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Antifungal activities of *Potentilla fruticosa* on 12 fungi spp.

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ABSTRACT

The antimicrobial activity of ethanol extracts of *Potentilla fructicosa* was examined on 12 phytopathogenic fungi. Among the 12 fungi, *Rhizoctonia cerealis, Botrytis cinerea, Alternaria alternata* and *Bipolaris sorokiniana* were strongly inhibited. The ethanol extracts was sequentially fractionated with petroleum ether, ethyl acetate, n-butanol and water and the fractions were tested for their antifungal activity against 4-pathogenic fungi (strongly inhibited by ethanol extract). It was found that the ethyl acetate (EAF) and n-butanol (BF) fractions inhibited the 4-fungi (*Rhizoctonia cerealis, Botrytis cinerea, Alternaria alternata* and *Bipolaris sorokiniana*) more than the other two. The EAF was further fractionated on a silica column using a petroleum ether /acetone gradient systems to obtain two compounds with strong inhibitory activity. These were identified as betulinic acid and olenolic acid by physical and spectral analysis. Furthermore, both compounds were obtained from *P. fruticosa* for the first time. While both these triterpenoids inhibited the *B. cinerea* only, while the olenolic acid inhibited the *B.sorokiiana* These two chemicals were also active against *Eschericia coli*, while Bacillus *subliits* was inhibited only by Betulinic acid. These results suggested that the two triterpenoids present in *P. fructicosa* were antifungal and had the potential for use in plant disease control.

Key words: Antibacterial activity, antifungal activities, fractions, pathogenic fungi, *Potentilla fruticosa*, triterpenoids

INTRODUCTION

Plant diseases caused by plant pathogensare reduces the crop production world wide (11). Among the plant pathogens, the fungi are most important (18). These pathogens are controlled using chemical fungicides but their use causes both environmental contamination and development of resistance in organisms (27). In recent years, therefore, attempts have been made to search for novel antifungal agents. Thus, plant materials used in traditional medicine have received some attention as source of biologically active antifungal compounds.(3,7,20,21,23,25) and many new antifungal agents from plants have been reported (7,21,25).

Potentilla fruiticosa (Rosaceae) is a plant used in traditional medicine and grows mostly in cold and humid areas (4). In China it grows in the high mountains in North west and South west (altitudes of 2000-4000 m). Its aerial parts are used in traditional medicine (5). The plant contains a variety of phytochemicals (flavonoids, phenolics and triterpenoids). So far, 12 flavonoids, 10 phenolics, 3 triterpenoids and 3 sterol compounds have been isolated and identified from this plant (6,8,9,10,15). There are several reports on

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the use of *P. fruitcosa* as antioxidant (2,16) in Fermented dutch sasauge (16) and in preventing lipid peroxidation (1). Its leaves are used as food additives and cosmetics (16,17) and its flower extracts have strong radical scavenging activity (15).

Recently, it was reported that the aqueous extracts of its aerial parts and ethanol extracts of its leaves have antibacterial activity against *Helicobacter pylori* (26), a variety of Gram positive and Gram negative bacteria and also *Candida albicans* (13). However there are no reports on the anti-fungal activity against any plant pathogenic fungi. We therefore, investigated its antifungal activities against several plant pathogenic fungi and we report here the isolation of two triterpenoids, which are both antifungal and antibacterial.

MATERIALS AND METHODS

Materials and chemicals

The aerial parts of *P. fruticosa* were collected in August 2010, from the Tai-bai area of Qinling Mountains, China and stored in the herbarium of Northwest A&F University, Yangling, China. Twelve plant pathogenic fungi and 3 bacteria used in this study are listed in Table 1. These were provided by College of Resources and Environment, Northwest A&F University, Yangling, China.

Table 1. List of fungi and bacteria used in this study

S.No	Microorganisma	Source (Crops, trees etc)	
		English name	Botanical name
	Fungi		
1.	*Alternaria brassicae* (Berk.) Sacc.	Mustard	*Brassica oleracea*
2.	*Thanatephorus cucumeris* (A.B. Frank) Donk	Cotton	Cotton
3.	*Verticillium dahliae* Kleb.	Cotton	Cotton
4.	*Alternaria solani* Sorauer	Tomato	*Solanum lycopersicum*
5.	*Botrytis cinerea* Pers.	Tomato	*Solanum lycopersicum*
6.	*Bipolaris sorokiniana* (Sacc.) Shoemaker	Wheat	*Triticum aestivum*
7.	*Rhizoctonia cerealis* E.P. Hoeven	Wheat	*Triticum aestivum*
8.	*Alternaria alternata* (Fr.) Keissl.	Apple	*Malus domestica*
9.	*Phacidiopycnis washingtonensis* Xiao & J.D. Rogers	Apple	*Malus domestica*
10.	*Physalospora Piricola* Nose	Apple	*Malus domestica*
11.	*Valsa mali* Miyabe & G. Yamada	Apple	*Malus domestica*
12.	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.	Grapes	*Vitis vinifera*
	Bacteria		
13.	*Bacillus subtilis* Ehrenberg	Soil	
14.	*Escherichia coli* Migula	Soil	
15.	*Staphylococcus aureus* Rosenbach	Soil	

These were cultured on Potato Dextrose Agar (PDA) medium at 27℃ for 72 h and the bacterial strains were grown on beef-extract peptone agar medium at 37 ℃ for 24h. All chemicals and solvents used were of analytical grade.

Extraction, isolation and characterization of plant chemicals

The plant material (17 Kg) was air-dried, pulverised to 30~40 mm and extracted 5x with 95% ethanol at room temperature by immersion method. The material was filtered through filter paper and the filtrates were combined and evaporated to dryness under vacuum. The dried ethanol extracts was resuspended in water and then subjected to extraction and separation of components as shown in Fig.1.

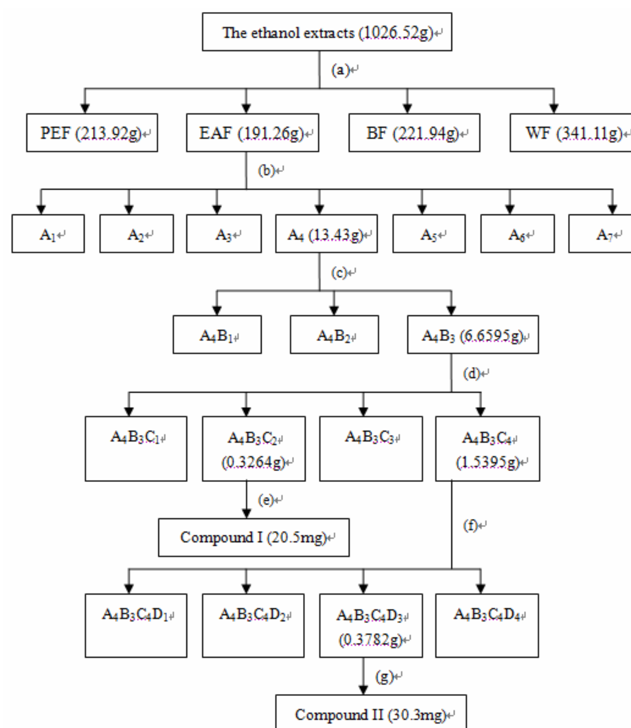

Figure 1. Schematic representation of franctionation procedure. PEF = Petroleum ether fraction, EAF = Ethyl acetate fraction, BF = n-Buthanol fraction, WF = Water fraction. Scheme: (a) 1). Suspended in water and then partitioned sequentially with petroleum ether, ethyl acetate and n-buthanol; 2). Collected separately and concentrated by a rotary evaporator to remove the solvent; (b) Silica gel column chromatography (100×10cm, 100-200 mesh), eluting with petroleum ether/acetone (95:5 → 0:100, v/v); (c) Silica gel column chromatography (50×5cm, 200-300 mesh), eluting with petroleum ether/acetone (80:20 → 50:50, v/v); (d) Silica gel column chromatography (40×2.5cm, 200-300 mesh), eluting with petroleum ether/ethyl acetate (80:20 → 50:50, v/v); (e) Silica gel column chromatography (30×1.5cm, 200-300 mesh), eluting with petroleum ether/ethyl acetate (5:1, v/v); (f) ODS column chromatography, eluting with MeOH-H_2O (30:70→90:10, v/v); (g) Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) column chromatography.

NMR spectra were obtained on a Bruker AVANCE III-500 spectrometer. Chemical shifts are given in δ (ppm) with respective solvent (pyridine-d_6 for compound Ⅰ, MeOD for compound Ⅱ) peaks as references. ESI-MS were measured on an Agilent 1100 Series LCQ Fleet mass spectrometer.

Determination of *in vitro* antifungal activity

The inhibition of fungal growth by various extracts and isolated compounds was studied by the hyphal radial growth assay with slight modifications (22). The ethanol extracts and various fractions to be tested and amphotericin B (standard) were dissolved in small amounts of DMSO and diluted in appropriate PDA medium (final concentration of DMSO < 1%), the total volume of the mixed medium was 10 ml in each plate. A 4 mm diameter disc of fungal inoculum was cut from the periphery of actively growing mycelium and placed in the centre of PDA plates (9 cm dia.) containing either ethanol extracts (10 mg/ml), four fractions (0.1-5.0 mg/ml), the two isolated compounds (50 μg/ml) and Amphotericin B (50 μg/ml). All the inoculated plates were incubated at 27 °C for 72h. The PDA plates without any test material were inoculated with the same fungi served as negative control and amphotericin B was used as positive control. There were three replicates for each combination of tested fungi and sample concentrations. The mean diameter of mycelial growth was recorded at the end of the incubation period and the inhibition (%) of growth was calculated as under (19):

$$\text{Mycelial inhibition (\%)} = (dc - dt) / dc \times 100$$

Where, dc: Mean diameter of fungal colony control dt: Mean diameter of fungal colony with treatments. EC_{50} value (mg/ml): Effective concentration at which the mycelial growth of fungus was inhibited by 50% and was obtained from the interpolation of logarithm regression analysis.

Determination of *in vitro* antibacterial activity

To determine the antibacterial activity of isolated compounds I and II, the disc-diffusion assay was used with slight modifications (14). Plates were prepared by pouring 10 ml of beef-extract peptone agar medium into sterile Petri plates (9 cm dia) and allowed to set. Molten beef-extract peptone agar held at 48℃ was inoculated with a broth culture (10^6~10^8 bacterial /ml) of the test bacterial strains and poured over base plates forming homogenous top layer. The two isolated compounds I and II and Norfloxacin (control) were dissolved in small amounts of DMSO and diluted with the appropriate quantity of sterile distilled water to reach final concentrations of 1mg/ml (final concentration of DMSO < 1%). The filter paper discs (6 mm dia) were sterilized by autoclaving and then soaked in 10μl of test solutions and solvent control. The impregnated discs were placed on the seeded top layer in plates. The Petri-dishes were inverted and incubated at 37℃ for 24h. Control plates had discs without test materials and served as negative control and Norfloxacin served as positive control. The average diameter of inhibition zones around the discs were measured visually (did not include the diameter of paper disc). The experiments were done in triplicate and results were expressed as mean ± S.D. of three experiments.

Statistical analysis

The data was expressed as the mean ± SD (standard deviation) and is the average of 3 values per experiment. Analyses were done using SPSS statistical programme (Version 13.0, SPSS Inc, Chicago, USA) and results with $P < 0.05$ were considered to be

statistically significant. When appropriate, the means were separated by using Duncan's test (P < 0.05) The EC_{50} was estimated for each fungus and each sample by using Probit analysis (SPSS statistic program, Version 13.0, SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Antifungal activities of ethanol extracts and fractions

In the primary screening study (Fig. 2), the antifungal activity of ethanol extracts (EE) against 12-plant pathogenic fungi was examined. The EE was inhibitory to all fungi tested to varying degree. However, its 10 mg/ml concentration was strongly inhibitory to *R. cerealis* (88.89±1.37 %), *B. cinerea* (78.00±2.00 %) and *A. alternata* (74.08±1.54 %) and moderately to *B. sorokiniana* (58.03±1.07 %).

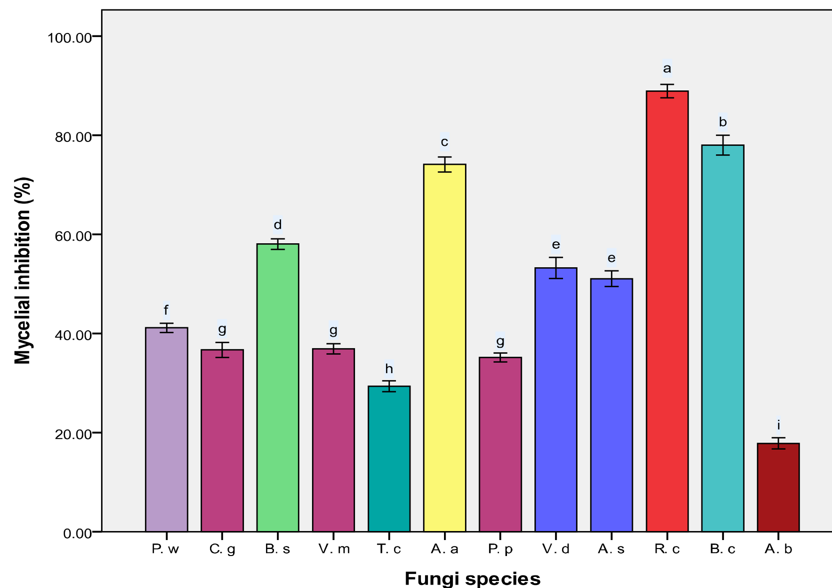

Figure 2. Antifungal activity of the ethanol extracts (10mg/ml) from *P. fruticosa* against 12 plant pathogenic fungi. *R.c.=Rhizoctonia cerealis, V.d.=Verticillium dahliae, B.s.=Bipolaris sorokiniana, A.s.=Alternaria solani, A.a.=Alternaria alternate, B.c.=Botrytis cinerrea, V.m.=Valsa mali, P.w.=Phacidiopycnis washingtonensis, A.b.=Alternaria bressicae, C.g.=Colletotrichum gloeosporioides, T.c.=Thanatephorus cucumeris, P.p.=Physalospora piricola*. Each value is the mean of three replications ± SD (standard deviation). For each fungal species, different letters (a-i) represent values that are significantly different according to Duncan's test (P<0.05).

To determine the most active component of ethanol extracts, its 4-fractions (petroleum ether, ethyl acetate, n-butanol and water fraction) were examined against the growth of 4-fungi (*R. cerealis*, *B. cinerea*, *A. alternate*, *B. sorokiniana*) (Table 2). Among the 4- fractions, the ethyl acetate fraction (EAF) and the n-butanol fraction (BF) showed

Table 2. Antifungal activity of ethanol extract (EE) and four solvent fractions (PEF, EAF, BF and WF) from *P. fruticosa* against 4- phytopathogenic fungi

Fractions	Fungal species [EC_{50} (mg/ml)]			
	B. cinerea	*R. cerealis*	*B. sorokiniana*	*A. alternata*
Ethanol extracts	0.978 a	0.806 b	1.417 a	1.167 a
Petroleum ether	2.982 b	1.867 c	3.750 b	3.272 b
Ethyl acetate	0.540 a	0.333 a	0.889 a	0.708 a
n-Buthanol	0.728 a	0.502 a, b	1.083 a	0.849 a
Water	3.130 b	2.137 c	4.058 b	3.677 b
Amphotericin B	0.079 a	0.044 a	0.130 a	0.107 a

The EC_{50} values in mg/ml were estimated for each fungal species and each sample by using Probit analysis. Columns, for each fungal species, having different letters (a - c) represent values that are significantly different according to Duncan's test (*P<0.05*).

strong antifungal activity than others against the tested fungi (*P<0.05*). The EC_{50} values for EAF against *R. cerealis, B. cinerea, A. alternata* and *B. sorokiniana* were 0.333, 0.540, 0.708 and 0.889 mg/ml, respectively while the EC values for BF were 0.502, 0.728, 0.849 and 1.083 mg/m, respectively.

Among the tested fungi, the growth of *R. cerealis* was significantly inhibited by all fractions while *B. sorokininan* was least inhibited (Table 2). When the effects of various solvent fractions were compared on the four fungi, it was found that EAF and BF fractions were strongly inhibitory than PEF. The strong inhibitory effects of EAF and BF may be due to presence of the active components with higher biological activity.

These results indicated that the phytochemicals in *P. fruticosa* were inhibitory to plant pathogenic fungi and that the EAF of the ethanol fraction was most active fraction.

Elucidation of structure of the compounds isolated from *Potentilla futicosa*

To determine the most active components inhibitory to fungi, the EAF was subjected to further fractionation as shown (Fig. 1) to yield two compounds.

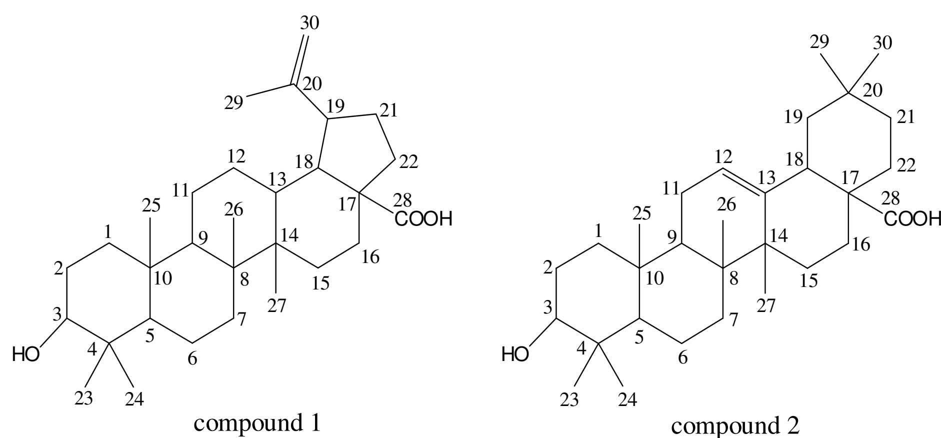

Figure 3. Structure of compound I and compound II

Compound I: It was obtained as white powder (Fig. 3). ESI-MS m/z (rel.int.): 455 [M-H]. According to the data showed in ESI-MS (m/z (rel.int.): 455 [M-H]), the molecular weight of compound I was 456. The ^{1}H-NMR spectrum of compound I showed signals for six methyl at δ_H =1.14(3H, s, H-23), 0.93(3H, s, H-24), 0.82(3H, s, H-25), 1.04(3H, s, H-26), 1.02(3H, s, H-27), 1.75(3H, s, H-29), and one ABX system at δ_H = 3.40(1H, dd, J=10.0, 5.0Hz, H-3), and terminal olefine at δ_H = 4.70(1H, d, J=1.6Hz, H-30a), 4.87(1H, d, J=1.6Hz, H-30b). The ^{13}C-NMR and DEPT spectra displayed 30 carbon signals including a carboxyl carbon, six quaternary carbons (one sp^2 and five aliphatic), six methane (one oxygenated and five aliphatic), eleven methylene (one olefinic and ten aliphatic) and six methyl groups. One methine carbon(δ_C =78.3) was ascribed to the carbon atom attached to one oxygen atom. ^{13}C-NMR(125MHz, *d$_6$*-pyridine): δ_C =39.4(CH_2, C-1), 28.3(CH_2, C-2), 78.3(CH, C-3), 39.6(C, C-4), 56.1(CH_2, C-5), 18.9(CH_2, C-6), 35.0(CH_2, C-7), 41.2(C, C-8), 49.9(CH, C-9), 37.7(C, C-10), 21.4(CH_2, C-11), 26.2(CH_2, C-12), 38.7(CH, C-13), 43.0(C, C-14), 30.4(CH_2, C-15), 33.0(CH_2, C-16), 56.8(C, C-17), 51.1(CH, C-18), 47.8(CH, C-19), 151.3(C, C-20), 31.3(CH_2, C-21), 37.7(CH_2, C-22), 28.8(CH, C-23), 16.4(CH_3, C-24), 16.6(CH_3, C-25), 16.6(CH_3, C-26), 15.1(CH_3, C-27), 179.1(COOH, C-28), 19.8(CH_3, C-29), 110.0(CH_2, C-30). Compound I was thus identified as betulinic acid by its physical and spectroscopic data. This is consistent with triterpenoid isolated from *Lycopus lucidus* Turcz. var. *hirtus* Regel. (24).

Compound II: It was obtained as white needle (Fig. 3). ESI-MS m/z (rel.int.): 455 [M-H]. According to the data showed in ESI-MS (m/z (rel.int.): 455 [M-H]), the molecular weight of compound II was 456. The ^{1}H-NMR spectrum of compound II showed signals for seven methyl at δ_H =1.15, 0.98, 0.94, 0.92, 0.91, 0.80, 0.78(3H, s, $CH_3\times7$), and one ABX system at δ_H =3.40(1H, dd, J=10.1, 5.3Hz, H-3), and one olefine proton at δ_H =5.28(1H, s, H-12). The ^{13}C-NMR and DEPT spectra displayed 30 carbon signals including a carboxyl carbon, seven quaternary carbons (seven aliphatic), five methane (one oxygenated and four aliphatic), eleven methylene (eleven aliphatic) and seven methyl groups. One methine carbon (δ_C =78.7) was ascribed to the carbon atom attached to one oxygen atom. ^{13}C-NMR(125MHz, MeOD): δ_C =38.5(CH_2, C-1), 27.6(CH_2, C-2), 78.7(CH, C-3), 38.6(C, C-4), 55.3(CH, C-5), 18.3(CH_2, C-6), 32.5(CH_2, C-7), 39.2(C, C-8), 47.6(CH, C-9), 36.9(C, C-10), 23.0(CH_2, C-11), 122.3(CH, C-12), 143.8(C, C-13), 41.7(C, C-14), 26.6(CH_2, C-15), 23.3(CH_2, C-16), 46.4(C, C-17), 41.2(CH, C-18), 45.9(CH_2, C-19), 30.6(C, C-20), 33.8(CH_2, C-21), 32.7(CH_2, C-22), 27.9(CH_3, C-23), 15.4(CH_3, C-24), 15.1(CH_3, C-25), 16.7(CH_3, C-26), 25.7(CH_3, C-27), 180.8(COOH, C-28), 32.9(CH_3, C-29), 23.3(CH_2, C-30). Compound II was therefore identified as oleanolic acid by its physical and spectroscopic data, which was consistent with the triterpenoid isolated from *Anemone tomentosa* (12).

Antimicrobial activities of isolated compounds I and II from *P. fruticosa*

The antimicrobial activity of two compounds isolated from *P. fruticosa* was examined against the four fungi and three bacteria.

Antifungal assay: In antifungal assay, compared with Amphotericin B(41.52±0.72%), compound I -betulinic acid, showed strong antifungal activity (46.49±0.94%), while the compound II - oleanolic acid, was slightly less active (41.44±0.78%) against *B. cinerea* at

concentration of 50 μg/ml (*P<0.05*). Both compounds were weakly antifungal than Amphotericin B against other fungi tested (Table 3).

Table 3. Antifungal activity of two triterpenoids isolated from *P. fruticosa* against 4 phytopathogens

Samples	Mycelial inhibition (%)			
	B. cinerea	*R. cerealis*	*B. sorokiniana*	*A. alternata*
Betulinic acid I	46.49±0.94 a	0 a	0 a	0 a
Oleanolic acid II	41.44±0.78 b	0 a	1.39±0.83 a	0 a
Amphotericin B	41.52±0.72 b	55.56±0.00 b	29.01±1.07 b	31.05±0.66 b

Antifungal activity of Amphotericin B (50μg/ml) and two triterpenoids (50μg/ml) was determined. Columns, for each fungal species, having different letters (a, b) represent values that are significantly different according to Duncan's test (*P*<0.05).

Anti bacterial assay: In the antibacterial assay, both the compounds at l mg/ml, inhibited the *E coli* but *B.subtilis* was inhibited only by betulini acid. Neither of the compounds inhibited the *S. aureus* (Table 4). The negative control (1% DMSO) didn't show any inhibition, while antibiotic control (Norfloxacin) showed strong inhibition.

Table 4. Antibacterial activity of two triterpenoids isolated from *P. fruticosa* against 3-bacteria

Compounds	Inhibition zone (mm)		
	E. coli	*B. subtilis*	*S. aureus*
Betulinic acid I	6.73±0.64 a	3.47±0.57 a	0 a
Oleanolic acid II	5.50±0.44 b	0 b	0 a
Norfloxacin	32.50±0.50 c	27.83±0.29 c	28.87±0.35 b

Antibacterial activity of Norfloxacin (1mg/ml) and two triterpenoids (1mg/ml) was determined. Columns, for each bacterial strain, having different letters (a-c) represent values that are significantly different according to Duncan's test (*P*<0.05).

CONCLUSIONS

This study showed that the phytochemicals from *P. fruticosa* were strongly antifungal against some plant pathogenic fungi. Two known triterpenoids, namely betulinic acid and oleanolic acid were isolated from the ethyl acetate fraction. This is the first report of their isolation from *P. fruticosa* . These two compounds exhibit strong antifungal activity against *B. cinerea* at concentration of 50μg/ml but had variable antibacterial activity. The antifungal activity was due to the presence of betulinic acid and oleanic acid. This plant material therefore has potential as source of antifungal compounds.

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