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Isolation of olean-12(13), 15 (16)-diene, olean-12(13), 15(16)-dien-3β-oland olean-15(16)-en-11α-ol from the pet-benzene extract of *Psidiumguajava* and their biocidal activity

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ABSTRACT

Phytochemical investigation of the pet-benzene extract of the leaves of *Psidiumguajava* was yielded the triterpenoids, olean-12(13), 15(16)-diene, olean-12(13), 15(16)-dien-3β-oland olean-15(16)-en-11α-ol. All the three compounds are reported for the first time from *Psidiumguajava*. The compounds were established by chemical characterized using IR, NMR, and Mass analysis by comparison with authentic sample. All the three triterpenoids showed moderate to good biocidal activity against some bacterial and fungal pathogens.

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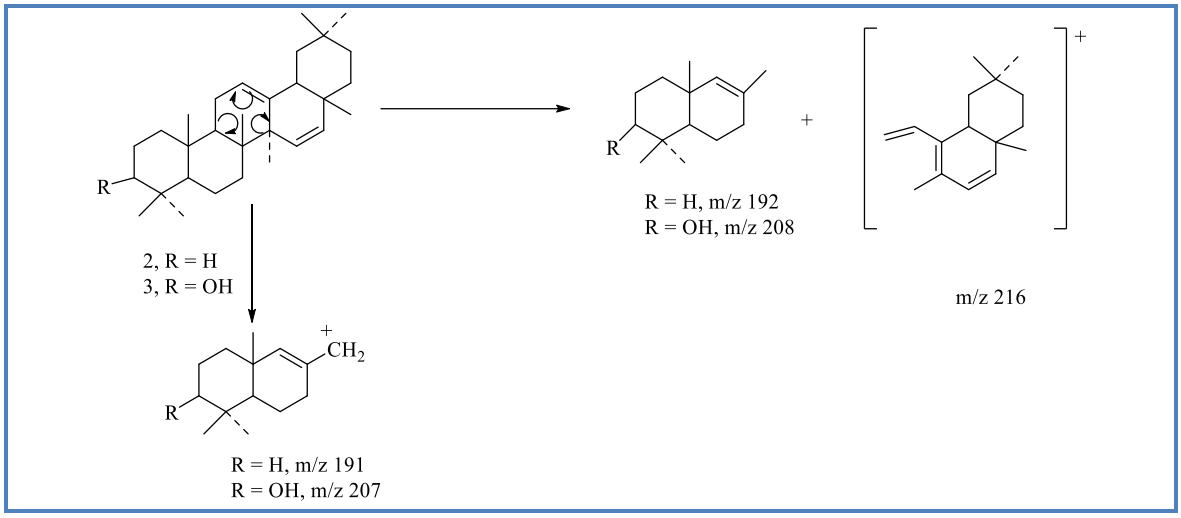
Graphical Abstract



Introduction

Plants contained a wide number of bioactive compounds [1, 2] that provide pharmacological effect. Natural products [3] are prone to have pharmacological [4] or biological activities and as a consequence; most traditional medicines as well as many modern medicines are based on them. The structural diversity of natural products exceeds that readily achievable by chemical synthesis [5-28]. Synthetic analogs can be prepared with improved potency and safety. As a result, natural products are often inspirational and used as starting points for drug discovery. In fact, natural products are the inspiration for approximately one-half of U.S. Food and Drug Administration-approved drugs [29, 30]. Himalayan region of Darjeeling hill and Terairegion are the rich source of wide variety of important medicinal plants [31-33]. Local people are still using these plants as folklore medicine. Among these plants, *Myrtaceae*are widely used in indigenous medicine from prehistoric ages. *Psidiumguajava* is an important representative of this family. Nowadays, because of the highly and efficient biological activities of the constituents isolated from *Psidiumguajava* [34-39], the plant draws much more attraction. Different parts of these plants have been used as folklore medicine. The phytoconstituents of the plant used for the treatment of various human ailments such as bronchitis, ulcers, bowels, eyesores, and cholera [35-37]. Leaf extract of *Psidiumguajava*has antibacterial, narcotic, antitussive, antioxidant, hemostaticproperties [38-41].

Based on these earlier reports recently we re-investigated the pet-benzene extract of the leaves of *P. guajava* and have isolated three more triterpenoids olean-12(13), 15(16)-diene(2), olean-12(13), 15(16)-dien-3β-ol(3) and olean-15(16)-en-11α-ol (4) along with β-sitosterol (5), ursolicacid (6), oleanolic acid (7), uvaol (8), guajanoic acid (9) [42], reported earlier. Isolation of olean-12(13), 15(16)-diene (2), olean-12(13), 15(16)-dien-3β-ol (3) and olean-15(16)-en-11α-ol (4) is reporting for the first time from this plant. The structure of all these compounds was established by chemical characterisation and by the analysis of spectroscopic (IR, NMR and Mass) data and also by comparison with authentic samples. Biocidal activities of these three compounds have also been carried out against some

bacterial and fungal pathogens in comparison to friedelin (1), a triterpenoid of same skeleton.

Isolation process

We collected fresh young mature leaves of *P. Guajava*from Darjeeling foothills during early summer, washed, shade dried and milled into coarse powder by a mechanical grinder. The powdered plant material was extracted with pet-benzene Soxhlet apparatus for 72 h The solvents were then removed under reduced pressure and a sticky brown residue was obtained. This residue was then purifiedby column chromatography using silica gel (60-120) mesh and suitable proportions of petroleum ether and ethyl acetate were used as the eluent.

Structure of all isolated and purified compounds were characterized by IR, NMR, and Mass. Five known compounds, *β*-sitosterol (5), ursolic acid (6), oleanolic acid (7), uvaol (8), guajanoic acid (9) [42], were identified by comparison (mixed m.p. Co-IR ; Co-TLC) with respective authentic samples. Apart from these known compounds, we could also be able to isolate three new triterpenoids of friedelin skeleton viz., olean-12(13), 15(16)-diene (2), olean-12(13), 15(16)-dien-3*β*-ol (3) and olean-15(16)-en-11*α*-ol (4) (Figure 1).

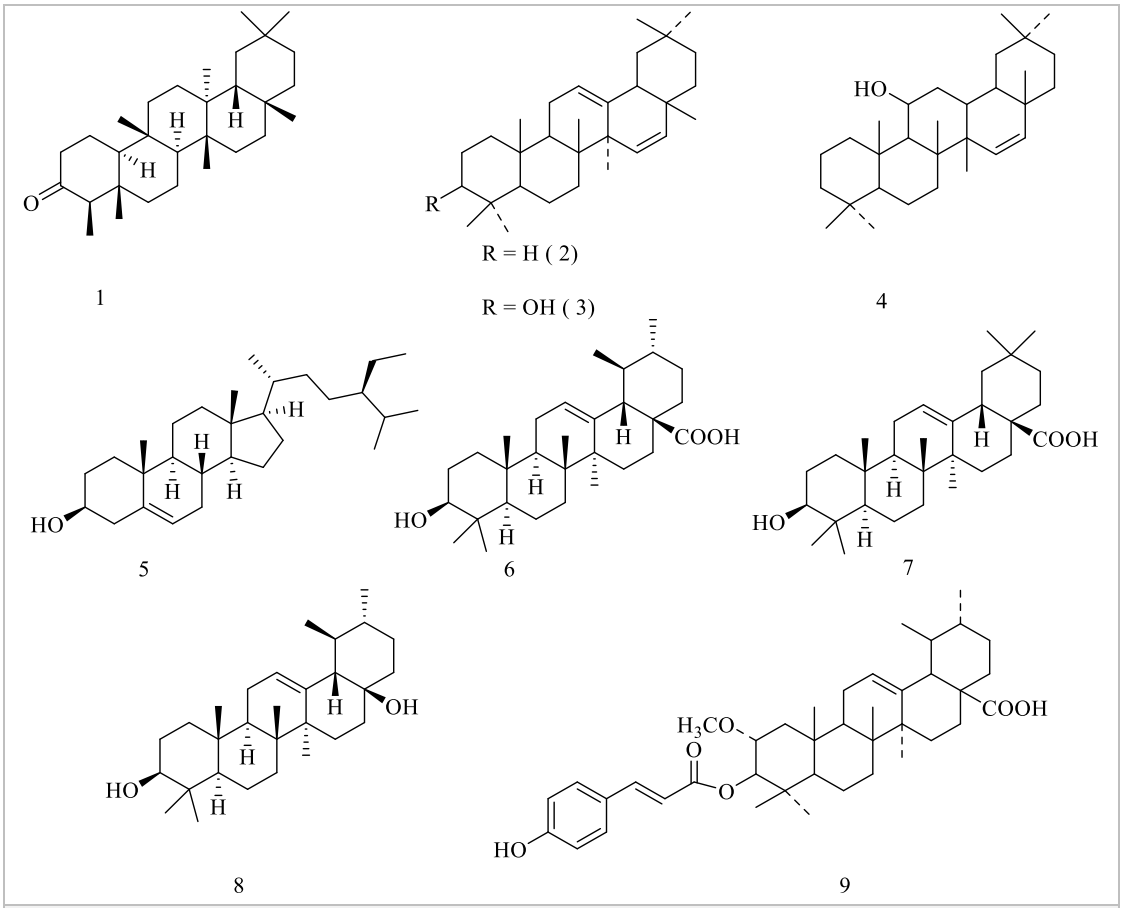


Figure 1. Friedelin (1), olean-12(13), 15(16)-diene (2), olean-12(13), 15(16)-dien-3*β*-ol (3), olean-15(16)-en-11*α*-ol (4), *β*-sitosterol (5), ursolic acid (6), oleanolic acid (7), uvaol (8), guajanoic acid (9)

Characterization of compound (2): identification of olean-12(13), 15 (16)-diene (2)

The chromatogram on elution with petroleum ether furnished a compound 2, analysed for $C_{30}H_{48}$, m.p. 202 °C. The IR spectrum of the compound 2showed peak at 820, 795, 780, 760 cm^{-1} for olefinic double bonds (C=CH- , -CH=CH-). The mass spectrum of the compound (Scheme 1) showed molecular ion peak at m/z 408, other peaks appeared at m/z 393, 216 (base peak), 201, 192, and 191. Thus from elemental analysis and mass spectrum, the molecular formula of the compound 2 is established to be $C_{30}H_{48}$. UV spectrum of the compound showed no absorption above 220 nm. The compound 2 gave yellow colouration with TNM test indicating the presence of olefinic double bond. Its PMR spectrum showed the presence of eight tertiary methyl group in the region 0.81 to 1.25 ppm as sharp singlet's, the AB-quartet centred at 5.38 ppm (J = 10 Hz) was assigned to the two olefinic proton at C-15 and C-16 (ABq, 2H, -CH=CH-), the multiplet centred at 5.36 ppm was assigned to olefinic proton at C-12 ($-CH_2-CH=$). The ^{13}C-NMR spectrum of the compound showed the presence of two doublets132.08 ppm and 134.32 ppm which have been assigned to C-15 and C-16 carbon bearing double bond (-HC=CH-), the doublet at 121.25 ppm and singlet at 142.57 ppm which have been assigned to C-12 and XC-13 carbons bearing double bond, 26 other peaks between 56.2 ppm to 15.30 ppm were also observed. Thus from spectral analysis the structure of the compound 2 was proposed as olean-12 (13), 15 (16)-diene (2).

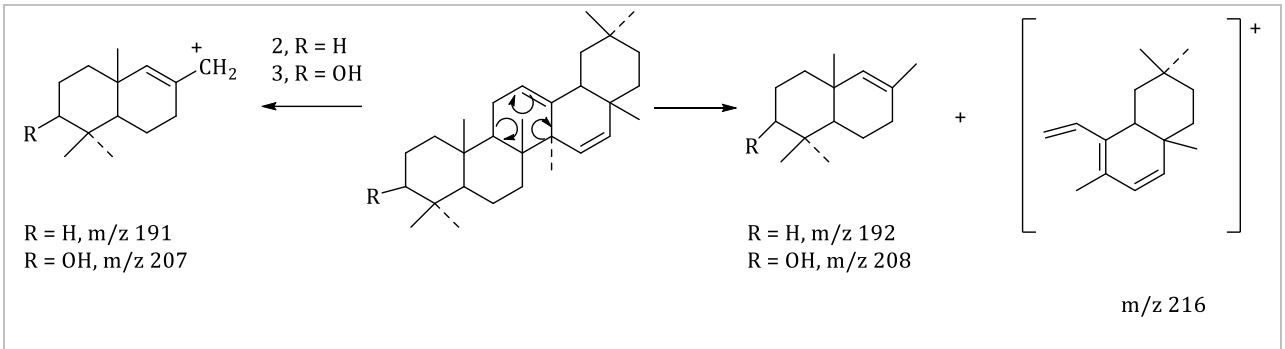
Characterization of compound (3): identification of olean-12 (13), 15(16)-dien-3β-ol (3)

Further elution of the chromatogram with petroleum ether-benzene (2:3) furnished a compound, (3), analysed for $C_{30}H_{48}O$, m.p. 235-36 °C. The compound 3gave yellow colouration with TNM indicating the presence of unsaturation. The IR spectrum of the compound (3) showed a broad peak at 3500-3200 cm^{-1} for hydroxyl functional group other peaks at 830, 800, 760, 750, 730 cm^{-1} could be attributed to the trisubstituted and disubstitutedolefinic double bonds. UV spectrum of the compound 3 showed no absorption above 220 nm. Mass spectrum OF compound 3 showed molecular ion peak at m/z 424 and the other peaks appeared at m/z 409, 406, 216 (base peak), 208, 207, 201. PMR spectrum of the compound 3 showed the presence of eight tertiary methyl as sharp singlets in the region 0.79 ppm to 1.19 ppm, a multipletcentered at 3.25 ppm was due to one proton germinal to hydroxyl group (H-C-OH), the AB-quartet centered at 5.36ppm (J = 10Hz) was assigned to the two olefinic protons at C-15 and C-16 (ABq, -HC=CH-, J=10 Hz), the triplet centered at 5.35 ppm was due to one olefinic proton at C-12 ($-CH_2-CH=$).The ^{13} C-NMR spectrum of the compound 3 showed the presence of two doublet at 131.93 and 134.48 ppm which have been assigned to C-15 and C-16 carbons bearing a double bond (-HC=CH-), the doublet at 120.98 ppm and singlet at 142.65 ppm have been assigned to C-12 and C-13 carbons bearing double bond ($-CH_{12}=C_{13}-$); the doublet at 79.01 ppm was due to carbon at C-3 position bearing hydroxyl group; 25 other peaks in the region 55 ppm to 15 ppm were also observed. From spectral analysis, the structure of the compound 3 was proposed as olean -12 (13), 15(16)-dien-3β-ol (3). Further the structure of the compound 3 was established as by preparation of its acetate, m.p. 198-99 °C (pyridine –acetic anhydride) which

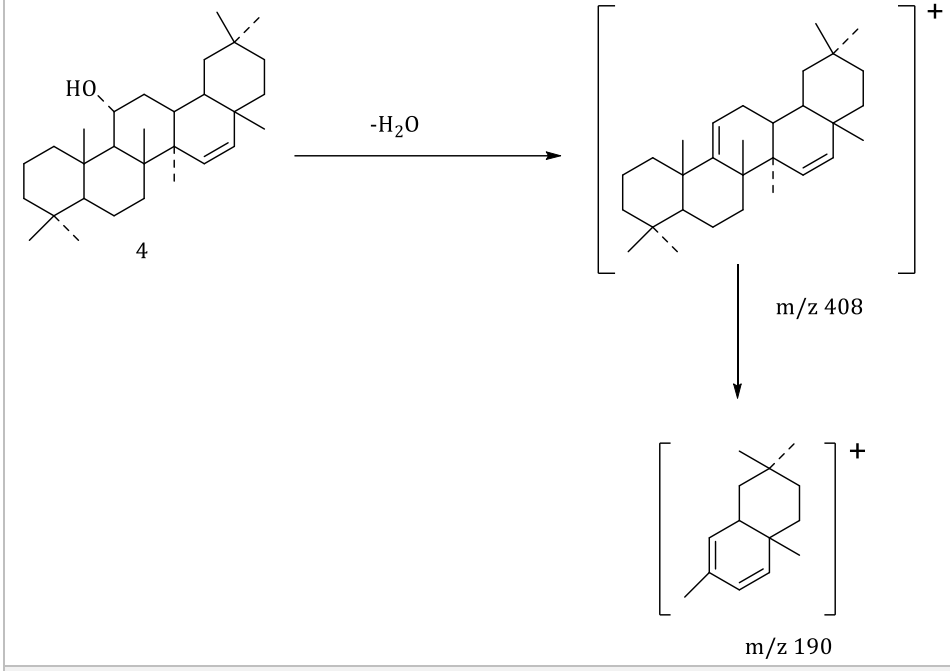
was found to be identical (mixedm.p., co-IR and co-TLC) with an authentic specimen of olean-12,15-dien-3*β*-yl acetate.

Characterization of compound (4): identification of olean-15(16)-en-11α-ol (4)

Further elution of the chromatogram with (1:3) furnished a compound 4, analysed for $C_{30}H_{50}O$, m.p. 205-6 °C .The compound 4 gave yellow colouration with TNM showing the presence of unsaturation (olefinic double bond). UV spectrum of the compound (4) showed no absorption above 220 nm. The IR spectrum of the compound 4 showed peaks at 3470 cm^{-1} for hydroxyl functional group other peaks at 780, 760 cm^{-1} were due to cis disubstituted olefinic double bond (-HC=CH-). The mass spectrum of the compound 4 showed molecular ion peak at m/z 426 which was consistent with molecular formula $C_{30}H_{50}O$. Other peaks appeared at m/z 408, 393, 288, 271, 270, 255, 204 and 190 (base peak) was also consistent with the structure 4 of the compound. The PMR spectrum of the compound 4 showed the presence of eight tertiary methyl groups which appeared as sharp singlets in the region 0.81 ppm to 1.18 ppm; the doublet of a triplet centered at 4.00 ppm was due to proton germinal to hydroxyl group (H-C-OH); AB-quartet centered at 5.32 ppm (J = 10 Hz) was assigned to the two olefinic protons at C-15 and C-16 (ABq, 2H, -HC=CH-, J = 10 Hz).^{13}C NMR spectrum of the compound 3 revealed the presence of only one olefinic double bond as represented by the two doublets at 135.00 and 131.9 ppm which have been assigned to C-15 and C-16 carbons bearing double bond (-HC=CH-), the presence of doublets at 68.5 was due to C-11 carbon bearing hydroxyl group; 27 other peaks in the region 58 to 18.5 ppm were observed. The presence of only one double bond suggested by ^{13}C NMR due to C_{15} –C_{16}olefinic double bond was confirmed by PMR spectrum that showed the presence of AB-quartet with J=10 Hz typical of a cis–disubstituted olefin. The proton of hydroxy function at C-11 appeared as a doublet of a triplet with J-values of 18 Hz. The large J-value indicate that the proton germinal to the hydroxyl group (-OH) is equatorially oriented. The acetoxyl group at C-3 position was lost by cleavage of the C=O bond. The structure of this compound 4was assigned as olean-15(16)-ene-11α-ol (4), which was confirmed by mass spectral data that consisted of the base peak at m/z 190 probably due to the fragment [scheme VIII]. Under the high energy condition, the hydroxyl group (-OH) is lost along with C_9-H forming a double bond between C-9 and C-11. Subsequent Retro-Diels-Alder cleavage resulted in fragment [scheme VIII].



Scheme 1. The mass spectrum of the compound 2



Scheme 2. The mass spectrum of the compound 4

Antimicrobial activity

Materials and methods

General introduction

In the present study, three fungal and four bacterial pathogens have been used. The details of the pathogens, their sources and their identification status have been presented in the following Tables (Table 1 and 2)

Table 1. Pathogenic fungal cultures used

Fungal culture	Source	Identified by	Identification No.
Colletotrichumgloeosporioides	Plant pathology lab (originally isolated from naturally infected brinjal leaf)	Indian Type Culture Collection, IARI New Delhi	ITCC-5446.02
Fusarium equiseti	Plant pathology lab (originally isolated from naturally infected tender brinjal stem)	Indian Type Culture Collection, IARI New Delhi	ITCC-6566.07
Curvulariaeragrostidis	Plant pathology lab (originally isolated from naturally infected tender brinjal stem)	Indian Type Culture Collection, IARI New Delhi	ITCC- 4150.2K

Table 2. Pathogenic bacterial cultures used

Culture	Source	Identification no./ Identified by	Bacterial culture
RS	American type culture collection	Procured from American type culture collection. Identification no. ATCC-B3123	***Ralstoniasolanacearum***
OB5	Plant Pathology Laboratory, Department of Botany, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr. A. Saha	***Xanthomonas* sp.**
TB3	Plant Pathology Laboratory, Department of Botan y, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr. A. Saha	***Pseudomonas syringae***
PB8	Plant Pathology Laboratory, Department of Botany, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr. A. Saha	***Erwiniacarotovora***

RS, OB5, TB3, PB8 were used as test pathogen throughout the present study

* Identification was done by biochemical characterization of the three bacteria

Maintenance of stock cultures

Colletotrichumgloeosporioides were grown in PDA media at 28±1 ºC in an incubator for 12 h The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Fusarium equiseti were grown in PDA media at 28±1ºC in an incubator for 48 h The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Curvulariaeragrostidiswere grown in PDA media at 28±1ºC in an incubator for 12 h**.** The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals.

Ralstoniasolanacearum, a Gram negative, nonsporing bacteria were grown in nutrient agar media at 37±1 ºC in an incubator for 24 h The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Xanthomonassp, a Gram negative bacteria were grown in nutrient agar media at 37±1ºC in an incubator for 24 h**.** The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time

maintenance. Throughout the present, study 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Pseudomonas syringae, a rod shaped, Gram negative, non sporeforming bacteria were grown in nutrient agar media at 37±1 ºC in an incubator for 24 h**.** The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Erwiniacarotovora, a Gram negative, rod shaped bacteria were grown in nutrient agar media at 37±1 ºC in an incubator for 24 h**.** The growth condition was aerobic. Finally, the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Spore germination bioassay

Ten days old sporulated fungal culture was taken and approximately 3-5 mL sterile distilled water was poured in the culture tube aseptically. Gentle scrapping was done by an inoculating needle on the agar surface. After the scrapping, the tube was shaken and the resultant mixture was strained through cheesecloth**.** The filtrate was used as spore suspension. The concentration of the spores in the suspension was adjusted by adding sterile distilled water following hemocytometer count.

All the test compounds were subjected to bioassay against three fungal pathogens (*C. gloeosporioides, F. equiseti*and *C. eragrostidis*). The spores of the pathogens were allowed to germinate in sterile distilled water drops mounted on sterile grease free slides kept in a humid chamber in case of control. In experimental sets 30 µL of each test compound (dissolved in DMSO) was placed on the center of a clean (grease free) microscopic slide. After that, spore suspension was mounted on the slides in the same place where the extract was applied. In solvent control set fresh solvent (DMSO) was placed and subsequently spore suspension was applied. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and the experimental slide was placed on the rods in a uniformly balanced position. Sterile distilled water was carefully poured in the petridish so that the bottom of the slide remained just above the water surface. The petridishwas then covered for maintaining humid condition and finally the petridishes were incubated at 28±1 ºC. After 48 h of incubation, the slides were stained with lacto phenol-cotton blue and were observed under microscope. Approximately, 200 spores were observed in each slide for germination. The entire experiment was repeated thrice.

Bioassay by disc diffusion method

2 mL of spore suspension was poured in a sterile petridish (90 mm diameter) and then 18 mL of the molten PDA medium was poured in the same petridish. The spore suspension and medium was mixed well and was allowed to solidify. After solidification of petridishes filter paper discs (Whatmann 40, 4mm in diameter r) were dipped in different concentrations of the test compounds (100, 200, 300, 400 and 500 ppm) and then were placed on the solidified plates. In solvent control sets, filter paper discs dipped in pure DMSO was placed. In distilled water control sets filter paper discs dipped in distilled water were placed on the solidified medium surface. Both experimental and control plates were incubated for period as required. Radial growth of each pathogen was measured. In case of bacterial pathogens nutrient agar was used instead of PDA.

Determination of minimum inhibitory concentration by agar cup method

For screening of inhibitory effect of botanicals against test pathogens both spore, germination bioassay technique and disc diffusion bioassay technique were followed. Minimum inhibitory concentrations (MIC) of all active components were measured following standard procedures as suggested by Portillo *etal*., 2005 [45].

A sensitive and quick petridish method as suggested by Eloff (1998) [43] was followed to determine the minimum inhibitory concentration of plant extracts against bacteria and fungi. Minimum inhibitory concentration (MIC) was determined by the micro dilution method using serially diluted test compounds. Various concentrations (Viz. 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm) of the plant extracts were prepared. Nutrient agar was used for the growth of bacterial strain and potato dextrose agar was used for the growth of fungi. By means of a cork borer (5 mm in diameter) a cup or well was made in a seeded (either by bacterial suspension or by fungal spore suspension) agar plate. The cup or well was filled with 50 µL tested compound and was incubated in the incubators meant for bacteria or fungus as applicable. The extract diffused from the cup to a certain extent and inhibited the growth of the pathogen if it contained any antimicrobial properties. It was found that the diameter of the inhibition zone was more when the concentration of the antimicrobial properties was more. The plates containing bacteria were incubated at 37±1°C for 48 h and the plates containing fungi were incubated at 28±1°C for 72 h. The antimicrobial activity was noted on the basis of the diameter of inhibition zone where no growth was found.

Results

Friedelin (1): As a reference sample, friedelin (1) a naturally occurring triterpenoid of same skeleton as the experimental compounds (2-4), was tested and the results are presented in Table 3a. It was observed that the antifungal activity of friedelin was effective at higher concentrations. Out of the five concentrations tested two concentrations (400 and 500ppm) showed antifungal activity (more than 90% inhibition) in all the three fungal pathogens (*Colletotrichumgloeosporioides, Fusarium equiseti*and *curvulariaeragrostidis*). Percent germination of spores gradually decreased with the increasing concentration of the compound. In all the three cases 500 ppm concentration was most effective than the other concentrations.

Antifungal activity of friedelinwas also determined by disc diffusion test. Results of the test have been presented in Table 3b. It was evident from the results that all the five concentrations tested were effective in case of *C. Gloeosporioides*and *F. equiseti*. The diameter of inhibition zones increased with increasing concentrations of the compound. However, growth of *C. eragrostidis* could not be controlled by any concentrations (100, 200, 300, 400, 500ppm) of friedelin (1) tested.

Antibacterial activity of friedelin (1) was also measured and has been presented in Table 3c and Growth of all the four bacteria (*R. solanacearum, Xanthomonassp, P. syringae, E. carotovora*) tested could be controlled significantly by the three different concentrations (300, 400, 500 ppm) of friedelin. The diameter of inhibition zones were gradually increased with increasing concentrations of the compound.

Table 3a. Percent inhibition of spore germination of *Colletotrichumgloeosporioides, Fusarium equiseti*and *Curvulariaeragrostidis* by friedelin (1). (When control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	85	15±1.06
	200	12-40	32	68±1.50
	300	12-27	21	79±1.20
	400	08-32	05	95±1.90
	500	08-24	04	96±1.04
Fusarium equiseti	100	12-24	84	14±1.72
	200	08-20	18	82±1.38
	300	08-20	17	83±1.92
	400	04-16	10	90±1.45
	500	04-12	05	95±1.40
Curvularia Eragrostidis	100	40-88	15	85±1.02
	200	32-80	11	89±1.30
	300	28-68	09	91±1.40
	400	24-56	07	93±1.55
	500	20-48	05	95±1.10

*Data after ± indicate standard error value

Table 3b. Antifungal activity of friedelin (1)

Fungal organism	Concentrations of compound (ppm)	Diameter of inhibition zone (cm)*
Colletotrichumgloeosporioides	100	0.8
	200	1.0
	300	1.1
	400	1.3
	500	1.5
Fusarium equiseti	100	0.7
	200	0.9
	300	1.0
	400	1.2
	500	1.3
Curvulariaeragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

*mean of three replications; [-]indicates no inhibition zone formed

Table 3c. Antibacterial activity of friedelin (1)

Bacterial organism	Concentrations of compound (ppm)	Diameter of Inhibition zone (cm)*
Ralstoniasolanacearum	Control	-
	100	0.4
	200	0.6
	300	0.9
	400	1.2
	500	1.4
Xanthomonassp	Control	-
	100	1.5
	200	1.7
	300	1.9
	400	2.1
	500	2.2
Pseudomonas syringae	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.6
	500	1.7
Erwiniacarotovora	Control	-
	100	1.1
	200	1.4
	300	1.7
	400	1.9
	500	2.2

*mean of three replications; [-] indicates no inhibition zone formed

Result of spore germination bioassay of olean-12(13), 15(16)-diene (2) has been presented in Table 4a. From the results it was observed that the antifungal activity of olean-12(13), 15 (16)-diene (2) was effective at all the five concentrations. But three concentrations (300, 400 and 500 ppm) showed significant antifungal activity (89-99% inhibition of spore germination) against the three fungal pathogens (*Colletotrichumgloeosporioides, Fusarium equiseti*and *Curvulariaeragrostidis*). Percent germination of spores gradually decreased with the increasing concentration of the compound. In all the three cases, 500 ppm concentration was more effective than the other concentrations.

From the results presented in the Table 4b and it was found that the antifungal activity of olean-12(13), 15(16)-diene(2)was very much significant. Out of five concentrations tested three concentrations (300,400,500 ppm) of the compound 2 were very effective and showed antifungal activity (inhibition zones diameter of 1.0-2.0 cm) against all the three tested fungal pathogens (*Colletotrichumgloeosporioides, Fusarium equiseti and Curvulariaeragrostidis*). Diameter of inhibition zones were increased with the increasing concentration of the compound.

Best antifungal activity was obtained against all the three fungus when 500 ppm concentration was used.

Table 4a. Percent inhibition of spore germination of *Colletotrichumgloeosporioides, Fusarium equiseti*and *Curvulariaeragrostidis of* olean-12(13), 15 (16)-diene (2) (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-48	23	77±1.10
	200	08-44	05	95±1.34
	300	08-40	03	97±1.58
	400	04-36	03	97±1.67
	500	04-28	01	99±1.87
Fusariumequiseti	100	12-20	27	73±1.60
	200	08-32	12	88±1.77
	300	08-32	11	89±1.98
	400	04-12	09	91±1.45
	500	04-12	06	94±1.65
Curvularia eragrostidis	100	36-100	13	87±1.20
	200	28-72	08	92±1.40
	300	24-68	07	93±1.44
	400	20-60	06	94±1.02
	500	16-44	04	96±1.19

*Data after ± indicate standard error value

Table 4b. Antifungal activity of Compound 2**.**

Fungal organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Colletotrichumgloeosporioides	100	0.6
	200	0.8
	300	1.0
	400	1.2
	500	1.3
Fusarium equiseti	100	1.3
	200	1.5
	300	1.6
	400	1.7
	500	1.9
Curvulariaeragrostidis	100	1.2
	200	1.5
	300	1.7
	400	1.8
	500	2.0

*mean of three replications

In Table 4c and the results of antibacterial activity of olean-12(13), 15 (16)-diene (2) against four bacteria (*R. solanacearum, Xanthomonassp, P. syringe* and *E. Carotovora*)

have been presented. Out of five concentrations tested, three concentrations (300, 400, 500 ppm) of the compound were proved to be significant (diameter of inhibition zones ranged from 1.2-2.3 cm). The diameter of inhibition zones were gradually increased with increasing concentrations of the compound 2. Highest antibacterial activity was observed at 500 ppm concentration of compound 2.

Table 4c. Antibacterial activity of Compound 2**.**

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone (cm)*
Ralstoniasolanacearum	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.8
	500	2.0
Xanthomonassp	Control	-
	100	1.2
	200	1.4
	300	1.7
	400	1.9
	500	2.2
Pseudomonas syringae	Control	-
	100	0.8
	200	1.0
	300	1.2
	400	1.5
	500	1.7
Erwiniacarotovora	Control	-
	100	1.2
	200	1.5
	300	1.8
	400	2.0
	500	2.3

*mean of three replications; [-] indicates no inhibition zone formed

Compound 3: From the results, presented in the Table 5a it was evident that the antifungal activity of Compound 3 was effective in controlling plant pathogens. In all the five concentrations tested showed antifungal activity against the three fungal pathogens (*Colletotrichumgloeosporioides, Fusarium equiseti and Curvulariaeragrostidis*). Inhibition of spore germination was gradually increased with the increasing concentrations of the compound. Although all concentrations of the compound were effective but 500 ppm concentration showed more than 95% inhibition of spore germination.

Table 5a. Percent inhibition of spore germination of *Colletotrichumgloeosporioides, Fusarium equiseti*and *Curvulariaeragrostidis* by Compound 3(when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-48	21	79±1.12
	200	12-48	17	83±1.79
	300	08-40	07	93±1.60
	400	04-36	06	94±1.77
	500	04-28	02	98±1.88
Fusariumequiseti	100	12-20	17	83±1.98
	200	08-16	12	88±1.75
	300	08-16	10	90±1.56
	400	04-12	06	94±1.36
	500	04-08	02	98±1.30
Curvularia eragrostidis	100	36-84	20	80±1.57
	200	32-72	12	88±1.10
	300	28-64	11	89±1.35
	400	24-56	10	90±1.90
	500	20-44	05	95±2.00

*Data after ± indicate standard error value

Antifungal activity of Compound 3was also tested by disc diffusion method on agar plates. Results presented in the Table 5b and showed that compound 3 was antifungal against *C. Gloeosporioides*and *F. equiseti* but it was not effective against *C.eragrostidis.* Compound-3 was tested for antifungal activity at five different concentrations (100, 200, 300, 400, 500 ppm). Compound 3 at concentration below 300 ppm did not show any antifungal activity. Diameter of inhibition zones ranged from 0.8-2.1 cm when concentrations of 300, 400 and 500 ppm of the compound 3 were tested against the two pathogens mentioned above.

The results of antibacterial activity of Compound 3presented in Table 5c and indicated that Compound 3 was effective against three bacteria (*R. solanacearum, Xanthomonassp, E. carotovora*). Out of five tested concentrations, two concentrations (400 and 500 ppm) of the compound were proved to be significant against all three bacteria.It was evident from the results that compound 3 was very less effective against *P. syringae.* Only 500 ppm concentration of the compound 3 showed its activity against *P. syringae.*

Table 5b. Antifungal activity of Compound 3**.**

Fungal organism	Concentrations of Compound (ppm)	Diameter of inhibition zone (cm)*
Colletotrichumgloeosporioides	100	-
	200	-
	300	0.8
	400	1.2
	500	1.4

	100	1.5
	200	1.5
Fusarium equiseti	300	1.6
	400	2.0
	500	2.1
	100	-
	200	-
Curvulariaeragrostidis	300	-
	400	-
	500	-

*mean of three replications; [-]indicates no inhibition zone formed

Table 5c. Antibacterial activity of Compound 3.

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone (cm) *
	Control	-
	100	0.4
	200	0.4
Ralstoniasolanacearum	300	0.8
	400	1.0
	500	1.3
	Control	-
	100	0.4
	200	0.8
Xanthomonassp	300	1.0
	400	1.2
	500	1.3
	Control	-
	100	0.4
	200	0.4
Pseudomonas syringae	300	0.4
	400	0.9
	500	1.1
	Control	-
	100	0.7
	200	0.9
Erwiniacarotovora	300	1.2
	400	1.3
	500	1.5

*mean of three replications; [-] indicates no inhibition zone formed

Compound 4: From the results presented in the Table 6a it was found that the antifungal activity of Compound 4 was positive. Out of five concentrations tested three concentrations (300, 400, 500 ppm) of the compound 4 were very much effective and showed antifungal activity (89-97% inhibition of spore germination) against all the three fungal pathogens (*Colletotrichumgloeospoerioides, Fusarium equiseti and Curvulariaeragrostidis*) tested. Percent germination was reduced gradually with increasing concentration of the compound. Although 300, 400 and 500 ppm

concentrations of the compound were effective but 500 ppm concentrations was most effective.

Table 6a. Percent inhibition of spore germination of *Colletotrichumgloeosporioides, Fusarium equisetai*and *Curvulariaeragrostidis* by compound4(when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	26	74±1.97
	200	08-40	12	88±2.01
	300	08-36	11	89±1.85
	400	04-32	08	92±1.66
	500	04-28	04	96±1.44
Fusarium equiseti	100	08-24	25	75±2.00
	200	08-20	15	85±1.73
	300	08-16	10	90±1.32
	400	04-16	03	97±1.56
	500	04-12	02	98±1.40
Curvularia eragrostidis	100	40-96	13	87±1.75
	200	32-80	11	89±1.30
	300	28-52	09	91±1.99
	400	24-48	06	94±1.45
	500	20-32	05	95±1.77

*Data after ± indicate standard error value

From the results presented in the Table 6b it was found that the antifungal activity of Compound 4 was very much effective against the pathogens tested. Diameter of inhibition zones ranged between 0.9-2.5cm against all the three tested fungal pathogens (*Colletotrichumgloeosporioides, Fusarium equiseti*and *Curvulariaeragrostidis*). Diameter of inhibition zones was increased with the increasing concentration of the compound.

Results of antibacterial activity of Compound 4 through disc diffusion method have been presented in Table 6c. Compound 4 showed significant activity in connection of 300ppm concentration and above. Diameter of inhibition zones of the effective concentrations ranged from 0.9-1.5 cm against the four test pathogens (*R. solanacearum, Xanthomonassp, Pseudomonas syringae, E. carotovora*).

Table 6b. Antifungal activity by Compound 4.

Fungal organism	Concentrations of Compound (ppm)	Diameter of inhibition zone (cm)*
Colletotrichumgloeosporioides	100	1.4
	200	1.6
	300	1.7
	400	1.9
	500	2.1
Fusarium equiseti	100	1.9
	200	2.0
	300	2.2

	400	2.3
	500	2.5
	100	0.9
	200	0.9
Curvulariaeragrostidis	300	1.1
	400	1.2
	500	1.4

*mean of three replications

Table 6c. Antibacterial activity of Compound4**.**

Bacterial organism	**Concentrations of compound (ppm)**	**Diameter of inhibition zone (cm)***
	Control	-
	100	0.4
	200	0.6
Ralstoniasolanacearum	300	0.9
	400	1.1
	500	1.3
	Control	-
	100	0.6
	200	0.8
***Xanthomonas*sp**	300	1.1
	400	1.2
	500	1.5
	Control	-
	100	0.4
	200	0.7
Pseudomonas syringae	300	0.9
	400	0.9
	500	1.2
	Control	-
	100	0.7
	200	0.8
Erwiniacarotovora	300	1.0
	400	1.2
	500	1.4

*mean of three replications; [-] indicates no inhibition zone formed

Discussion

The present study was based on the phytochemical investigations and antimicrobial activities of theleaves of the plant *P. Guajava.* This medicinal as well as anti-microbial propertiesof the plant is well documented [41].

Friedelin a triterpenoid (special group of secondary metabolites) was isolated from the plant *Quarcussuber*. *Q. suber* is a plant commonly called Cork Oak, belongs to the family Fagaceae (Beech family)and tested as a mother compound.

The compound 1, chemical name friedelin showed antibacterial activity and anti fungal activity. In disc diffusion studies growth of two fungi (*Colletotrichumgloeosporioids, Fusarium equiseti)* was controlled by compound-1.

Curvulariaeragrostidis could not be controlled in disc diffusion test even at 500 ppm concentration of friedelin. It showed little activity against *Ralstoniasolanacearum*at lowest concentration (100 ppm). In contrast, three other bacteria (*Xanthomonassp, P. syringae, E. carotovora*) tested were controlled significantly at 100 ppm concentration of friedelin (1).

Compound 2 was stronger than the mother compound friedelin(1), as evident from the result of spore germination bioassay and disc diffusion bioassay against three different fungi (*Colletotrichumgloeosporioids, Fusarium equiseti*and *Curvulariaeragrostidis)*.In contrast to the mother compound (friedelin) which could not check the growth of *Curvulariaeragrostidis* in disc diffusion test, compound-2could control *Curvulariaeragrostidis* at all the five concentrations (100, 200, 300, 400 & 500 ppm) tested. Stronger antibacterial activity of compound 2 was also evident from the disc diffusion test against the four bacterial pathogens (*R. solanacearum, Xanthomonas*sp.,*P. Syringae*and *E. carotovora*).

The second compound coded as compound 3 was tested for antifungal and antibacterial activity. Compound 3 could check spore germination of all the three fungi (*Colletotrichumgloeosporioides, Fusarium equisetai*and *Curvulariaeragrostidis*) moderately at five different concentrations of the compound tested. In disc diffusion test the compound showed no activity against *Curvulariaeragrostidis*. The compound showed poor activity against *Colletotrichumgloeosporioides* but showed high activity against *Fusarium equiseti.* In case of bacteria only *E. carotovora* was checked at 100 ppm concentration. Growth of all three other bacteria (*Ralstoniasolanacearum, Xanthomonas*sp*. & Pseudomonas syringae*) were controlled only at higher concentrations of the compound 3. Thus, compound-3 was less antimicrobial than compound 2.

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Conflict of Interest

We have no conflicts of interest to disclose.

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