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Punica granatum Peel as Antifungal and Antibacterial Source

A. S. Abdel-Aty*, S. M. Ahmed, M. A. Desheesh and A. M. Hussain

Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt

* Corresponding author E-mail: sabry2000@yahoo.com, ahmed.abdelatty@alexu.edu.eg

Abstract

The fruit peel of *Punica granatum* was collected, extracted and evaluated for its antibactericidal activity on *Agrobacterium tumafaciens, Erwinia amylovora and Pseudomonas solanscearum* and its fungicidal effects on *Rhizoctonia solani*, *Phytohthora. infestans*, *Alternaria alternate* and *Fusarium oxysporum*. Its combination with a standard bactericide (streptomycin sulfate) and a standard fungicide was also studied. The GC-Mass spectroscopy (GC-MS) identification of the bioactive constituents was also carried out. The obtained *P. granatum* extract was less effective against the three tested bacteria than the standard bactericide although their combination completely prevented *A. tumefaciens*, *E. amylovora* and *P. saloranacearum* growth. Generally, the fungicidal effects differed as a function of the treated fungus owing to different sensitivity of the fungus to the extracted bioactive components. *P. infestans* was the most sensitive, followed by *F. oxysporum* to this combination. *A. alternata* was the least sensitive fugus in this respect.

P. granatum crude extract weakly stimulated the PPO activity of *P. infestans* and *A. alternate*. Twenty compounds were identified in the extract of *P. granatum* peel sample. including 1,2,3-Trihydroxy benzene (6.20%), Epicatechin-gallate (1.02%), Catechin (1.00%), Methyl-dodecanoate (2.90%), Methyl-tetradecanoate (2.70%), Methyl-hexadecanoate (35.4%), n-Hexadecanoic acid (6.8%), 9-Octadecenoic acid methyl ester (18.40%), Methyl-octadecanoate (5.90%), 6-Tridecyl-tetrahydro-2H-pyran-2-one (3.70%), Methyl-linoleiate (1.68%), Octadecanoic acid (1.9%), Eicosane (1.87%), Heptacosane (0.98%), Unknown (1.25%), Unknown (1.28%), 1,3,5-Trimethyl-2-octadecyl-cyclohexane (0.58%), 9-Octadecenamide (0.76%), Unknown (0.7%) and A glucoside derivative (2.50%).

Keywords: *P. granatum, A. tumafaciens, E. amylovora and P. solanscearum R. solani, P. infestans, A. alternate, F. oxysporum*, GC-MS

1. Introduction

Fungi and bacteria are among the most important plant pathogens causing with more than 20% crop losses [1]. Synthetic pesticides are powerful to eliminate these pathogens but with dangerous impact to environment and humans. Thousands of ecofriendly biologically active constituents are contained in plants and so their identification has been gaining immense attention. These active components in plants (phytochemicals) are helpful against pathogenic microorganisms in replacement of antibiotics [2].

Since ancient times, people of numerous civilizations knew pomegranate as edible fruits in disease therapy as cardiovascular health, cancer prevention, anti-inflammatory, antimicrobial activities, anti-parasitic and others [3]. Pomegranate peel inhibits a wide range of pathogens, including viruses, bacteria, fungi, and mold [4]. The ethanolic extracts of pomegranate seed and peel inhibited *P. aeruginosa* and *S. aureus*, the peel extract had a stronger antibacterial effect than the seed extract [5]. The peel extract of pomegranate (*Punica* grenatum) has activity against white garden snail, *Thiba pisana* [6]. The antibacterial potential of the cold, hot aqueous extracts and ethanolic extracts of the peels of *Punica granatum* against *Staphylococcus aureus* (Gram positive bacteria), *Escherichia coli* and *Psendomonas aeruginesa* (Gram negative bacteria) was determined [7] and they reported that all of the tested extracts showed a strong activity against the three microorganisms. While [8] reported that the ethanolic extracts had antifungal activities *in vitro* against *Alternaria alternata, Fusarium oxysporum, Phoma destructiva, Rhizoctonia solani*, and *Sclerotium rolfsii* [9] found that aqueous extract of *Punica granatum* recorded significant antifungal property against eight important species of *Aspergillus* such as *A. candidus, A. columnaris, A. flavipes, A. flavus, A. fumigatus, A. niger, A. ochraceus,* and *A. tamarii.* [10] appeared that the plant extracts from *Punica grantum* caused strong *in vitro* antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* bacteria. The 80% aqueous methanolic extract of peels (WME) was a potent inhibitor for Food-borne diseases such as listeriosis and diseases caused by the emergence of multi-drug resistant pathogens (e.g. *Staphylococcus aureus*) *Listeria monocytogenes, S. aureus, Escherichia coli* and *Yersinia enterocolitica* [11].

*Corresponding author e-mail: ahmed.abdelatty@alexu.edu.eg.; sabry2000@yahoo.com.
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So, the fruit peel of *Punica granatum* was collected, extracted and evaluated for its anti-bactericidal activity on *Agrobacterium tumafaciens, Erwinia amylovora and Pseudomonas solanscearum* and its fungicidal effects on *R. solani*, *P. infestans*, *A. alternata* and *F. oxysporum* fungi. Its combination effect with a standard bactericide (streptomycin sulfate) and a standard fungicide (azoxystrobin) was studied. GC-Mass spectroscopy (GC-MS) identification of the bioactive constituents was carried out.

2. Materials and Methods

2.1. Collection and extraction of the tested plant sample

The *Punica granatum* (pomegranate) (Punicaceae) were purchased from the local market, transferred to the laboratory, cleaned from dust and the fruit peels were separated, puleverized (100 gm) and soaked in 300 ml of 70% aqueous acetone twice for a week in each time at room temperature in the dark and filtered. The obtained filtrates were combined and the organic solvent was completely evaporated under vacuum at less than 50°C and the concentration of the extracted materials was determined.

2.2. Antibacterial effect measurement

2.2.1. Bacterial strains and the standard bactericide

Three phytopathogenic bacteria; *A. tumerfaciens*, *E. carotovra* and *P. solanacearum* were provided by the Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. Streptomycin sulfate (El-Nile Company for Chemical Industry and Drugs, Egypt) was used as a standard bactericide.

2.2.2. Determination of MICs on the treated bacteria

The minimum inhibitory concentrations (MICs) were determined [12]. After solidification of bacterial culture grown in a nutrient broth for 18 hours (approximately10^8 CFU/ml was planted in three replicates from the treated bacteria on the agar surface at 27 ºC for 24h. Control and streptomycin sulfate were concurrently carried out for comparison.

2.2.3. Effect of plant extract combination with the standard bactericide

Eight combinations of the obtained crude plant extract of *P. granatum* peels and the standard bactericide (streptomycin sulfate) were evaluated against the tested bacteria. The used plant extract MIC and streptomycin sulfate MIC were used at these eight combinations: 1.0 : 1.0; 0.5 :1.0; 1.0 : 0.5; 0.5 : 0.5; 0.5 : 0.25; 0.25 : 0.25; 0.5 :1.0 and 0.25 :1.0. All combinations were evaluated against all of the tested bacteria and the growth inhibition was also recorded.

2.3. Fungicidal activty measurements

2.3.1. Tested fungi

F. oxysporum, *R. solani*, *A. alternata* and *P. infestans* were obtained from the Fungicide Bioassay Laboratory, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Egypt.

2.3.2. Fungicidal activity measurement

Antifungal activity of the obtained *P. granatum* plant crude extract was tested using the radial growth technique [13] at 50, 100, 250, 500, 1000, and 2000 µg ml^{-1} in triplicate. Control was concurrently conducted. Inhibition percentage of mycelial growth was calculated [14]. EC_{50} was determined [15]. Azoxystrobin (Amistar 250 SC, Syngenta) was used for comparison as a standard fungicide.

2.3.3. Effect of combination between plant extracts and azoxystrobin

Six combinations of the obtained *P. granatum* plant crude extract with the standard fungicide (azoxystrobin) were evaluated. These combinations were: 1.0 EC_{50} with 0.5 EC_{50} ; 0.5 EC_{50} with 0.5 EC_{50}; 0.25 EC_{50} with 0.5 EC_{50}; 1.0 EC_{50} with 0.25 EC_{50}; 0.5 EC_{50} with 0.25 EC_{50} and 0.25 EC_{50} with 0.25 EC_{50} of the extract and the standard fungicide, respectively. All combinations were evaluated and the growth inhibition percent (I %) was recorded.

2.4. GC-MS identification of the most active crude extract

GC–MS analysis was done in the High Institute of Health, Alexandria University, Egypt on a Trace GC Ultra/Mass Spectro-photometer ISQ (Thermo Scientific) using A ZB-5MS Zebron capillary column (30 m, 0.25 mm, 0.25 µm; Agilent). Helium served as a mobile phase with 39.0 cm/sec, 1.0 ml/min flow rate at 160.0 kPai. Direct injection in acetone (1µl of 0.5 mg/ml), splitless, inlet temperature 250^O C. Column Oven Temp.: 70.0^O C. The oven temperature program: 1 min at 70^O C, temperature shifted up to 250^O C with 15^O C /min. The temperature was held for 2, 2, 2 and 5 min. at 70, 90, 150 and 200^O C, respectively, followed by holding 10 min. at 250^O C, EI at 70 ev. Mass conditions continued to 32 min, m/z range of 50.00-600.00. Peak area percent was used for obtaining quantitative data with the Excalibur 2.0 software (Thermo Technologies) without correction. The compounds in each sample were identified by comparison of their mass spectral pattern and their linear retention indices (RIs) based on a homologous series of alkanes (C_8-C_{24}) with those of authentic references and the MS libraries (NIST and Wiley) database under identical GC-MS conditions.

2.5. Statistical analysis

The experiment was conducted using a Completely Randomized Design (CRD). All statistical analysis were carried out using SPSS, 20 Software was used for data analysis. Inhibition percents were calculated [16] was considered statistically significant.

3. Results and Discussion

3.1. Bactericidal activity

3.1.1. Bactericidal activity of the original tested plant extract.

As recorded in Table (**1**), streptomycin sulfate as a standard bactericide caused the most inhibitory effects against *A. tumefaciens, E. amylovora* and *P. solaracerum* with minimum inhibitory concentrations (MIC's) values equaled 1, 1 and 32 µg ml^{-1}, respectively. The *P. granatum* crude extract was less effective against the three treated bacteria with MIC values equaled 500 µg ml^{-1}.

3.1.2. Combination effect of the extract with streptomycin sulfate.

It was indicated that *P. granatum* (fruits peels) extracts mixed with streptomycin sulfate completely prevented *A. tumefaciens, E. amylovora* and *P. saloranacearum* growth at all the tested crude extracts and streptomycin sulfate rates. The tested plant extract when mixed with the used standard bactericide (streptomycin sulfate) approved to be more toxic than when used alone (Table **2**). So enhancement effect has been obtained by using the plant extract combined with the used standard bactericide.

3.2. Fungicidal activity

3.2.1. Fungicidal activity of the original tested plant crude extract

As shown in Tables (**3a** and **3b**), the hyphal growth of the treated soil born fungi (*R. solani* and *F. oxysporum*) were inhibited. Against *R. solani*, the *P. granatum* fruit peel extract appeared less effective with 123.0 µg/ml EC_{50} than the used standard fungicide, azoxystrobin with 12.3 µg/ml EC_{50} value. On *F. oxysporum***,** the tested extract appeared less active than azoxystrobin with no significant diference between the two tested soil born fungi.

Regarding the hyphal growth of the treated air born fungi (*A. alternata* and *P. infestans*), the tested plant extract of *P. granatum* fruit-peel inhibited them with effective concentration of 50% of the hyphal growth (EC_{50}) equaled 59.3 and 78.6 µgml^{-1} with no significance between the two treated air born fungi, whenever the standard fungicide, azoxystrobin caused its inhibition with EC_{50} value equaled 13.7 µg ml^{-1} and 7.1 µg ml^{-1} EC_{50} values on *A. alternata* and *P. infestans* fungi, respectively. Generally, the fungicidal effects differed as a function of the treated fungus owing to different sensitivity of the fungus to the extract bioactive components.

3.3. Effect of plant extracts combination with azoxystrobin

As recorded in Table (**4**), mixing *P. granatum* extract (1.0 EC_{50}) with azoxystrobin (0.5 EC_{50}) completely inhibited *F. oxysporum* hyphal growth. So against *F. oxysporum*, mixing azoxystrobin at 0.5 or 0.25 EC_{50} increased the inhibitory effect with increasing the tested extract concentration.On *R. solani*, *P. granatum* was less effective. Azoxystrobin at 0.25 EC_{50} increased the inhibition effect with increasing the extract concentration. Azoxystrobin at 0.5 EC_{50} was more active than at 0.25 EC_{50} in all cases.

On *P. infestans*, All extract mixtures with 0.5 EC_{50} azoxystrobin achieved 100% inhibition of *P. infostans* hyphal growth. At 0.25 EC_{50} of azoxystrobin, the inhibition effect was increased with increasing the extract concentration. So, *P. infestans* hyphal growth proved to be highly sensitive to be inhibited by the tested mixtures of plant crude extracts with the standard fungicide, azoxystrobin. Among the treated fungi, *A. alternata* was the most resistant to all mixtures of as they exhibited very low inhibition percents in comparison to the other treated fungi.

From the obtained results, *P. infestans* was highly sensitive to the tested mixture between the crude plant extract and azoxystrobin. *A. alternata* was less sensitive towards the tested mixtures. It was clear that *P. infestans* was the most sensitive, followed by *F. oxysporum* to this combination with 49.4% - 76.5% inhibition range. *A. alternata* occupied the last place in sensitivity to this combination. So, it could be said that using the mixtures of plant extracts increased the fungicidal activity of azoxystrobin against the treaterd fungi in agreement with [17].

3.4. Effect on the fungal polyphenol oxidase (PPO) enzyme

From the ontrol activity values in Table (**5**), the effect on polyphenol oxidase (PPO) enzyme in *P. infestans* exhibited that the liquid fraction of the tested *P. granatum* crude extract weakly stimulated the PPO activity of *P. infestans* with 27.8, 14.8, 12.3 and 0.7% stimulation in descending order with increasing the tested concentrations (50, 100, 200 and 300 µgml^{-1}), respectively. On the other hand, its solid fraction weakly inhibited PPO enzyme of *P. infestans* with 0.8, 12.7, 19.0 and 20.4% inhibition in descending order with increasing the tested concentrations. However, against the polyphenol oxidase in *A. alternate* as listed in Table (**5**), Both liquid and solid fractions of *P. granatum* crude extract caused relatively the same stimulation effect against the enzyme in *A. alternata* with range 29.1 - 5.5% stimulation at the tested concentration except the highest one 300 µgml^{-1} of solid fraction caused weak inhibition of the tested enzyme with 11.5% inhibition.

3.5. GC-MS identification of P. granatum crude extracts

Due to the effect of *P. granatum* crude extract, which exhibited complete growth prevension of all the treated bacteria at all combination ratios, it was identified for its constituents. As through the concentration of its crude extract, some solid

materials were separated (filtered off) and known as solid fraction and the remained part was called as liquid fraction. Both two fractions were individually identified for their components using GC-MS.

3.5.1. Identification the P. granatum liquid fraction

The elution curve of the *P. granatum* liquid fraction is shown in Figure (**1**) and Table (**6**), It is well known that beside the fatty acid content, the pomegranate peel contains hydrohyzable tannins and flavonoids [18].

At 6.07 min., a compound with 127 molecular weight was confirmed as 1,2,3-trihydroxybenzene that may be produced from gallotannins (gallic acid connected to a glucose) or a flavanol moiety based on[5] as they isolated gallocatechin from the pomegranate peel at 6.08 min retention time. Worth mentoining that the gallocatechin is easily hydrohyzable to catechin and gallic acid that loses c=o fragment to 1,2,3-trimethylbenzene, which can be fragmented through loss of a hydroxyl (OH) group and the ring fission pathways. Alao [5] emphasized our obtained data.

At 8.27 min., a flavonol derivative was eluted and identified as 3- *o*-galloyl-epicatechin in agreement with [5]. The obtained mass spectrum of this compound showed fragments at m/z 152,139,124,109, which indicated the hetero aromatic ring fission (retro diels alder (RDA)) fishion and the separation of the two aromatic rings (ring A and B). Fragments at m/z = 77, 54 are owed to the aromatic ring fission emphasizing the structure to be 3-*o*-galloylepicatechin compound.

At a retention time of 9.59 min. (1.87%), a compound was eluted. Through its mass spectrum, it was noticed that it has a molecular weight more than 280, and it doesn't show a 149 m/z signal (absence of a glucose moiety). It has a peak at m/z 290 (1%), which may tend to be a flavonol compound. The 290 m/z molecular ion either loses H_2O molecule to 272 m/z fragment or goes through the retro diels alder fission of the hetero aromatic ring to m/z 139 and 124 fragments that can undergo to be fragmented to m/z 109 and 77 that gives 52 m/z fragment from the produced phenyl ion. The hetero aromatic ring fission leads also to produce m/z 136, 163 and 138 m/z fragments. Based on our obtained fragmentation data and in agreement with [19], this compound could be identified as catechin. Also [5] proved its presence in the *P. granatum* peel at a difference in retention time between it and epicatechin gallate similar to our obtained data.

At 11.50 min., a methyltetradecanoate was identified at a molecular weight of 242, while at 15.85 min., the methylhexadecanoate compound was identified at a molecular weight of 270, respectively. At 16.47 min. the n-hexadecanoic acid was eluted and its mass spectrum ensured its structure. In the fact this n-hexadecanoic acid may be referred to the hydrolysis of ascorbic acid –2,6-dihexadecanoate compound that has been reported in pomegranate peel [5].

The 10-octadecenoic methyl ester (Mw = 296 and methyloctadecanoate (Mw=298) were identical in the liquid and the solid fractions at 17.51 and 17.69 min. comparing with 17.63 and 17.83 min, respectively (0.1 min. delay). This delay is owed to the different sample solubility or injection process. This difference is too small to be minded and it should be neglected. The presence of octadecanoate esters (methyl or ethyloctadecanoate) in our analysis of the abtained data agreed with [5] as they proved them in the pomegranate (*P. grenatum*) peel contents.

The pyran-2-one derivative was eluted at 18.11 min. retention time with 282 molecular weight and identified as in the solid fraction as 6-tridecyl-tetrahydro-2H-pyran-2-one.

A methylated unsaturated fatty acid with two double bonds (dienoic acid) with 294 molecular weight, from which it could be deduced that the free fatty acid structure is $C_{18}H_{32}O_2$ by subtracting the methyl group. The mass spectrum of this compound revealed through its fragmentation pathways the position of these two double bonds. The obtained predicted data from the instrument (GC-MS) don't give the proper positions of the two unsaturation positions and so the propability did not exceed 16.8%. Linoleic acid was identified to be a *P. granatum* peel constituent ([5]. Following the linoleic acid structure (octadeca- 9,12–dienoic acid) lead us to reach the identity between its methyl ester derevative and the obtained mass spectrum. The fragmentation of the parent molecular ion at m/z 294 by loss CH_3O fragment gives m/z 263 and 31 fragments. While loss of $COOCH_3$ gives m/z 235 and 59 fragments, these obtained four fragments appeared due to breaking the bonds around C=O in the caroboxylic group. Breaking down the bonds around the C=C bond between carbons 9 and 10 gave m/z 157, 137, 183 and 111 fragments, whereas breaking down around the -C=C- bond between carbon 12 and 13 produced m/z 197, 97, 223 and 71 fragments. From the m/z 71, aliphatic chain fragments at 57 and 43 were produced through loss of CH_2 groups successively.

At a retention time of 19.40 min., a compound was eluted from its molecular weight through the spectrum, it may be around 400, through fragments appeared on its spectrum it looks like losing CH_2 group(s), which means that the compound has a hydrocarbon chain as it gave fragments at 57, 71, 84, 98, 112, 139, 167 etc. (Table **6**).

This prediction tends to the heptacosane compound ($C_{27}H_{56}$), a saturated carbon chain. This identification goes with [5] who identified the compound heptacosane from the ethanolic extract of pomegranate peel and seeds at a retention time of 19.15 min.

3.5.2. 3.2. Identification of the P. granatum solid fraction

The elution curve of the solid fraction was figured in Figure (**2**) and Table (**7**), from which it could be deduced that the solid fraction components were identified to have fatty acids esters (in methyl ester form) at the retention time rage (11.45 - 18.44) min. At a retention time of 11.45 min., a fatty acid derivative was identified as dodecanoic acid methyl ester (methyl dodecanoate) ($C_{13}H_{26}O_2$) based on its reported

molecular weight (214) and fragmentation pathways through the cleavage of bonds around the carbonyl group (C=O) in the carboxylic group giving m/z= 31 and 183, 59 and 155 fragments. Breaking the aliphatic long chain bonds produce fragments at m/z 74 (the base peak), 88, 143, 171, 185 and 199 that are related to the protonated $^+CH_2$-$COOCH_3$, $^+CH_2$-CH_2-$COOCH_3$, $^+CH_2$-$(CH_2)_5$-$COOCH_3$, $^+CH_2$-$(CH_2)_7$-$COOCH_3$, $^+CH_2$-$(CH_2)_8$-$COOCH_3$, $^+CH_2$-$(CH_2)_9$-$COOCH_3$ fragments, respectively. In the same manner of fragmentation pathways, the next identified compound was the methyl tetradecanoate (molecular weight 242,

$C_{15}H_{30}O_2$ was separated at 13.76 min. retention time. By explaining its fragmentation pathways, the parent molecular ion gave its fragments at m/z 199, 185, 171, 143, 74 (the base peak) and 57 fragments.

Methyl hexadecanoate ($C_{17}H_{34}O_2$) with a 270 molecular weight and Ethyl hexadecanoate ($C_{18}H_{36}O_2$; 284 molecular weight) were separated at 15.92 and16.48 min. retention time, respectively. These two compounds were also identified on their molecular weights and previously mentioned fatty acid fragmentation pathways giving their fragments at 227, 185, 171, 143, 88 and 74 (the base peak) m/z in comparison to m/z 255, 241, 213, 171, 88, 74 and 57 (the base peak) fragments, respectively. The fragment at m/z 255 might be referred to the M-C_2H_5 is a descriptive fragment for the ethyl hexadecanoate compound comparing with the methyl hexadecanoate component. At a retention time of 16.61 min., a free n-hexadecanoic acid was reported through the mass spectra. This compound was ascertained for its structure by its fragmentation as its base peak at m/z 73 is referred to $^{+}CH_2$-CH_2-COOH fragment ion. Its parent molecular ion at 256 m/z was fragmented to m/z 227, 185 and 171 fragments, which are owed to M-C_2H_5, M-$^{+}CH_2$-$(CH_2)_3$-CH_3, M-CH_2-$(CH_2)_4$-CH_3 fragment ions, respectively through the aliphatic chain breaking mechanism by successive loss of CH_2 group. Worth mentioning that this n-hexadecanoic acid may be produced through the hydrolysis either methyl or ethyl hexadconoate components or the ascorbic acid dihexadecanoate that was reported by[5] to be found in the pomegranate fruit extract.

An unsaturated fatty acid derivative with 296 molecular weight was eluted at a retention time of 17.64, which was predicted to be methyl octadec-9-enoate. The mass spectrum of this compound ensured its structure as its molecular ion peak appeared at 296 as a very weak signal. It loses OCH_3 moiety to give 265 m/z fragment or loses $COOCH_3$ moiety to 237 m/z fragment. Another key of its fragmentation is the breaking around the C=C bond to give two major fragments: CH_3-$(CH_2)_7$-CH=CH- at m/z 139 or -CH=CH-$(CH_2)_7$-$COOCH_3$ at m/z 183, which loses -C=C- fragment to fragments at m/z 113 and 157, respectively. Fragmentation through the aliphatic carbon chain leads to fragments at m/z 84 (the base peak) and 85 that are due to $^{+}CH_2$-CH_2-$COOCH_3$ and CH_3-$(CH_2)_4$-CH_2^{+} fragment ions. Fragments at m/z 89 and 99 are related to $^{+}CH_2$-$(CH_2)_2$-$COOCH_3$ and CH_3-$(CH_2)_5$-CH_2^{+} fragment ions, respectively.

At 17.83 min. retention time, a compound with 298 molecular weight was predicted to be methyl octadecanoate. Through its fragment pathways, the parent molecule (298,6) loses CH_3O to give m/z 276 fragment. Fragments at m/z 255, 227, 199, 171, 143, 98, 87 and 74 are referred to the successive breaking of the aliphatic chain as explained above.

At 18.30, a pyranone derivative was the most predicted. This predication could be accepted [20] reported a tetrahydro pyran-2-one derivative as a component in the pomegranate fruit peel. At the same time the side aliphatic chain (tridecyl) group can be fragmented through the open chain breaking down to fragments at m/z 142, 128, 100, 86 and 72. Also the cleavage between the aliphatic group and the hetero-aromatic ring gives a fragment at 126 m/z, which loses a CO group to m/z 98. The parent molecular ion loses a water molecule to m/z 264 fragment, which loses CO molecule to m/z 235 fragment. The fragment at m/z 169 resulted from 6-propyl pyrane-2-one derivative.

At 18.44 min. retention time, octadecanoic acid was revealed based on the general fatty acid fragmentation pathways. This compound may be due to the hydrolysis of methyl octadecanoate that has been identified before.

At 18.59 min., non-fatty acid was eluted with a molecular weight equals 282 as predicted. This compound could be identified as Eicosane (C_{20} H_{42}) as it was reported to be found in the pomegranate extract [21]. It is fragmentation pathway was carried out through loss of CH_2 groups successively (aliphatic chain fragmentation pathway).

At 21.51 min., 1,2,3-trimethyl-2-octadecylcyclohexane was predicted to be eluted. The fragmentation pathways revealed the $C_{18}H_{37}$ moiety through the aliphatic chain breaking down the m/z fragment appeared due to the un-substituted cyclohexane moiety. The octadecenamide was explained at 22.26 min. and it was ensured by its possible fragmentation processes through loss of NH_2CO-NH_2 fragment and the breaking around the -CH=CH- group as well as the normal fragmentation of the C_8H_{15} group in agreement with [22].

At 25.75 min., a glycoside derivative (or an ellagitannin derivative) was isolated. It showed fragmentation pathways that illustrate the typical fragmentation processes of glucose as it gives m/z= 179 indication the glycosidic bond cleavage between glucose and its aglycone. Also, the 149 m/z fragment (the base peak) points to loss of CHOH group from the 179 m/z fragment. The identified components of the pomegranate solid fraction are shown in Table (**7**).

From the obtained results, as a whole, twenty compounds were identified in the extract of *P. granatum* peel sample. These compounds are arranged according to the retention time in agreement with [19] and [5] in the following order: 1,2,3-Trihydroxy benzene (6.20%), Epicatechin-gallate (1.02%), Catechin (1.00%), Methyl-dodecanoate (2.90%), Methyl-tetradecanoate (2.70%), Methyl-hexadecanoate (35.4%), n-Hexadecanoic acid (6.8%), 9-Octadecenoic acid methyl ester (18.40%), Methyl-octadecanoate (5.90%), 6-Tridecyl-tetrahydro-2H-pyran-2-one (3.70%), Methyl-linoleiate (1.68%), Octadecanoic acid (1.9%), Eicosane (1.87%), Heptacosane (0.98%), Unknown (1.25%), Unknown (1.28%), 1,3,5-Trimethyl-2-octadecyl- cyclohexane (0.58%), 9-Octadecenamide (0.76%), Unknown (0.7%) and A glucoside derivative (2.50%).

The obtained data agreed with previous studies referring the remarkable antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels to the presence of tannins, flavonoids, phenols alkaloid, saponins, and terpenoids [23-24]. Its phytochemicals elements cause a wide range of clinical applications [25]. However, [26] referred the antibacterial effects of *P. granatum* extract to valoneic acid dilaction, monogalloyl-hexoside, hexahydroxy diphenoyl hexoside and coumaric acid.

Regarding the side effect on human, *Punica granatum* L (pomegranate) is safe medicinal plant used for generations in treating ulcers, diarrhea, and male infertility with anti-diabetic, anti-tumor, anti-inflammatory, anti-malaria, anti-fibrotic, anti-fungal, anti-bacterial, anti-obesity , alleviation of non-alcoholic fatty liver disease, metabolic syndrome, dental infections , anti-parasitic as anti-amoebic, anti-anthelmintic, cryptosporicidal, leishmanicidal, giardiacial, and other anti-parasitic characteristics, a "blood tonic and other effects [27-30] (Maphetu *et al*., 2022; Muhialdin *et al*., 2023; Al-Said *et al*., 2009; Jurenka, 2008). Its inhibitory potential on *E. coli* and *K. pneumonia* is an effective management of UTIs [31]. The methanolic peel extract inhibited

S. aureus, *S. Typhimurium*, *S. Dysenteriae* and *E. coli* as causes of serious gastrointestinal infections, leading to hemorrhagic diarrhea threatening young children and the elderly life [32]

Due to the results against the treated plant pathogenic fungi and bacteria and the positive interaction with human being, the pomegrenate peel could be of high agricultural importance in pest control without negative impact against both human and environment.

Table (1): Minimum inhibitory concentrations (MICs, µg ml^{-1}) of the crude extract

Tested plant extract	MIC (µg ml^{-1})		
	A. tumefaciens	*E. amylovolora*	*P. solanacearum*
P. granatum (Fruit peel)	500	500	500
Streptomycin sulfate	1	1	32

Table (2): Bactericidal effect of the P. granatum peel extract combinations with the standard bactericide, streptomycin sulfate.

Treated bacteria	**Bacterial growth at diferent MIC ratios***							
	1:1	**0.5:1**	**1:0.5**	**0.5:0.5**	**0.5:0.2**	**0.25:0.25**	**0.5:0.1**	**0.25:0.1**
A. tumefacies	-	-	-	-	-	-	-	-
E. amylovora	-	-	-	-	-	-	-	-
P. saloranacearum	-	-	-	-	-	-	-	-

*Ratio, Plant extract: Bactericide +: bacterial growth presence; –: absence of bacterial growth

Table (3 a): Effect of the tested P. granatum peel extract on R. solani and F. oxysporum

Treatment	*Rhizoctonia solani*				*Fusarium oxysporum*			
	EC_{50} µgml^{-1}	95% Conf. Limits		Slope ±SE	EC_{50} µgml^{-1}	95% Conf. Limits		Slope ±SE
		Lower	**Upper**			**Lower**	Upper	
***P. granatum* extract**	**123.0**	**73.5**	**185.4**	**2.41 ± 0.19**	**191.0**	**147.7**	**240.6**	**3.79 ± 0.23**
Azoxystrobin	**12.3**	**7.8**	**16.9**	**0.91 ± 0.18**	**3.0**	**0.72**	**5.4**	**0.83 ± 0.19**

Table (3 b): Effect of the tested P. granatum peel extract on the treated air born fungi

Air born fungi	***Alternaria alternata***				***Phytophthora infestans***			
	EC_{50} µgml^{-1}	95% Conf. Limits		Slope ±SE	EC_{50} µgml^{-1}	95% Conf. Limits		Slope ±SE
		Lower	**Upper**			**Lower**	Upper	
***P. granatum* extract**	**59.3**	**45.9**	**72.6**	**1.92 ± 0.19**	**78.6**	**66.0**	**91.2**	**2.37 ± 0.21**
Azoxystrobin	**13.7**	**8.0**	**20.5**	**0.73 ± 0.18**	**7.1**	**5.9**	**8.3**	**2.57 ± 0.25**

Table (4): Effect of the crude extract and azoxystrobin combinations against the treated fungi; shown as hayphal growth inhibition%.

Treated fungus	Average hyphal growth inhibition %					
	*1.0 : 0.5	0.5 : 0.5	0.25 : 0.5	1.0 : 0.25	0.5 : 0.25	0.25 : 0.25
F. oxysporum	100.0	76.5	74.1	56.5	49.4	31.8
R. solani	44.7	41.2	29.4	29.4	23.5	3.9
P. infestans	100.0	100.0	100.0	74.5	96.1	25.9
A. alternata	35.3	29.4	25.9	5.9	15.3	21.2

*Ratio, Plant extracts EC_{50}: azoxystrobin EC_{50}

Table (5): Effect of P. granatum extract on polyphenol oxidase (PPO) in P. infestans and A. alternata; shown as control activity%.

Concentration	*P. infestans*		*A. alternata*	
	liquid	solid	liquid	solid
0	100.0^{b}	100.0^{a}	100.0^{d}	100.0^{d}
50	127.8^{a}	99.2^{a}	129.1^{a}	129.6^{a}
100	114.8^{ab}	87.3^{ab}	121.4^{b}	121.4^{ab}
200	112.3^{ab}	81.1^{b}	111.7^{c}	104.4^{cb}
300	100.7^{b}	79.6^{b}	105.5^{cd}	88.5^{c}

Different letters indicate significant differences among treatments according to LSD (P= 0.01)

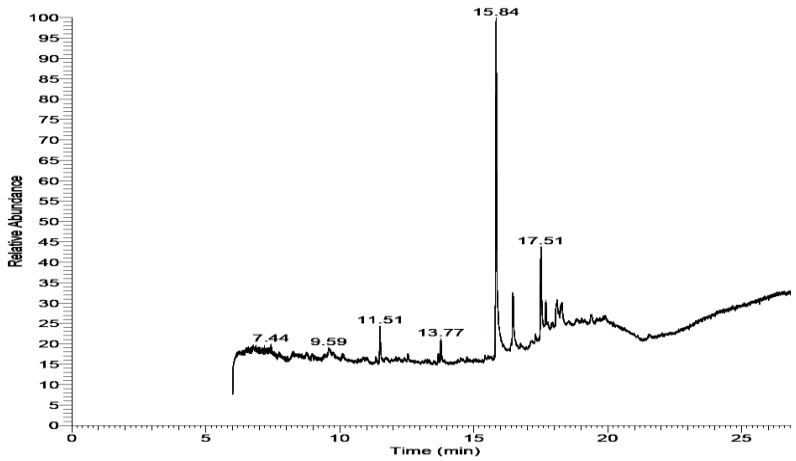
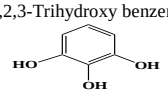
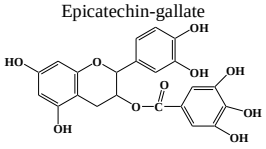
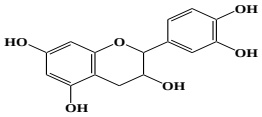
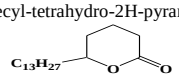


Figure (1): Elution curve of *Punica granatum* extract (liquid fraction)

Table (6): GC-MS identification of P. granatum liquid fraction constituents

No	Rt min.	Area %	MW	Compound	Ms spectral data as m/z (relative abundance)
1	6.07	12.29	$C_6H_6O_3$ 126	1,2,3-Trihydroxy benzene 	
2	8.27	2.04	$C_{22}H_{17}O_{10}$ 442	Epicatechin-gallate 	m/z: 290 (1.0), 268 (2), 225 (2), 180 (4), 152 (4), 146 (15),139 (3), 124 (12), 109 (17), 95 (37), 77 (12), 65 (7), 54 (70)
3	9.59	1.87	$C_{15}H_{13}O_6$ 290	Catechin 	m/z: 290 (M^+, 1.0), 272 (1), 253 (4), 219 (12), 152 (6), 136 (6), 123 (12), 109 (14), 95 (36), 77 (45), 65 (62), 54 (90)
4	11.50	4.48	$C_{13}H_{26}O_2$ 214	Methyl-dodecanoate CH_3-$(CH_2)_{10}$-$COOCH_3$	m/z: 214 (M^+, 4.0), 185 (8.0), 171 (14), 143 (22), 88 (52), 74 (100), 59 (8)
5	13.78	3.43	$C_{15}H_{30}O_2$ 242	Methyl-tetradecanoate CH_3-$(CH_2)_{12}$-$COOCH_3$	m/z: 242 (M^+, 4), 199 (18), 143 (82), 74 (100), 57 (43)
6	15.85	39.78	$C_{17}H_{34}O_2$ 270	Methyl-hexadecanoate CH_3-$(CH_2)_{14}$-$COOCH_3$	m/z: 270 (M^+, 4), 251 (2), 227 (6), 199 (10), 185 (10), 171 (8), 143 (24), 88 (72), 74 (100), 59 (8), 57 (20)
7	16.47	9.11	$C_{16}H_{32}O_2$ 256	n-Hexadecanoic acid CH_3-$(CH_2)_{14}$-$COOH$	m/z: 256 (M^+, 12), 239 (6), 224(12), 213 (32), 185 (28), 171 (6), 143 (22), 83 (42), 74 (36), 59 (60), 57 (72)
8	17.51	13.02	296	9-Octadecenoic acid methyl ester CH_3-$(CH_2)_6$-C=C-$(CH_2)_8$-$COOCH_3$	m/z: 296 (M^+, 2), 265 (12), 222 (14), 180 (14), 143 (10), 127 (15), 88 (46), 74 (70), 57 (100)
9	17.69	3.42	$C_{19}H_{38}O_2$ 298	Methyl-octadecanoate CH_3-$(CH_2)_{16}$-$COOCH_3$	m/z: 298 (M^+, 6), 255 (18), 199 (17), 143 (32), 88 (74), 74 (100), 59 (26), 57 (36)
10	18.11	5.33	$C_{18}H_{34}O_2$ 282	6-Tridecyl-tetrahydro-2H-pyran-2-one 	m/z: 282 (M^+, 2), 264 (10) , 235 (4), 169 (14), 142 (12), 128 (24), 100 (23), 98 (99), 58(32)
11	18.30	3.36	$C_{19}H_{34}O_2$ 294	Methyl-linoleiate CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-$COOCH_3$	m/z: 294 (M^+,22), 262 (8) , 235 (2), 129 (27), 98 (62), 81 (100), 71 (96), 57 (62)
12	19.41	1.96	$C_{27}H_{56}$ 380	Heptacosane CH_3-$(CH_2)_{25}$-CH_3	m/z: 358 (2), 324 (6), 281 (10), 221 (8), 167 (12), 139 (16), 112 (14), 98 (33), 84 (52), 71 (54), 57 (100)
Total		**100.0**			

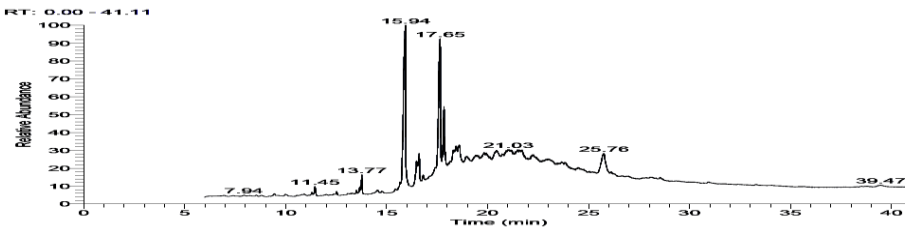
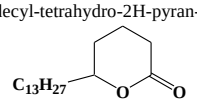
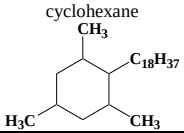


Figure (2): Elution curve of P. granatum extract (solid fraction)

Table (7): GC-MS identification of P. granatum solid fraction constituents

No	Rt min.	Area %	MW	Compound	Ms spectral data as m/z (relative abundance)
1	11.45	1.19	$C_{13}H_{26}O_2$ 214	Methyl-dodecanoate CH_3-$(CH_2)_{10}$-$COOCH_3$	m/z: 214 (M^+, 4.0), 185 (8.0), 171 (16), 143 (14), 88 (66), 74 (100), 57 (22)
2	13.77	1.97	$C_{15}H_{30}O_2$ 242	Methyl-tetradecanoate CH_3-$(CH_2)_{12}$-$COOCH_3$	m/z: 242 (M^+, 18), 199 (68), 185 (34), 171 (16), 143 (82), 74 (100), 57 (43)
3	15.92	30.92	$C_{17}H_{34}O_2$ 270	Methyl-hexadecanoate CH_3-$(CH_2)_{14}$-$COOCH_3$	m/z: 270 (M^+, 10), 227 (24), 185 (12), 171 (16), 143 (46), 88 (78), 74 (100), 57 (6)
4	16.50	3.65	$C_{18}H_{36}O_2$ 284	Ethyl-hexadecanoate CH_3-$(CH_2)_{14}$-$COOC_2H_5$	m/z: 284 (M^+, 18), 255 (12), 241 (48), 213 (38), 171 (18), 115 (24), 88 (62), 74 (64), 57 (100)
5	16.71	4.48	$C_{18}H_{36}O_2$ 256	n-Hexadecanoic CH_3-$(CH_2)_{14}$-$COOH$	m/z: 256 (M^+, 17), 227 (6), 191 (12), 185 (26), 171 (24), 111 (12), 73 (100), 57 (54)
6	17.63	23.76	$C_{19}H_{36}O_2$ 296	9-Octadecenoic acid methyl ester CH_3-$(CH_2)_7$-C=C-$(CH_2)_7$-COO CH_3	m/z: 296 (M^+, 2), 265 (4), 180 (12), 166 (14), 123 (40), 99 (62), 98 (50), 84 (100), 83 (98), 71 (48)
7	17.83	8.19	$C_{19}H_{38}O_2$ 298	Methyl-octadecanoate CH_3-$(CH_2)_{16}$-$COOCH_3$	m/z: 298 (M^+, 8), 255 (16), 227 (6), 199 (16), 171 (4), 143 (24), 88 (54), 74 (100), 57 (24)
8	18.30	2.04	$C_{18}H_{34}O_2$ 282	6-Tridecyl-tetrahydro-2H-pyran-2-one 	m/z: 282 (M^+, 2), 264 (10) , 235 (4), 169 (14), 142 (12), 128 (24), 100 (23), 98 (99), 58(32)
9	18.44	3.81	$C_{18}H_{36}O_2$ 284	Octadecanoic acid CH_3-$(CH_2)_{16}$-$COOH$	m/z: 284 (M^+, 8), 241(12) , 185 (16), 97 (44), 71 (78), 57 (100)
10	18.59	3.75	$C_{20}H_{42}$ 282	Eicosane CH_3-$(CH_2)_{18}$-CH_3	m/z: 282 (M^+, 2), 185 (6), 155 (14), 111 (12), 97 (36), 85 (34), 71 (64), 57 (100)
11	20.44	2.51		Unknown	
12	21.03	2.36		Unknown	
13	21.51	1.17	$C_{27}H_{54}$ 378	1,3,5-Trimethyl-2-octadecyl-cyclohexane 	m/z: 306(2), 257 (4), 225 (12), 169 (8), 153 (14), 112 (26), 84 (72), 71 (100), 57 (50)
14	22.26	1.53	$C_{18}H_{35}NO$ 281	9-Octadecenamide CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-$CONH_2$	m/z: 281(M^+, 4), 269 (8), 253 (12), 224 (8), 171 (22), 139 (24), 111 (74), 70 (44)
15	23.07	1.20		Unknown	
16	25.75	4.93	---	A glucoside derivative	m/z:, 167 (20), 151 (10), 149 (100), 113 (10), 48 (6), 57 (28)
Total		**97.46**			

4. Conclusions

P. granatum (fruits peels) extracts showed persuasive antifungal and antibacterial activities against the treated pathogens affecting the tested enzyme. Twenty compounds were identified in the extract of *P. granatum* peel sample. The pomegranate peel could be of high agricultural importance in pest control without negative impact against both human and environment.

5. Conflicts of interest

There are no conflicts to declare

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