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Antifungal potential of two new triterpenoidal glycosides from the *Albizia kalkora* against *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

We isolated two new triterpenoidal glycosides from *Albizia kalkora* (Roxb.) Prain and assessed their fungicidal effects against plant pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causing wilt in tomato crop. The new triterpenoidal glycosides [albizioside A **(1)** and albizioside B **(2)]** were isolated from the chloroform soluble fraction of *A. kalkora* extract. Their structures were elucidated by IR, 1D and 2D-NMR and mass spectrometry. *In-vitro* antifungal studies showed that compound **1** had promising antifungal properties causing 89.3 % growth inhibition as compared to 90.0 % inhibition by synthetic fungicide benomyl at 200 mg mL^{-1}. Compound **2** showed 73.3 % inhibition in fungal growth at the same concentration. This study concluded that both the phytochemicals may be developed as natural fungicides to avoid the harmful effects of synthetic fungicides. Furthermore, derivatives of both the phytochemicals may also enhance their antifungal properties.

Key words: Albizioside A, albizioside B, *Albizia kalkora,* 1D and 2D-NMR, extract, Fabaceae, *Fusarium oxysporum,* fungicides, phytochemicals, tomato, triterpenoidal glycosides.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a major economic crop and a model plant for research (29). The soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* causes vascular wilt in tomato causing huge economic losses (16,23). The pathogen is difficult to control through plant breeding due to its ability to develop strategies to overcome plant resistance (2). Generally, soil-borne diseases are difficult to control. Moreover, synthetic fungicides used to control plant pathogens also pollute the environment (28). Hence now scientists are exploring new environment-friendly strategies to control soil-borne plant pathogens (4,24,27). Among these, the use of natural antifungal compounds from plants is gaining much importance against soil-borne phytopathogens (7,18,19).

Albizia kalkora (Roxb.) (Fabaceae family), is a deciduous plant of tropical and subtropical regions (Figure 1). *Albizia* is a major genus of this family that has about 167 species distributed worldwide (1,10,11). It is 3-8 m tall tree native to Asia. In Pakistan, it is common in Bahawalpur region (17). It is used in Chinese herbal medicine to treat bacterial and viral infections, burns, swellings, bruises, nasal hemorrhage and inflammations. It is also used as remedy for dog bites and bee stings (21). The pharmacological and phytochemical studies showed that the plant has many biological active constituents inhibitory against fungal pathogens (8). This study aimed to investigate phytochemical components of *A. kalkora* extract and their antifungal potential against *F. oxysporum* f. sp. *lycopersici*.

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Figure 1. *Albizia kalkora*

MATERIALS AND METHODS

Collection of plant material

The studies were conducted at School of Chemistry, University of Punjab Lahore, and HEJ, University of Karachi, Pakistan during 2020 and 2021. Different parts of *A. kalkora* (15 kg) were collected from Cholistan Desert, Bahawalpur, Pakistan (28.5° N, 70.5° E), altitude : 112 m, annual rain fall : 100-250 mm, maximum and minimum temperatures : 50 °C and 0 °C, respectively (15). The taxonomical verification of the plant was confirmed from Cholistan Institute of Desert Studies (CIDS), and The Department of Botany, The Islamia University Bahawalpur.

Extraction and isolation

The collected plant material was dried, crushed, ground and extracted with methanol. The methanolic extract was evaporated under reduced pressure and the gummy material (0.5 kg) was partitioned between water, *n*-butanol, ethyl acetate, chloroform and *n*-hexane soluble fractions. The chloroform fraction of *A. kalkora* was chromatographed over silica gel in a column, which was eluted with *n*-hexane-chloroform and chloroform-methanol mixtures with increasing polarity to obtain 15 major fractions labeled as A^1-A^{15} (A=*Albizia*). Fraction-A^5 was assessed by TLC to check how many spots were present as well as its retardation (R_f) values. Fraction-A^5 showed four major spots on TLC and this fraction was re-chromatographed over silica gel column chromatography eluting with solvent system *n*-hexane-chloroform and re-chromatographed over PTLC with solvent system *n*-hexane-ethyl acetate (4.5:5.5), finally obtained two pure compounds **1** and **2**.

Sugar analysis and acid hydrolysis of glycosides/saponins

The glycosides were hydrolyzed by chemical method. Compounds **1** and **2** (5 mg of each) glycoside and 10 % HCl aq. were refluxed for 3.0 h at 100 °C, cooling, separate aglycone moiety, recrystallized from chloroform and identified further by comparison with their spectroscopic data. The aqueous hydrolysate was recrystallized with silver carbonate and concentrated. The sugar was found to be D-glucose and L-rhamnose in compound **1** and two molecules of D-glucose in compound **2** by using different solvent systems.

Compound 1: Albizioside A : Colourless amorphous solid; M.P. 283-285 ^oC; $[\alpha]_D^{20}$ +77.5 (*c*= 0.2, $CHCl_3$); soluble in chloroform, ethyl acetate, acetone and methanol; IR (KBr) ν_{max} cm^{-1}: 3380(OH), 1720 (C=O), 1660 (C=C), 1074(C-C), 820(C-H bending) ; **EIMS:** *m/z* (rel. int. %): $[M\text{-Glycosides}]^+$ 488 (25), 470 (22), 458 (53), 452 (23), 437 (43), 422 (31), 375 (20), 264 (69), 226 (75),133 (23) , 91 (23), 45 (15); **FABMS:** $[M+1]^+$ at *m/z* 797, 651, 489**;** **FAB-HRMS:** $[M+1]^+$ at *m/z* 797.4630 (Calculated for $C_{42}H_{69}O_{14,}$ 797.4706); ^{1}H-NMR (400 MHz, C_5D_5N) and ^{13}C-NMR (100 MHz, C_5D_5N) see Table 1.

Compound 2: Albizioside B : Colourless gummy solid ; M.P. 272-274 ^oC; $[\alpha]_D^{20}$ +62.5 (c= 0.2, $CHCl_3$); IR (KBr) ν_{max} cm^{-1} : 3375(OH), 1720 (C=O), 1650 (C=C), 1074 (C-C), 820 (C-H bending); **EIMS:** *m/z* (rel. int. %): $[M\text{-glycosides}]^+$ 486 (12), 469 (35), 441 (66), 422 (34), 370 (54), 262 (89), 224 (91), 91 (100), 45 (25); **FABMS:** $[M+1]^+$ at *m/z* 811, 649, 487; **FAB-HRMS:** $[M+1]^+$**:** at *m/z* 811.3611 (Calculated for $C_{42}H_{67}O_{15}$ 811.9681); ^{1}H-NMR (400 MHz, C_5D_5N) and ^{13}C-NMR (100 MHz, C_5D_5N) (Table 1).

Instrumentation and characterization

Melting point apparatus model Buchi 535 was used to examine the melting points of extracted compounds with the help of glass capillaries. The optical rotations of purified compounds were measured by digital polarimeter (JASCO DIP-360). All the chemicals for antimicrobial activity were purchased from Sigma Aldrich (MO, St. Louis, USA). The ultra violet spectrophotometer (Hitachi U-3200) was used to obtain the UV Spectra. Infrared spectra were recorded on infrared spectrophotometer (Agilent Technologies Cary-630). Nuclear Magnetic Resonance spectrometers (Bruker AM-400 and AM-500) were used to record ^{1}H-NMR in $CDCl_3$, C_5D_5N and CD_3OD using TMS as standard and ^{13}C -NMR spectra in $CDCl_3$, CD_3OD and C_5D_5N at 100 and 125 MHz whereas 2D-NMR (COSY, NOESY, HMQC and HMBC) were recorded in $CDCl_3$, CD_3OD and C_5D_5N at 400-500 MHz in the same instrument. Silica gel by E-Merck (40-400 mesh) was used to perform column chromatography. E-Merck 20x20 cm, 0.5mm thick Pre-coated silica gel GF-254 preparative plates were used for thin layer chromatography. Pre-coated PR-18 gel (E-Merck) glass was used for preparative TLC. GC-18A equipped with FID was used for Glc. Standard carbohydrates (D- Glucose and L-rhamnose) were purchased from Sigma Chemical Corporation (St. Louis Missouri, USA).

Antifungal bioassay

Fusarium oxysporum f. sp. *lycopersici* (FOL) was isolated from infected tomato roots collected from tomato fields in village Okara Punjab, Pakistan (30.68^o N, 73.74^o E), altitude: 112 m, annual rain fall: 509 mm. Warm dry climate with mean maximum and minimum temperatures as 45 ^oC and 3 ^oC, respectively. Tomato roots were surface sterilized with 1 % $NaOCl_{(aq)}$ followed by several washings with d.H_2O. Potato dextrose agar (PDA) growth medium was used for culturing and subsequent purification of FOL at 27 ± 1 ^oC for 10 days (3). Microscopic morphological characters were identified as stated in the literature (26)**.** Purification and identification of the fungus was done at Department of Plant Pathology, University of the Punjab, Lahore, Pakistan.

Two treatments of isolated phytochemicals and two controls (benomyl as synthetic fungicide (+ve control) and without any compound or fungicide (–ve control) were designed. Eight concentrations (1.5625 3.125, 6.25, 12.5, 25, 50, 100 and 200 mg mL^{-1}) of each compound were tested individually, replicated thrice in completely randomized design to assess their effects on fungal growth using food poison technique. Fungal discs (3 mm) from 7-days old FOL culture plate were inoculated on poisoned PDA plates and incubated in Wise Cube temperature-controlled incubator at 27 ± 1 °C. Average diameter of all replicates from each treatment was measured on 8th day when plates were covered with FOL mycelia in –ve control. Colony morphology and microscopic characters were noted and FOL growth was assessed by comparing with uninduced FOL culture (6).

$$\text{Growth Inhibition (\%)} = \frac{\text{(Fungal Growth in Control)} - \text{(Fungal Growth in treatments)}}{\text{Fungal Growth in Control}} \times 100$$

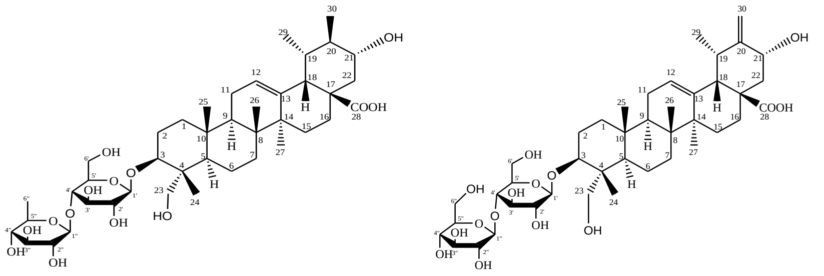
Statistical analysis

Standard errors were calculated for growth inhibition measured for three set of experiments in each case. These means were statistically compared by one-way ANOVA using computer software Minitab 17.

RESULTS AND DISCUSSION

Compound 1 - Albizioside A

The colourless solid (powder) compound **1** was isolated from *A. kalkora* and purified by sophisticated chromatographic techniques. The structure was elucidated by physical, chemical and spectroscopic analysis. The melting point and $[\alpha]_D^{23}$ were determined as 283-285 °C and +77.5° (c = 0.2, $CHCl_3$) respectively. Chemical analysis showed positive bromine water test for unsaturation, litmus and ferric chloride test indicated the presence of carboxylic acid moiety, whereas, salkowki's test and Liebermann-Burchard's test confirmed the triterpenes nature. The glycosidic nature of compound **1** was confirmed by Molisch's test (14).



Albizioside A (**1**) Albizioside B (**2**)

Figure 2. Structures of new compounds isolated from *Albizia kalkora*

The FT-IR spectroscopic analysis showed strong adsorption band for hydroxyl moiety at 3380 cm^{-1}, carbonylic moiety at 1720 cm^{-1} and olefinic (C=C) moiety at 1660 cm^{-1} The FAB-HRMS gave $[M+1]^+$ at m/z = 797.4630 (Calcd. for $C_{42}H_{69}O_{14}$ 797.4706). The

FAB mass spectrum showed prominent peaks at *m/z* 797, 651, 489 and EIMS [M-Glycosides]$^+$ gave retro Diels Alder cleavage of ring C at *m/z* 266 and *m/z* 224(19,8).

Table 1. The ^{1}H-NMR- (400 MHz) and ^{13}C-NMR- (100 MHz) data of isolated compound11 and 2- in C_5D_5N with- δ- in ppm and- J -in Hz

Carbon Position	**Compound 1**		**Compound 2**	
	^{1}H-NMR(δ)	**^{13}C-NMR(δ)**	**^{1}H-NMR(δ)**	**^{13}C-NMR(δ)**
1	1.49 (m)	37.2	1.49 (m)	37.2
2	1.71 (m)	26.9	1.71 (m)	26.9
3	3.18 (dd, J = 9.4, 6.3 Hz)	72.9	3.72 (dd, J = 11.0, 7.0 Hz)	72.9
4	-	42.0	-	42.0
5	0.80 (m)	48.1	0.80 (m)	48.1
6	1.45 (m)	17.9	1.45 (m)	17.9
7	1.39 (m)	32.8	1.39 (m)	32.8
8	-	40.1	-	40.1
9	1.69 (dd J = 10.9, 6.8 Hz)	45.7	1.69 (dd J = 10.9, 6.8 Hz)	45.7
10	-	36.5	-	36.5
11	2.20 (m)	22.5	2.20 (m)	22.5
12	5.52 (t , J = 6.0 Hz)	124.0	5.52 (t , J = 6.0 Hz)	124.0
13	-	140.1	-	140.1
14	-	43.1	-	43.1
15	1.88 (m)	28.6	1.88 (m)	28.6
16	1.94 (m)	29.2	1.94 (m)	29.2
17	-	48.9	-	48.9
18	2.99 (d, J = 4.4 Hz)	51.5	2.99 (d, J = 4.4 Hz)	51.5
19	1.68 (d, J = 4.4 Hz)	39.6	1.68 (d, J = 4.4 Hz)	39.6
20	1.23 (m)	36.3	-	150.2
21	4.30 (dd J = 6.0, 4.2 Hz)	69.1	4.30 (dd J = 6.0, 4.2 Hz)	69.1
22	2.16 (dd J = 6.1, 4.2 Hz)	39.0	2.16 (dd J = 6.1, 4.2 Hz)	39.0
23	3.35 (d, J = 11.3 Hz), 4.00 (d, J = 11.3 Hz)	64.6	3.35 (d, J = 11.3 Hz), 4.00 (d, J = 11.3 Hz)	64.6
24	1.07 (s)	21.5	1.07- (s)	21.5
25	0.82 (s)	24.5	0.82- (s)	24.5
26	1.24 (s)	22.4	1.24- (s)	22.4
27	1.04 (s)	20.3	1.04- (s)	20.3
28	-	180.6	--	180.6
29	1.06 (d, J = 6.5 Hz)	17.0	1.06 (d, J = 6.5 Hz)	17.0
30	0.99 (d, J = 10.2 Hz)	18.8	4.98- (d, J = 10.2 Hz)	109.1
1′	5.92 (d, J = 7 .1 Hz)	102.67	5.56- (d, J = 7.2 Hz)	102.67
2′	4.31 (m)	79.52	4.33- (m)	79.52
3′	4.26 (m)	75.28	4.22 - (m)	75.28
4′	3.60 (m)	74.81	3.52 - (m)	74.81
5′	3.65 (dd, J = 11.5, 4.2 Hz)	71.52	4.56- (dd, J = 11.5, 4.2 Hz)	71.52
6′	4.42 (dd, J = 11.5, 4.1 Hz)	62.20	4.38- (dd, J = 11.5, 4.1 Hz)	62.20
1″	5.92 (d, J = 6.8 Hz)	98.0	5.98- (d, J = 7.1 Hz)	98.26
2″	4.92 (m)	79.79	4.88- (m)	78.39
3″	4.92 (m)	76.12	4.87 - (m)	76.39
4″	4.92 (m)	71.05	4.88- (m)	65.88
5″	4.20 (m)	71.23	4.16- (dd, J = 11.4, 4.1 Hz)	71.65
6″	1.60 (d, J = 6.9 Hz)	19.59	1.54- (dd, J = 11.4, 4.1 Hz)	60.69

The proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy exhibited that four tertiary methyls were observed as singlet (s) peak at δ 1.07, 0.82, 1.24, and 1.04 of each protons, while two doublets were seen for two methyls at *δ* 1.06 (*J* = 6.5 Hz) and 0.99 (*J* = 10.2 Hz). The olefinic proton appeared as triplet peak at *δ* 5. 52 (*J* = 6.0 Hz, H-12) as characteristics for Δ^{12} for ursane class of compounds. The hydroxy methine protons were observed at *δ* 4.30 (dd *J* = 6.0, 4.2 Hz, H-21) and *δ* 3.35 (d, *J* = 11.3 Hz), 4.00 (d, *J* = 11.3 Hz, H-23). The characteristic NMR signals for anomeric protons were observed as doublet at *δ* 5.01 (*J* = 7 .1 Hz, H- 1′) and *δ* 5.92 (*J* = 6.8 Hz, H- 1″).

The hydroxymethine signals of sugar moiety resonated at *δ* 4.99-3.65 (8H, m). A characteristic methyl signal in a glycosidic moiety was appeared at *δ* 1.60 (d, *J* = 6.9 Hz). Acid hydrolysis of compounds **1** also yields L-rhamnose and D-glucose as carbohydrate components and white amorphous powder of aglycone**.** The above evidences clearly indicated that compound **1** was clearly evident from the loss of 162 amu from the molecular ion peak and the loss of 178 amu from the resulting fragment in positive ion of FAB mass spectrum that the L-rhamnose was attached with D-glucose which itself was attached to aglycone. The HMBC and H-H COSY spectra determine the sugar linkage sequence. In HMBC spectrum J^3 correlation of *δ* 5.01(H-1′) with *δ* 84.05 (C-3), *δ* 5.92 (H- 1″) with *δ* 74.81(C-4′) and *δ* 1.60 (H-6″) with *δ* 71.05 (C-4″) were observed. The absolute configuration of glucose and rhammnose were assumed to be D and L respectively because only these forms are found in terpenoid glycoside. These results confirmed the structure of compound **1** (Albizioside A) as 3*β*, 21*α*, 23*α*-trihydroxy ursane-12(13), 20(30)-diene-28-oic, 3-O-*β*-D-glycopyranosyl- (4→1)-α-L-rhamnopyranoside. Structure of Albizioside A is presented in Figure 2.

Compound 2 - Albizioside B

The colourless gummy solid compound **2** was isolated from *A. kalkora* and purified by sophisticated chromatographic techniques. The structure was elucidated by physical, chemical and spectroscopic analysis. The melting point and $[\alpha]_D^{23}$ were 272-274 °C and +62.5° (*c* = 0.2, $CHCl_3$), respectively. Chemical analysis showed positive bromine water test for unsaturation, litmus and ferric chloride test indicated the presence of carboxylic acid moiety whereas salkowki’s test and Liebermann-Burchard’s test confirmed the triterpenes nature. The glycosidic nature of compound **2** was confirmed by Molisch’s test (14).

The FT-IR spectroscopic analysis showed strong adsorption band for hydroxyl moiety at 3375 cm^{-1}, carbonylic moiety at 1720 cm^{-1} and olefinic (C=C) moiety at 1650 cm^{-1}. The FAB-HRMS gave $[M+1]^+$ at *m/z* = 811.3611 (Calcd. for $C_{42}H_{67}O_{15}$ 811.9681). The FABMS spectrum showed prominent peaks at *m/z* 811, 649 and 487 confirmed two sugar moieties and EIMS $[M\text{-glycosides}]^+$ retro Diels Alder cleavage of ring C at *m/z* 262 and *m/z* 224 confirmed the position of olefinic moiety (8,19)**.**

The proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy exhibited that four tertiary methyls were observed as singlet (s) peak at *δ* 1.07, 0.82, 1.24, and 1.04 of each three protons while two doublets were seen for two methyls at *δ* 1.06 (*J* = 6.5 Hz) and 4.98 (*J* = 10.2 Hz). The one olefinic proton appeared as triplet peak at *δ* 5. 52 (*J* = 6.0 Hz) at (H-12) and another olefinic protons observed at *δ* 4.98 (d, *J* = 10.2 Hz, H-30) characteristics for Δ^{12} for ursane class of compounds. The hydroxy methine proton were observed at *δ* 4.30 (dd *J* = 6.0, 4.2 Hz, H-21) and *δ* 3.35 (d, *J* = 11.3 Hz), 4.00 (d, *J* = 11.3 Hz, H-23). The characteristic NMR signals for anomeric proton were observed as doublet at *δ* 5.01 (*J* = 7 .2 Hz, H- 1′) and *δ* 5.98 (*J* = 6.8 Hz, H- 1″). The hydroxymethine signals of disaccharides appeared at *δ* 4.88-3.52 (8H, m). A characteristic methyl signal in a glycosidic moiety was appeared at δ 1.54 (dd, J = 11.4,4.1 Hz). Acid hydrolysis of compounds **2** yields two molecules of D-glucose as carbohydrate components and white amorphous powder of aglycone.

The above data clearly indicated that compound **2** was clearly evident from the loss of 178 amu from the molecular ion peak and the loss of 178 amu from the resulting fragment in positive ion of FAB mass spectrum that the D-glucose was attached with D-glucose which itself was attached to aglycone. The HMBC and ^{1}H-^{1}H COSY spectra determine the sugar linkage sequence. In HMBC spectrum J^3 correlation of δ 5.01 (H-1′) with δ 72.9 (C-3), δ 5.98 (H- 1″) with δ 74.81 (C-4′) and δ 1.60 (H-6″) with δ 71.05 (C-4″) were observed. The absolute configuration of both glucose moieties was assumed to be D configuration because only this form of glucose is found in terpenoid glycoside. These results confirmed the structure of compound **2** (Albizioside B) as 3β, 21α, 23α-trihydroxy-12(13)-ursene-28-oic, 3-O-β-D-glycopyranosyl- (4→1)-α-D-glucopyranoside. Structure of albizioside B is presented in Figure 2.

Antifungal bioassay of isolated phytochemicals

Antifungal analysis revealed the highly significant inhibitory effects of isolated phytochemicals on FOL growth (25). Ten concentrations of each phytochemical and

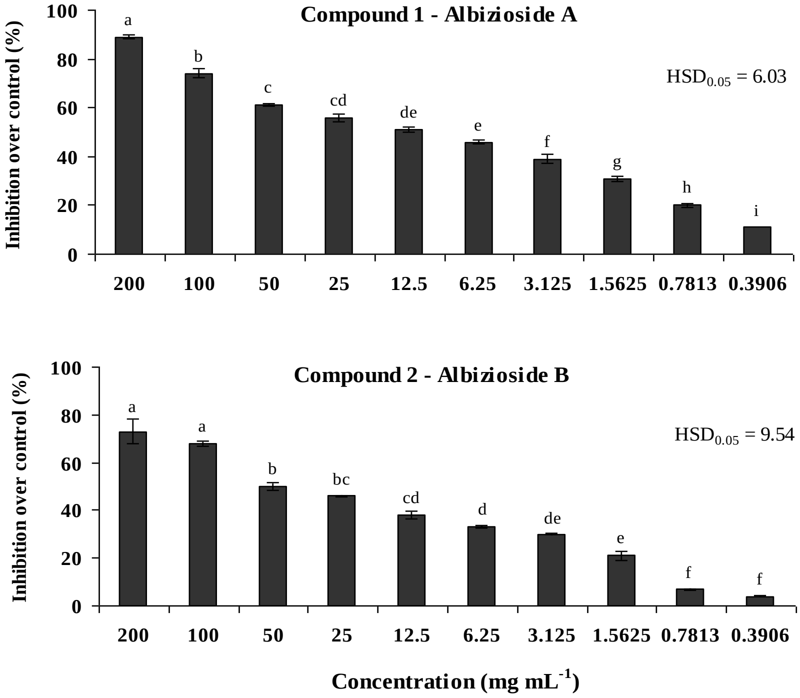


Figure 3. Inhibitory potential of various concentrations of compound **1** and **2** against growth of *Fusarium oxysporum* f. sp. *lycopersici.* Vertical bars standard errors of means. Different letters on bars show significant difference (P = 0.05) as determined by Tukey's HSD Test.

synthetic fungicide were treated against FOL. In general, all the concentrations of both phytochemicals significantly suppressed the growth of FOL. Generally, inhibition in growth due to phytochemicals was gradually increased by increasing their concentrations. Compound **1** showed 89.33 % inhibition as same as benomyl 90 % at 200 mg mL^{-1} concentration, while, compound **2** showed 73.3 % inhibition at the same concentration (Figure 3) (13). Correlation between antifungal activity and chemical structure of isolated phytochemicals showed that compound **1** [3*β*, 21*α*, 23*α*-trihydroxy ursane-12(13), 20(30)-diene-28-oic, 3-O-*β*-D-glycopyranosyl- (4→1)-α-L-rhamnopyranoside] and compound **2** [3*β*, 21*α*, 23*α*-trihydroxy-12(13)-ursene-28-oic, 3-O-*β*-D-glycopyranosyl- (4→1)-α-D-glucopyranoside] affected the growth of FOL due to the presence of hyroxy group (C-21), carboxylic moiety (C-17) and sugar moieties. It may be predicted that these triterpenoids saponins attacked the fungal cell wall and cell membrane (composed of *β*-1, 3-glucan, squalene, ergosterol, *β*-1, 6-glucan and chitin). Furthermore, these moieties can interact with enzymatic mode of action, which involves the fungus reproduction. The hydrogen bonding and hydrophobic interactions between ligands (phytoconstituents) and FOL enzymes play key role to control the growth of FOL. Lower inhibition of compound 2 two may be less interactions of ligand and enzymes (22).

CONCLUSIONS

In this study, two new saponins, albizioside A and albizioside B were isolated from *A. kalkora*. These saponins exhibited potent inhibitory activity against *F. oxysporum* f. sp. *lycopersici* when compared with standard fungicide. Albizioside A was highly antifungal and its efficacy was similar to synthetic fungicide benomyl. It reduced the fungal growth by 89. % as compared to 90.0 % reduction by the fungicide. However, the Albizioside B was less effective and suppressed fungal growth only by 73.3 %.

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We are thankful to Dr. Imran Anjum, Department of Chemistry, Govt. Sadiq Egerton College Bahawalpur, Punjab, Pakistan who helped us to collect plant and its taxonomical verification. Department of Plant Pathology, Faculty of Agricultural Sciences, University of Punjab Quid-i-Azam Campus, Lahore provided us facilities to perform antifungal activities.

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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