloroform for HPLC Grade Ltd.
- Water was obtained by following purification in a deionized water system.
  - Nutrient Agar, Bio-Pharm. Tech.
  - MacConkey Agar, Bio-Pharm. Tech.

Collection of Materials

Pomegranate husk materials were collected of ripe pomegranate fruits and washed from the soil with water. The crusts were then separated from the rest of the fruit. The crusts were placed on large filtration papers at room temperature. The crust was dried and grinded in a small electric mill. After that the dry powder was obtained, until they are used.[32,33]

Extraction

Preparation of pomegranate husk extracts

The method described globally was adopted by dissolving 400 g of pomegranate husk powder in 1 L of water. It was mixed well with an electric mixer and then placed in a trembled incubator at 25°C for 24 h to increase the extraction ratio. When the filtrate was collected and concentrated in the rotary vacuum evaporator at a temperature of 40°C, the concentrated extract was placed in dishes with a large surface area. The remaining water was dried in an electric oven at a temperature of 40°C until the water completely evaporated and obtained dry powder. The powder was placed in tightly sealed containers and marked with PVC and frozen under the 4°C until to use.[34-36]

Extraction Methods

The method of extracting all active compounds from pomegranate husks was completed in two main steps by the separatory funnel; the first step was completed by mix solvent ethanol:chloroform:water (40:40:10). A number of organic compounds were obtained from the extraction process

from the organic and the aquatic layer, three compounds which were obtained in the organic layer are 1-docosene, 2-piperidinone, N-[4-bromo-n-butyl]-, and eicosane and four compounds from the aquatic layer are tributyl acetyl citrate (TBAC), O-veratramide, methyl palmitate (MP), and butanamide, N-decyl-N-methyl-.

Phytochemical Screening of Pomegranate Husk Extract

The pH value of the pomegranate husk extract was determined by mixing 10 g of dried powder with 50 ml of distilled water, and then, the solution was filtered. The pH was measured by pH-meter. After that, some qualitative chemical tests were conducted to determine some of the aggregates and active ingredients.

Alkene (1-decosene) has been detected using nitrous reagent, and this reagent was similar to reagent 2-piperidinone, N-[4-bromo-n-butyl]-. 1-decosene was measured in the wavelength 260 nm with ultraviolet (UV)-vis spectrophotometer versus the blank. Alkane (eicosane) was measured using double-beam UV-vis with fixed wavelength 375 nm. The polyhydric compound (TBAC) that have multi hydroxid groups with the standard reagent in the wavelength 410 nm was measured using double-beam for UV-Vis against blank. The aromatic (hydroxy-carbamide O-veratamide) was measured at 230 nm. Fatty acid (MP) was measured in 310 nm. The aliphatic amide (butanamide, N-decyl-N-methyl-) was measured in the wavelength 210 nm with UV-vis spectrophotometer. All the maximum wavelengths that have been determined for all active compounds after scanning are measured by the same UV spectrum device - which contains a dual-beam package.

Isolation and Purification of O-Veratramide (2,3-Dimethoxymethybenzamide)

After isolating the two layers, organic and aquatic from each other, an isolation was done for the compound O-veratramide (2,3-dimethoxybenzamide) from the aquatic layer, being the compound to be studied biologically against different types of agricultural pests. A special method was used to isolate and purify the O-veratramide from the aquatic layer containing this compound using different solvents such as methanol, chloroform, ethyl acetate and hexane, each solvent used separately to precipitation, analyze and estimate the compound.

Chemical Detection Results

Chemical detection results indicated seven active compounds in the organic layer and aquatic layer extracted from pomegranate husks. Four extracts from the aquatic layer are TBAC, O-veratramide, MP, and butanamide, N-decyl-N-methyl and three organic extracts are 1-docosene, 2-piperidinone, N-[4-bromo-n-butyl]-, and ecosane. The

results obtained were confirmed by GC-Mas technique (GC-MS) and UV diagnostic results.

Determination of O-veratamide (2,3-Dimethoxybenzamide)

Using a standard solution of O-veratamide, direct measurements were made for plant extracts in volume (1.0 mL) for different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5) µg/ml. O-veratamide absorption was measured at a wavelength of 230 nm. The maximum wavelength is determined after scanning with the same UV device against deionized water as blank. The concentration of O-veratamide was calculated from R^2 values obtained from the linear regression equation obtained from Quercetin calibration. TPCs were determined using linear regression equation; the standard chart of O-vertamide was obtained. The concentration of O-vertamide extract was calculated after purification. The mean SD ($n = 3$) was expressed a mg/g from O-vertamide equivalent from dry extract. The chemical composition of O-vertamide is not currently accurately classified in our databases, nor has information about this compound been identified including synonyms, ID numbers, and we do not have complete information about this chemical. The molecular mass in the GC-Mass measurements does not specifically refer to the O-vertamide structure due to the interaction of this compound with fragments generated by cracking the same mass formed due to the very high temperature of the GC-Mass. Therefore, the parent mass is shown at 281.

Determination of 1-docosene

Nitrous reagent was used to determine the 1-docosene in the various organic crude extracts. 1-docosene standard was used as a reference standard (10–50 µg/mL) for plotting calibration curve R^2. A volume of 1.0 mL from the plant extract (50 µg/mL) was mixed with 1.0 mL of nitrous reagent (diluted 1:10 with deionized water) and was neutralized with 3 mL of HNO_3 acid (1 % w/v) + two drops from Br^2 solution. The reaction mixture was kept in the dark at room temperature for 1 h with intermittent shaking. The absorbance of the resulting nutty dark color was measured using double-beam UV-visible spectrophotometer at a fixed wavelength of 254 nm. The TPCs were determined using linear regression equation obtained from the standard plot of 1-docosene, and this compound was calculated in mean SD ($n = 3$) and expressed as mg/g 1-docosene equivalent of the dry extract.

A Similar Identification of Amide 2-Piperidinone, N-[4-Bromo-n-Butyl]-

By a standard solution of 2-bisperididone, N-[4-bromo-n-butyl], the plant extract was direct measured in volume 1.0 mL and different concentrations (2.0, 4.0, 6.0, 8.0, and 10.0 µg/ml). The 2-piperidinone compound, N-[4-bromo-n-butyl]- was estimated at a peak absorption of 260 nm with

UV spectrometer against the deionized water as blank. The amount of 2-piperidinone, N-[4-bromo-n-butyl] was calculated from the linear regression equation obtained from Quercetin calibrations. TPCs were determined using the linear regression equation obtained from the standard plot of 2-piperidinone, N- [4-bromo-n-butyl]. This compound was calculated as mean SD ($n = 3$) and was expressed in mg/g from 2-piperidinone, N- [4-bromo-n-butyl]- equivalent of the dry extract.

Determination of Eicosane

Nitrous reagent was used to determine the Eicosane various from organic crude extracts. A pure powder from eicosane was used as a reference standard at concentration range (10–50 μg/mL) for plotting calibration curve. A volume of 1.0 mL of the plant extract (50 μg/mL) was mixed with 1.0 mL of nitrous reagent (diluted 1:10 with deionized water and was neutralized with 3 mL of HNO_3 (1% w/v) without bromination. The reaction mixture was kept in the dark at room temperature for 1 h with intermittent shaking. The absorbance of the resulting light yellow color was measured using double-beam UV-Vis spectrophotometer that is fixed wavelength 375 nm. The TPCs were determined using linear regression equation obtained from the standard plot for eicosane. This compound was calculated as mean SD ($n = 3$) and expressed as mg/g eicosane equivalent of dry extract.

Determination of TBAC

TBAC in crude extracts was determined by the reported procedure. The plant extracts were used to find a standard calibration curve. Briefly, 10 mg of plant extracts were dissolved in 80% ethanol and then diluted to 10, 20, 30, 40, and 50 μg/mL. The diluted standard solutions of plant extracts in volume 1.0 mL for different concentrations were separately mixed with 1.0 ml of 99% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 mol/L potassium acetate, and 3.0 mL of deionized water in a test tube. The test tubes were incubated for 1 h at room temperature to complete the reaction. The reaction mixture was measured in the wavelength 410 nm with double-beam UV-visible against blank. A typical blank solution contained all reagents except ferric chloride which is replaced by the same amount of deionized water. The amount of TBAC was calculated from linear regression equation obtained from the extract plant calibration. The TPCs were determined using linear regression equation obtained from the standard plot of TBAC. This compound was calculated as mean SD ($n = 3$) and expressed as mg/g for TBAC equivalent of dry extract.

Estimation of MP

Using the standard solution of MP, the direct measurement of the plant extract (1.0 ml) was measured for different concentrations (5, 10, 15, 20, and 25 μg/mL). The absorbance

of the MP compound was measured at the maximum wavelength 310 nm determined after scanning in the same apparatus that has double-beam versus the deionized water as blank. The amount of MP was calculated from the linear regression equation obtained from the calibration curve. TPCs were determined using the linear equation obtained from the standard scheme of MP. SD was calculated for MP ($n = 3$) and expressed as mg/g for the dry extract.

Determination of Butanamide, N-Decyl-N-Methyl-

Using the standard solution of butanamide, N-decyl-N-methyl, direct measurement of plant extracts (1.0 ml) at a different concentration (1, 2, 3, 4, and 5 μg/ml) of 0.1 ml sample. The uptake of butane-N-decyl-N-methyl-compound was measured at the maximum wavelength of 210 nm specified after scanning in the same device containing a double pack versus deionized water as empty. The amount of butanamide, N-decyl-N-methyl was calculated from the linear regression equation obtained from the calibration curve. TPCs were determined using the linear equation obtained from the standard plan of butanamide, N-decyl-N-methyl. SD was calculated for butanamide, N-decyl-N-methyl ($n = 3$) and expressed as mg/g for the dry extract.

GC-Mass Analysis

In general, studies indicate that all extracting compounds can be analyzed more precisely by applying GC-MS (mass chromatography, MSDCHEM \ 1 \ METHODS \ MUAFAQ.M) to determine negative M/Z. Fragments allow easy identification of composite parts by mass spectrum. To shorten the total time for GC analysis of these compounds, the cavity column should be long and narrow as 10 m × 0.250 I.m, I.D, 0.25 μm, SS., Inlet He and recommended optimal conditions for separation and diagnosis of all extraction compounds as listed in Table 1.

Chemical Composition of Extracts by GC-MS Analysis

The compounds were studied through GC-mass [Table 1 and Figures 2-4] to create a molecular ion for each extract. It has been found that its molecular ion equals the weight of the formula of the minus one or more. The graphs confirm the weight of all compound molecules that give a good indication of the isolation and identification of all chemical compounds extracted separately.

Data Analysis

Data analysis is expressed as mean ± SD. (t) was tested for ANOVA and was used to analyze the level of statistical significance between groups. $P < 0.05$ was considered to be statistically significant.

Table 1: Data analysis parameters for separation and specific determination of pomegranate husks extract in GC-Mass spectrum

Column	HP-5MS, 5% phenyl methyl Sillox (1629.5)
	30 m×0.250 µm I.D. × 0.25 µm, SS., Inlet He
EMV mode	Gain factor (1.00)
Resulting EM voltage	1306
Low mass	28
High mass	441
Threshold	150
Minimum quality for all narcotics	(90–97%)
Flow rate	1 ml/min
Run time	24 min
Hold up time	1.5388 min
Solvent delay	3.00 min
Average velocity	36.796 cm/s
Temperature	Initial 70°C to maximum 375°C
Pressure	8.81 Psi

GC: Gas chromatography
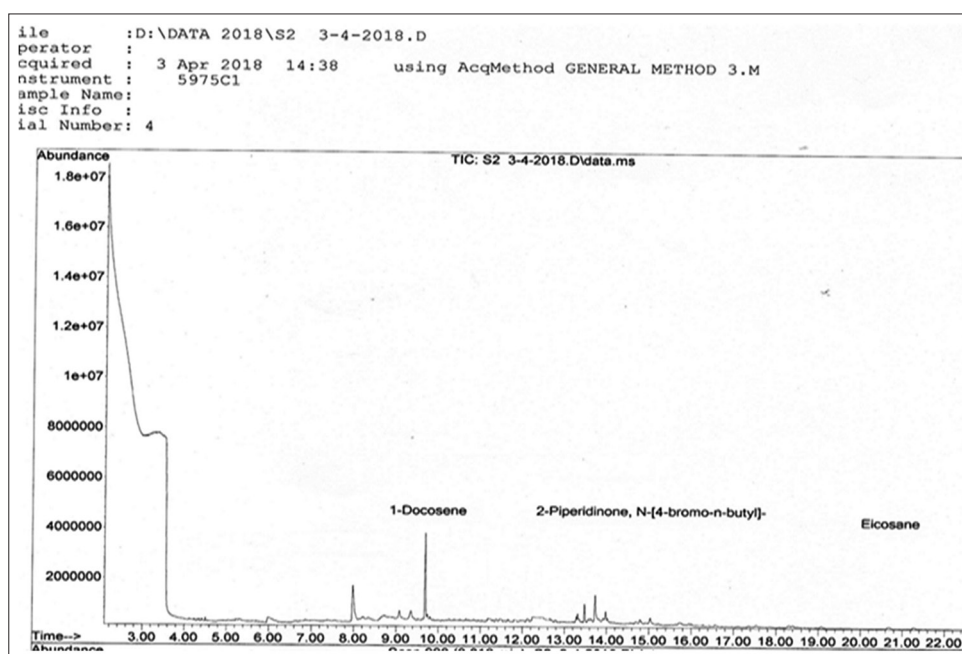
Figure 2a: Gas chromatography-mass spectrum for three compounds in organic layer

Miscellaneous Extraction Compounds

A number of biologically active compounds were obtained from the organic and aquatic layers in the pomegranate extract as shown in Table 2 and Figure 2. The process was performed using different solvents. Active extract O-veratramide was obtained with high bioefficacy against some types of bacteria.[37,38]

Percentage obtained from extracts

Approximately 20 g (5%) dry mass of all extract components was obtained from extracting 400 g of pomegranate husks after 16 h of continuous hot extraction in Soxhlet extract using ethanol solvent. The Copshan division method was used for raw alcohol extracts. Table 3 shows the different extraction ratios when using different solvents such as hexane, chloroform, ethyl acetate, and methanol.

O-vertamide Crude

The weight ratio of O-vertamide is calculated using the following linear regression equation obtained from the standard O-vertamide.
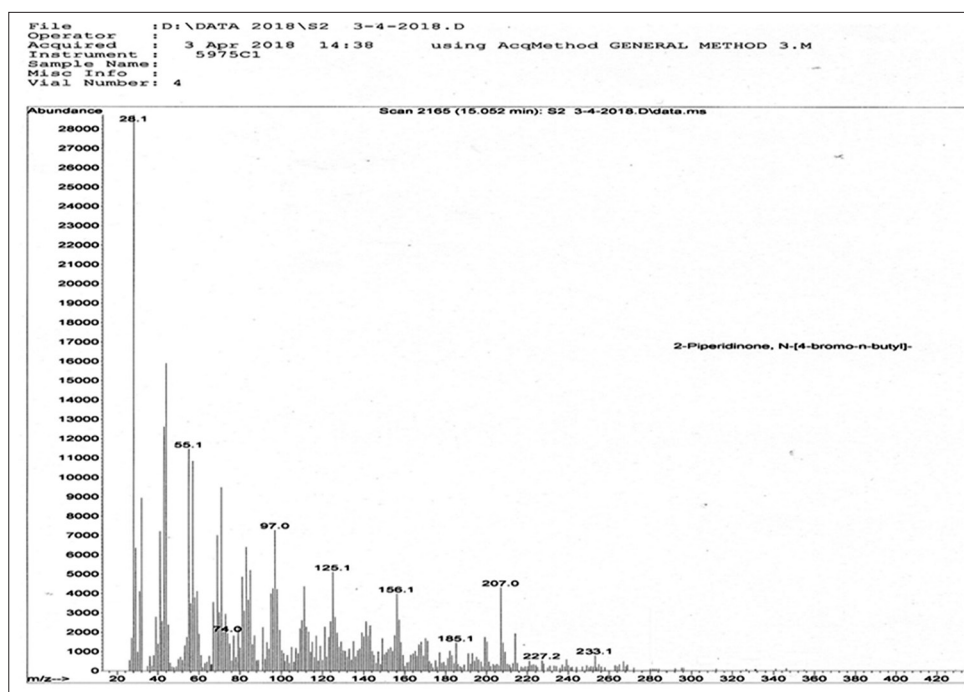
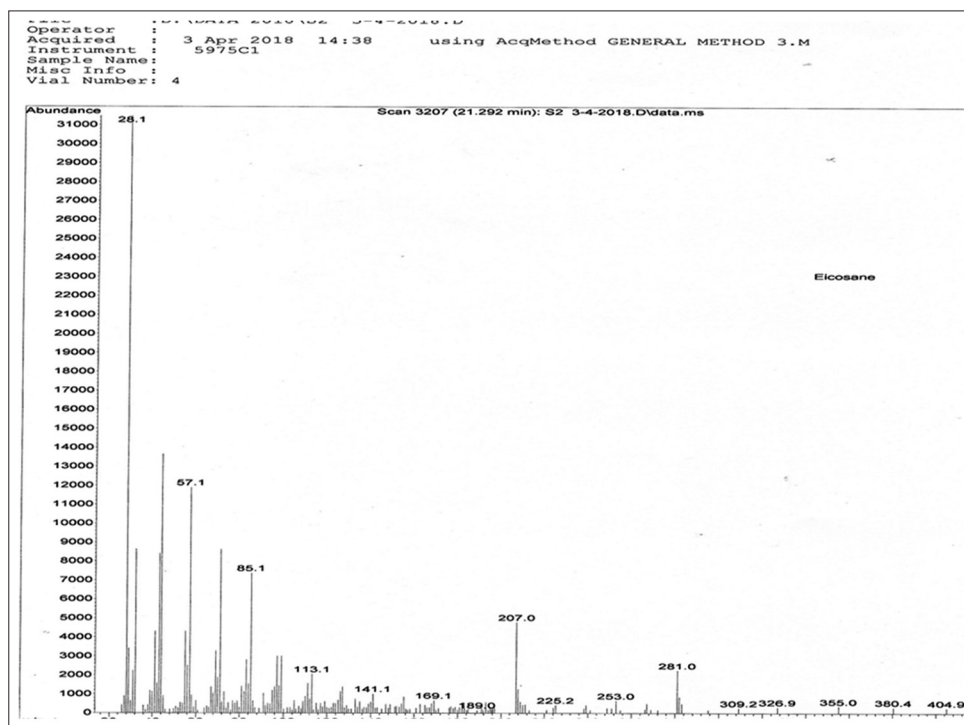**Figure 2b:** mass spectrum for 2-piperidinone, N-[4-bromo-n-butyl]

Figure 2c: mass spectrum for eicosane

Table 2: Miscellaneous extraction compounds in organic and aquatic layers							
Phase	1-Docosene	2-piperidinone, N-[4-bromo-n-butyl]-	Eicosane	TBAC	O-Vertamide	MP	Butanamide, N-decyl-N-methyl-
Organic layer	+	+	+	-	-	-	-
Aquatic layer	-	-	-	+	+	+	+

TBAC: Tributyl acetyl citrate, MP: Methyl palmitate
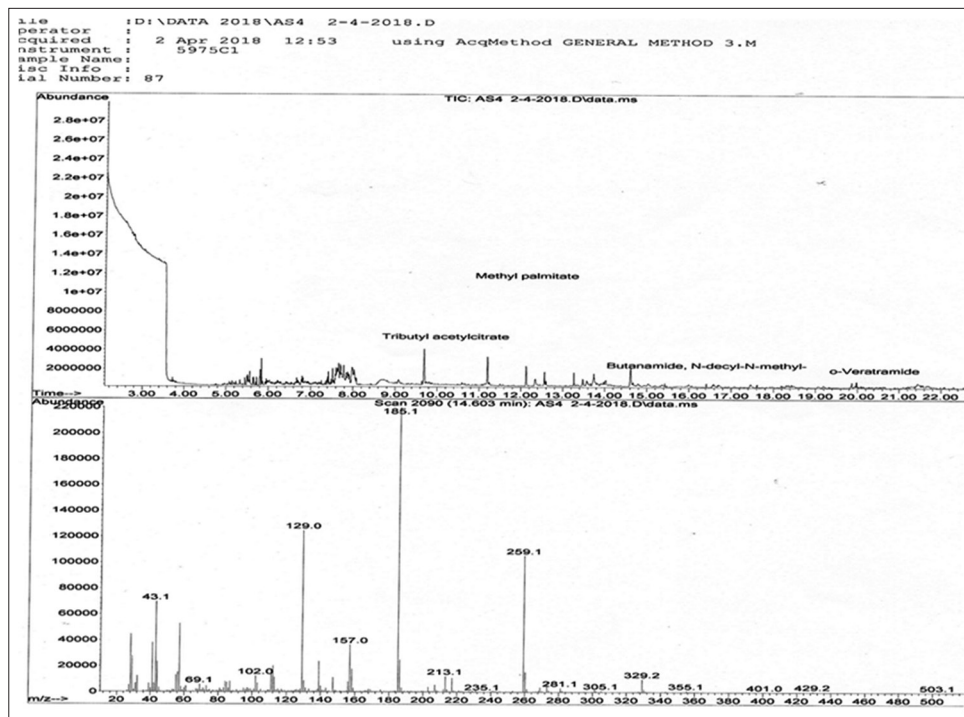
Figure 3: GC-mass spectrum for four compound in aqua layer,
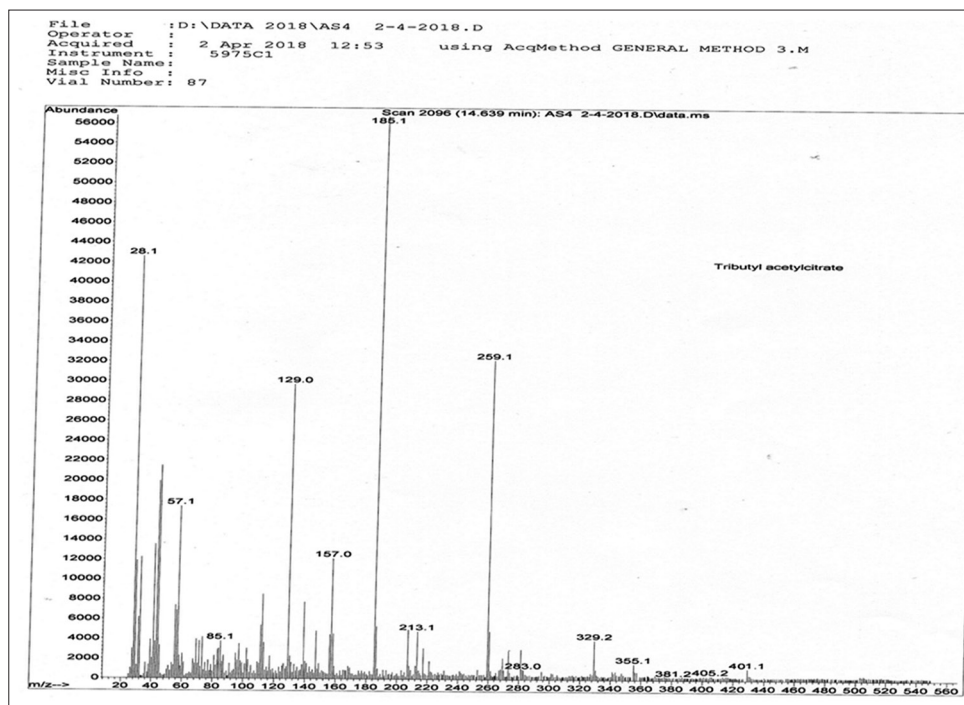
Figure 3a: mass spectrum for tri-butyl acetyl citrate

$$y = 0.012x+0.030, R^2 = 0.9988$$

Where y is absorbance and x is the amount of O-vertamide that calculated by the microgram unit.

The crude extract of O-vertamide in methanol, chloroform, ethyl acetate, and hexane was 89 ± 0.5, 71.18 ± 1.65, 95.12 ± 0.30, and 80.28 ± 0.13, respectively, and the statistical

significance was calculated for all values of the obtained results ($P = 0.001$).

Ethyl acetate extract was found to contain the highest amount of O-vertamide compound followed by methanol, hexane, and chloroform. However, little quantity O-vertamide compounds were detected in chloroform extract. A significant difference
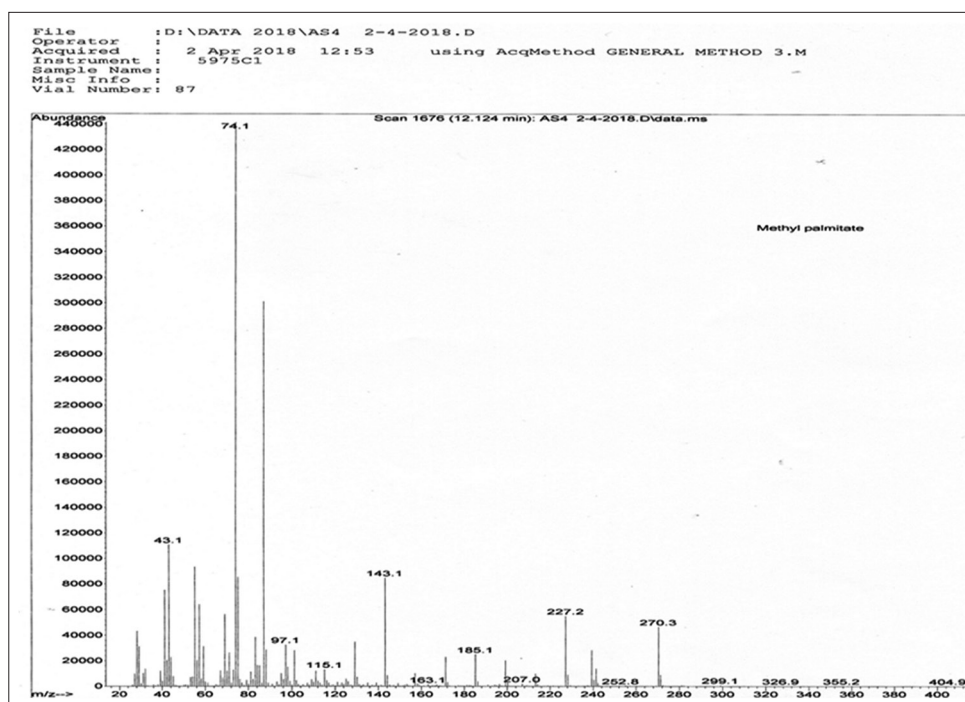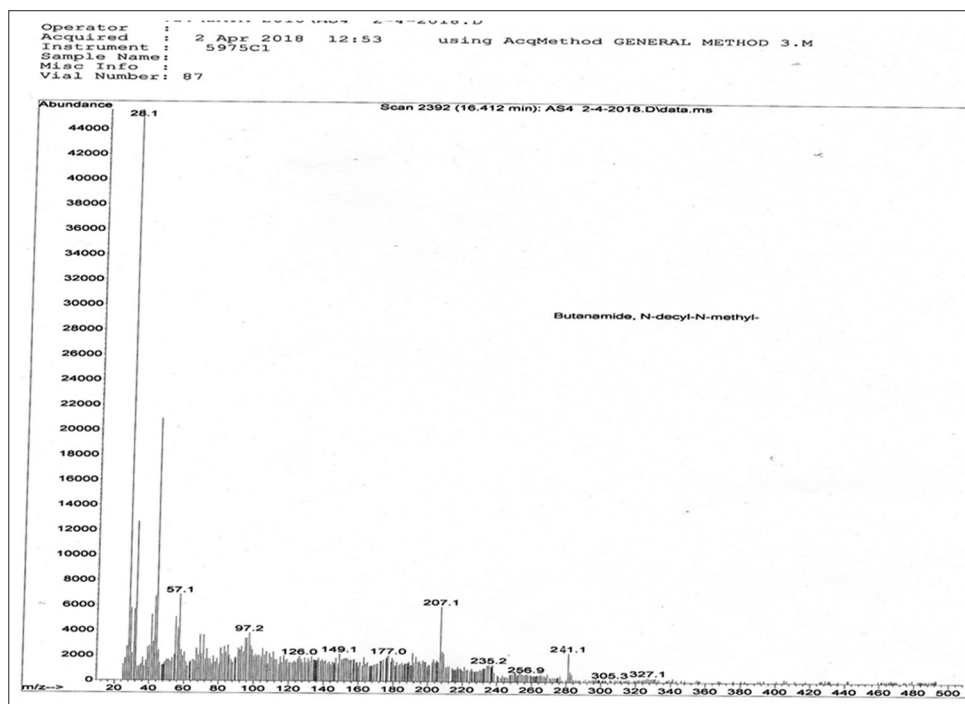
Figure 3b: mass spectrum for methyl palmitate

Figure 3c: mass spectrum for butanamide, N-decyl-N-methyl

Table 3: Amount and % yield of organic and aqua extracts from pomegranate husks

Extract	Amount (g)	Yield (w/w) %
Methanol	7.8	39
Chloroform	1.1	5.5
Ethyl acetate	7.1	35.5
Hexane	4	20

in the aquatic layer contents for different extractions was observed by the ANOVA parameter test.

Determination of Antimicrobial Activity

The susceptibility of the compound (2,3-dimethoxybenzamide) was tested on four types of common bacteria associated with food *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia*
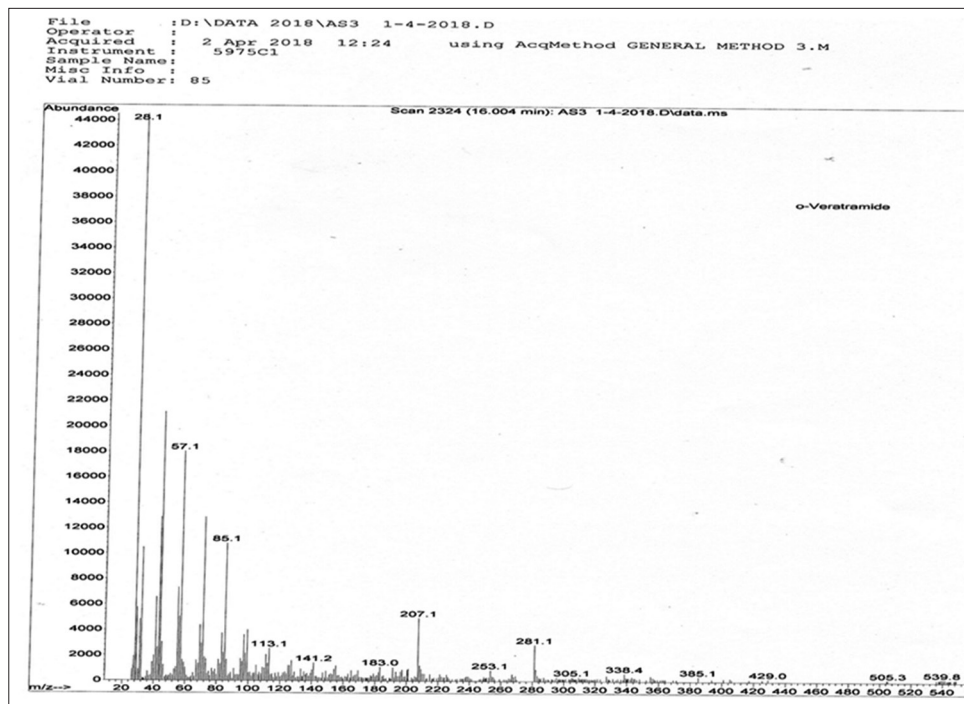
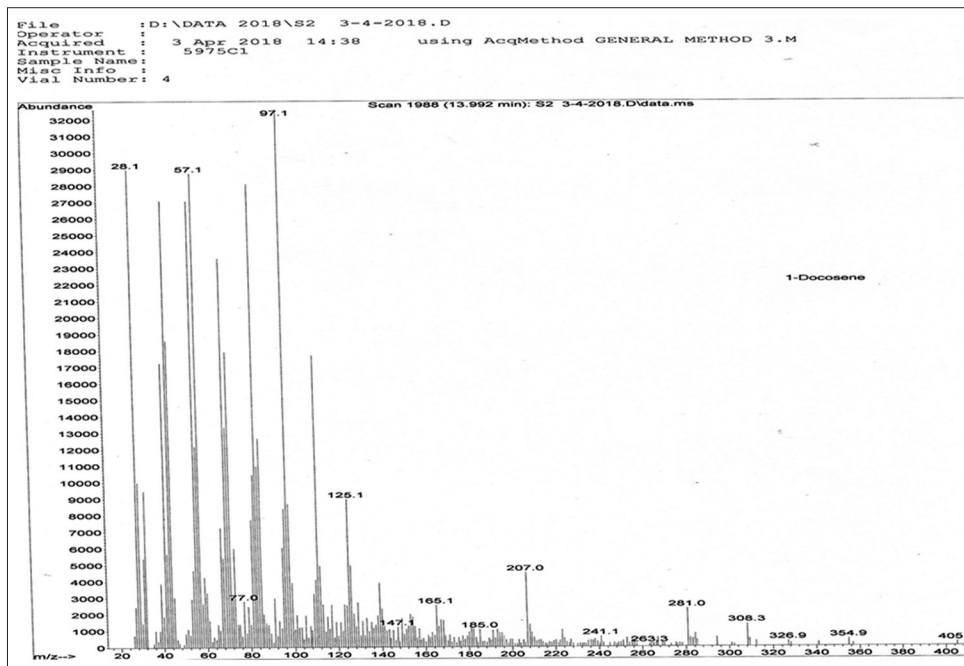**Figure 3d:** mass spectrum for O-veratramide (2,3-dimethoxymethybenzamide)

Figure 4: (a) Mass spectrum for 1-docosene

coli, and *Klebsiella pneumonia.* Four types of bacterial species are used for these studies; the ATCC number of species is as follows: *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), *K. pneumonia* (ATCC 13882), and *E. coli* (ATCC25922). The activated bacteria were suspended according to the method described by Jianu *et al*., (106 cells/ml) and using the method of dissemination (10 µg/disk), the incubation of the dishes was carried out for 24 h at a temperature of 10 µg/37°C, after incubation, the diameter of the inhibition area was measured in millimeters of bacterial strains, and the test was performed on three replicates.[39,40]
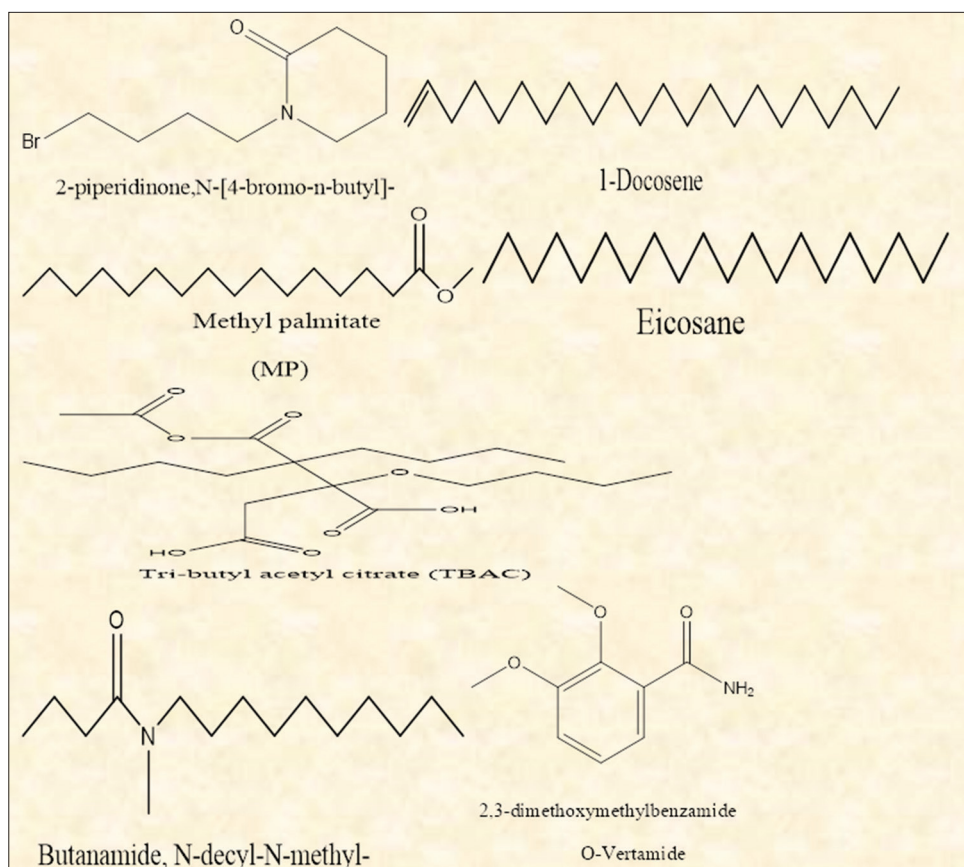
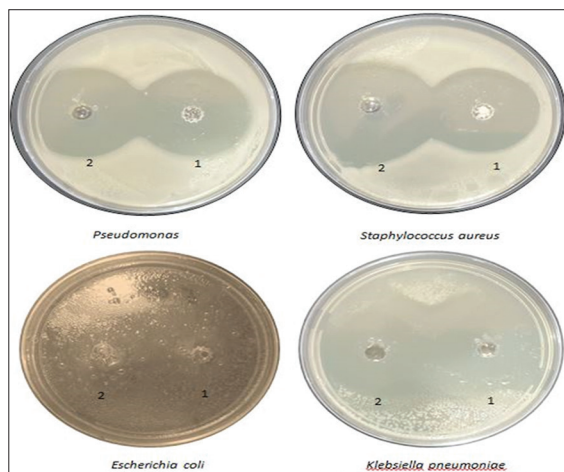**Figure 4:** (b) compounds obtained from the organic and aquatic layers

Figure 5: The results of the antibacterial examination (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia,* and *Escherichia coli*)

RESULTS AND DISCUSSION

Figure 5 shows the results of the antibiotic test (*S. aureus*, *P. aeruginosa*, *K. pneumonia,* and *E. coli*) using concentrations of 2,3-dimethoxybenzamide (10 µl and l5 µl) referred to 1 and 2, respectively, and it is noted that the highest inhibition of the compound was on the *K. pneumonia* bacteria to form a large area of the dish followed by *S. aureus*, which the area of inhibition of the sample (concentration 1) by 27mm, while the diameter of inhibition of the sample (concentration 2) of the same bacteria diameter of 34mm higher, the amount of inhibition. For Pseudomonas, it was observed that they were close to the concentration (1 and 2) by 28mm and 30mm respectively, while the effect of inhibition of the compound for both concentrations on *E.coli* with concentration (1 and 2) was 12mm and 17mm respectively compared with other types of bacteria under study. Other bacteria were studied with an inhibition of concentrations (1 and 2) which was 12 mm and 17 mm, respectively.[3,41-44]

CONCLUSIONS

In this study, seven active compounds were extracted and isolated in pomegranate extract. Their chemical composition was determined by GC-MS, one of the compound as O-veratramide used against different types of Gram-positive and Gram-negative bacteria. The presence of biologically active compounds in pomegranate husks demonstrated the importance of pharmacy. However, more studies will be required to find its biological activity and biological character.

ACKNOWLEDGMENTS

CONFLICTS OF INTEREST

There are no conflicts of interest for anyone.

DEDICATION

Give my work humility to the faculty of pharmacy and professors specialized in the fields of chemistry and biology.

FUNDING OF RESEARCH

AUTHOR'S CONTRIBUTIONS

This research was done individually in the laboratories of the College of Agriculture, University of Basrah. This research was completed over a period of 3 months with serious and continuous work, and therefore, excellent results were obtained in finding an easy and sensitive way to estimate the O-vertamide compound in extract from pomegranate husks and study of its biological effect.

Ethics: We undertake to address any ethical or health issues that may arise after the publication of this manuscript.

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