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Antimicrobial Chalcones from the Seeds of *Persicaria lapathifolia*

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Abstract

Bioassay-guided sequential extraction of the seeds of *Persicaria lapathifolia* followed by chromatographic separations resulted in the identification of three chalcones; flavokawain B (1), pinostrobin chalcone (2) and pashanone (3). The structures of the isolated compounds were established based on NMR and MS spectroscopic data. Disk diffusion method was employed to evaluate the antibacterial and antifungal activities of the isolated compounds against two bacterial and four fungal strains. The chloroform extract and the compounds demonstrated significant antibacterial activities. Compound 2 showed the highest activity against *Staphylococccus aureus* bacterial strain and *Trichoderma* spp fungal strain. Its antifungal activity (22 mm) against *Trichoderma* spp is even greater than that of the reference drug, clotrimazole (20 mm).

Keywords: Polygonaceae; *Persicaria lapathifolia*; Seeds; Chalcones; Antibacterial; Antifungal

Introduction

Infectious diseases are the major public health problem in the world. Despite the enormous resources expended during the last four decades through the use of high-throughput screening and combinatorial chemistry, genomics, and vaccine development to combat these diseases, it remains a public health and economic problems because of the emerging of resistance pathogens that limits the therapeutic uses of many of the drugs that are in the market [1,2]. In this era of emerging of resistance pathogens, the discovery of new lead compounds with novel mechanism of action from natural source cannot be over emphasized, especially from plants which have documented traditional uses to treat these diseases [3].

Persicaria lapathifolia (family Polygonaceae) is among the medicinal plant that has been commonly visited by traditional healers in some parts of Ethiopia. Despite the wide usages of this plant in the traditional circles for the treatment of various ailments, the phytochemical information pertaining to the seeds of this plant and its microbial activity has not been addressed. Therefore, as part of our ongoing program in search for new antimicrobial compounds from African traditional medicinal plants [4,5], here we report the isolation of three compounds (1-3) along with their antibacterial and antifungal activities from the seeds of *P. lapathifolia*.

Results and Discussion

The seed of *P. lapathifolia* was sequentially extracted with chloroform and methanol. The chloroform extract was subjected to column chromatography for further purification following its promising antibacterial activity, and afforded three compounds 1-3, (Figure 1).

Compound 1 was isolated as yellow amorphous solid from the fraction eluted with 5% ethyl acetate in petroleum ether. The positive mode ESI-MS spectrum showed molecular ion peak of *m/z* 307 $[M+Na]^+$ and 591 $[2M+Na]^+$, both corresponding to the molecular formula $C_{17}H_{16}O_4$, which indicated ten degrees of unsaturation. The UV (λ_{max} 219 and 342 nm) spectrum revealed absorptions for a conjugated carbonyl moiety.

The ^{1}H NMR spectrum showed signals for ten protons (Table 1) including the highly downfield shifted signal at δ_H 14.32 for phenolic hydroxy group involved in hydrogen bonding. The presence of a typical carbonyl carbon signal at δ_C 192.7 in ^{13}C NMR spectrum and a *trans*-oriented double bond at δ_H 7.79 (1H, *d*, *J* = 15.6 Hz, H-α) and 7.91 (1H, *d*, *J* = 15.6 Hz, H-β) confirmed the existence of chalcone skeleton. The presence of five mutually coupled multiplet aromatic protons at δ_H 7.39-7.60 confirmed by HMBC and COSY analyses has indicated the presence of mono-substituted aromatic ring A. In ring B, two upfield shifted (due to di-*ortho* oxygenation) and *meta* coupled proton signals at δ_H 5.96 (1H, *d*, 2.0) and 6.10 (1H, *d*, 2.0) were assigned to H-3' and H-5', respectively, which otherwise fully substituted with two methoxy (δ_H 3.90 and 3.82) and one hydroxy (δ_H 14.32) groups.

The ^{13}C NMR spectrum (Table 1) showed signals for 17 carbon atoms, accounted for two methoxy groups, nine methine and six quaternary carbon atoms. The position of the methoxy groups, δ_H 3.82 and 3.90 were established at C-4' (δ_C 168.5 and C-6' (δ_C 166.5), respectively based on their HMBC correlations to vicinal carbon atoms. Whereas, the hydroxy group (δ_H 14.32) involved in hydrogen bonding was placed at C-2' (δ_C 162.6) *peri* to the carbonyl group. These data are consistent with the compound being 2'-hydroxy-4',6'-dimethoxychalcone; trivial name, flavokawain B, which was previously reported from *P. ferrugineum* [6].

The second compound (2) was isolated as red amorphous solid. It has melting point 144-145 °C and UV spectrum maximum (λ_{max}) at 221, 288 and 335 nm. The ESI-MS showed a sodium adduct ion $[M+Na]^+$ at *m/z* 293 and $[2M+Na]^+$ at *m/z* 563, which is consistent with the molecular formula of $C_{16}H_{14}O_4$.

The ^{1}H NMR spectral (Table 1) features of compound 2 is almost identical to that of compound 1 with the two downfield shifted proton signals at δ_H 8.26 and 7.80 (*J* = 15.6 Hz) for *trans*-configured α, β-unsaturated chalcone moiety and five mutually coupled aromatic

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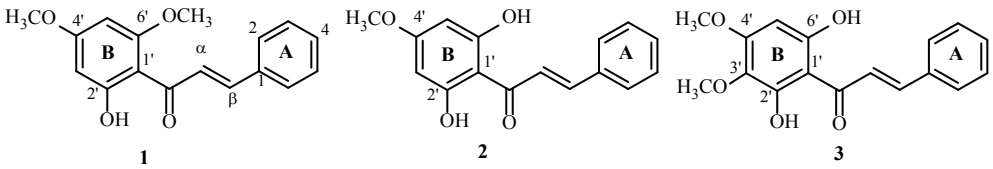

Figure 1: Structures of the isolated compounds.

Position	1		2		3	
	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C
1		135.7		136.5		135.6
2/6	7.60 (2H, *m*)	128.4	7.44 (2H, *m*)	129.2	7.61 (2H, *m*)	128.6
3/5	7.42 (2H, *m*)	128.9	7.70 (2H, *m*)	129.8	7.41 (2H, *m*)	129.0
4	7.39 (1H, *m*)	127.6	7.43 (1H, *m*)	128.4	7.39 (1H, *m*)	127.6
α	7.79 (1H, *d*, 15.6)	130.1	7.80 (1H, *d*, 15.6)	131.0	7.81 (1H, *d*, 15.6)	130.3
β	7.91 (1H, *d*, 15.6)	142.3	8.26 (1H, *d*, 15.6)	143.0	7.91 (1H, *d*, 15.6)	142.7
1'		106.4		106.2		106.6
2'		162.6		165.5		159.0
3'	6.10 (1H, *d*, 2.0)	93.9	6.04 (1H, *s*)	94.7		128.5
4'		168.5		167.3		155.5
5'	5.96 (1H, *d*, 2.0)	91.3	6.04 (1H, *s*)	94.6	6.07 (1H, *s*)	90.0
6'		166.5		165.5		159.2
3'-OCH_3					3.91 (3H, s)	61.0
4'-OCH_3	3.82 (3H, *s*)	55.9	3.82 (3H, *s*)	55.9	3.93 (3H, s)	56.2
6'-OCH_3	3.90 (3H, *s*)	55.6				
2'-OH	14.32 (1H, *s*)		12.02 (2H, *s*)		14.36 (2H, *s*)	
CO		192.7		193.5		193.4

Table 1: ^1H (500 MHz) and ^{13}C (125 MHz,) NMR data of compounds **1**, **3** (in $CDCl_3$) and **2** (in acetone-d_6).

Strains		Crude extract		Compounds			Controls	
		CE	ME	1	2	3	G	C
Bacterial strains	*E. coli*	12	10	-	18	12	19	-
	S. aureus	15	8	9	-	13	17	-
Fungal strains	*Aspergillus spp*	30	30	10	15	20	-	23
	Trichoderma spp	14	18	-	22	18	-	20
	Fusarium spp	20	20	10	19	20	-	22
	Penicillium spp	18.5	16.5	-	10	14	-	15

Table 2: *In vitro* antibacterial and antifungal (Diameter of Zone of Growth Inhibition (mm)) activities of the extracts and the isolated compounds. (**Key**: These results are average results of three experiments. **-**: Not active; CE: Chloroform Extract; ME: Methanol Extract; G: Gentamycin, C: Clotrimazole).

protons (δ_H 7.43-7.70) for the phenyl ring A. The only notable difference is observed in ring B, where the two upfield shifted *meta* coupled protons (δ_H 5.96 and 6.10) in compound 1 being replaced with chemically equivalent two proton singlet (δ_H 6.04) in compound 2, suggesting that ring B is symmetric.. Furthermore, the presence of only one methoxy protons at δ_H 3.82 and a chelated hydroxyl proton at δ_H 12.02 allowed the placement of these functionality at C-4' (δ_C 167.3) and C-2' (δ_C 165.5), respectively, supporting the aforementioned argument. Therefore, the two protons at δ_H 6.04 were equivocally assigned to H-3' and H-5'. The ^{13}C NMR spectrum (Table 1) showed signals for 16 carbon atoms including one methoxyl group, nine methine and six quaternary carbon atoms. Therefore, based on these spectroscopic data, compound 2 was identified as 2',6'-dihydroxy-4'-methoxychalcone, trivial name pinostrobin chalcone, previously reported from bark of *Lindera umbellate* [7].

Compound 3 was isolated as orange solid with melting point of 134-135°C and UV absorption maximum (λ_{max}) at 221 and 342 nm. The ESI-MS ion peaks at *m/z* 323 for $[M+Na]^+$ is in agreement with the molecular formula $C_{17}H_{16}O_5$. The ^1H and ^{13}C NMR (Table 1) spectral pattern were similar to that of compound 1 except for the absence of only one protonated aromatic carbon in ring B of compound 3. Similar to compound 1 and 2, the presence of five mutually coupled aromatic protons (δ_H 7.39-7.61) for the ring A *trans*-oriented two olefinic protons (δ_H 7.81 and 7.91) were also confirmed for 3., Moreover, Two methoxy groups (δ_H 3.93 and 3.91), a shielded singlet aromatic proton (δ_H 6.07) and a chealeted hydroxyl proton (δ_H 14.36) constitute ring B of compound 3.

The ^{13}C NMR (Table 1) spectrum showed signals for 17 carbon atoms including signals for two oxygenated methyl groups, eight methine and seven quaternary carbon atoms. The placement of the methoxy group at δ_H 3.91 (δ_C 61.0) was deduced from the downfiled chemical shift value ($_C$ 61.0) indicating its devoid of planarity due to the presence of di-*ortho* substitution and hence placed at C-4' (δ_C 155.5). This allowed the placement of the other methoxy group δ_H 3.93 (δ_C 56.2) at C-3' (δ_C 128.5), which was further confirmed by the HMBC correlation. Thus, based on the above evidence, compound 3 was found

to be 2′,6′-dihydroxy-3′, 4′-dimethoxychalcone, trivial name pashanone (3), a compound previously isolated from *Polygonum ferrugineum* and *Polygonum hydropiper* [8,9].

The crude extracts (chloroform and methanol) and the isolated compounds (1 - 3) were *in vitro* assayed against two bacterial and four fungal strains (Table 2). The activities of the extracts and the isolated compounds were comparatively assessed by the diameter of zone of inhibition in millimeters and zones of inhibition more than 6 mm were taken into consideration.

Both extracts showed marginal inhibitory activities against both tested bacterial strains, with the highest activity. However, the inhibition displayed on both Gram-negative and Gram-positive bacteria for the isolated compounds were good with variable degree of potency between the tested compounds. Compound 1 showed marginal activity against Gram-negative bacteria (*Escherichia coli)* while it had little or no inhibitory activity against Gram-positive bacteria (*Staphylococccus aureus*). Whereas, compound 2 exhibited highest zone of growth inhibition (18 mm) on Gram-positive bacteria strain (*Staphylococccus aureus*), which is comparable to that of reference drug, gentamycin (19 mm) and has no activity against *Escherichia coli*. This is may be related to the polar/none polar nature of the compounds, as the two compounds differ from one another by presence of methoxy and free phenol at position C-6′. It is therefore, believed that compound 1 having a non-polar methyl ether moiety at C-6′ could pass the outer lipid membrane of the Gram-negative bacteria. Whereas, the Gram-positive bacteria which has no such outer membrane was expected to be most susceptible to the more polar compound 2. Compound 3 showed comparable activity against both strains, as it possesses both polar and none polar groups.

Evaluation of antifungal activity revealed that both chloroform and methanol extracts had significant activities against all the fungal strains with inhibition zone diameters ranging from 14–30 mm as compared to the reference drug (clotrimazole), which displayed inhibition zone diameter ranging between 15 mm (*Penicillium* spp) and 23 mm (*Aspergillus* spp). The better activity of the crude extracts could be related to the synergistic interactions of several secondary metabolites present in the extracts, which cannot be seen when pure compounds are evaluated alone. The isolated compounds showed variable activity against the fungal strains with the highest activity is observed for compound 2 (22 mm), which is even greater than that of the reference drug, clotrimazole (20 mm) against *Trichoderma* spp. In general, the current study revealed potential application of extracts and the compounds from the seeds of *P. lapathifolia* for the treatment of infectious diseases, which is in line with the traditional application of the plant.

Experimental Section

General

Rotary evaporator (Heidolph, USA) for solvent evaporation, UV chamber (LF-206.LS, EEC) for detection of spots on TLC plate, Melting point apparatus (MFB 590010T, Griftin, Britain); UV-Visible photo-spectroscopy (JENWAY 6705, UK), Column chromatography was performed on silica gel (0.06–0.2 mm) size. Analytical TLC was performed on Merck pre-coated silica gel 60 F_{254} plates. IR spectra were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Electron Corporation, Madison, WI, USA). ESI-MS was done on a Micromass AC-TOFmicro mass spectrometer (Micromass, Agilent Technologies 1200 series, Tokyo, Japan). 1D (^{1}H, ^{13}C) NMR and 2D (COSY, HSQC, HMBC, NOESY) NMR spectra were recorded on an Avance 500 MHz spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 298 K using the residual solvent peaks as a reference.

Plant material

The seed of *P. lapathifolia* was collected from Jimma University main campus, Oromia regional state, Ethiopia in October 2016. The plant material was identified and the voucher specimen has been deposited in Jimma University Herbarium. The collected plant material was chopped into smaller pieces and shade dried at room temperature.

Extraction and isolation

The air dried plant material was ground to small size to facilitate easy solvent penetration and sequentially extracted with chloroform and methanol. The extracts were then screened for their antimicrobial activity. The chloroform extract showed better antibacterial activity and was selected for further isolation of the bioactive compounds. The chloroform crude extract (32 g) was adsorbed on 50 g silica gel and applied on column chromatography already packed with silica gel. The column was eluted with petroleum ether, with increasing gradient of ethyl acetate to afford 24 major fractions *ca*. 250 mL each. Fractions 2-5 (5% EtOAc in petroleum ether) were combined and purified by small column chromatography (column size: 80 cm length and 4 cm diameter) on silica gel (250 g; eluent: increasing gradient of ethyl acetate in petroleum ether) to give compound 1 (11.9 mg) and compound **3** (8.2 mg). Whereas, compound 2 (7.3 mg) was isolated from fractions 8-22 (10% ethyl acetate in petroleum ether) with similar repetitive small column chromatography.

Bioassay (agar disk diffusion method)

The crude extracts and isolated compounds were evaluated for *in vitro* antibacterial activities against two bacterial strains (*Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923) and the antifungal activities against four fungus strains (*Aspergillus* spp, *Trichoderma* spp, *Fusarium* spp and *Penicillium* spp) by disc diffusion method. The bacterial strains were obtained from Microbiology laboratory, Biology Department, Jimma University. Whereas, the pathogenic fungi were isolated from the infected tomatoes sample collected from cropping area, where there is high plantation of tomatoes using the standardized dilution factors as per the protocols. Nutrient agar, Rose Bengal agar and Potato dextrose agar were used for isolating these fungi. A single colony of fungi was taken from potato dextrose agar (PDA), Nutrient agar and Rose Bengal agar and it was placed on the slide and analyzed. Wet mount was added to slide and mixed with the transferred fungal colony, and then it was covered with cover slip. In addition, oil immersion was added in the wet mount and the morphology of fungi was observed through microscope. Finally, based on their morphology and fungal identification keys, the isolated fungi were characterized.

Agar disk diffusion method [10] was used to evaluate the antibacterial and antifungal activities of both crude extract and isolated compounds on nutrient agar. Briefly, the stock cultures were maintained on the nutrient agar slants which were stored at 4°C. Agar cultures of the test microorganisms were prepared according to manufacture instruction. The test solutions were prepared by dissolving 0.01 g ratio of plant extracts to achieve final stock concentrations of 100 mg/mL in DMSO. Freshly, grown liquid culture of the test pathogens solution of having similar turbidity with 0.5 McFarland were seeded over the Mueller-Hinton Agar medium with sterile swab. Sterile Whatman filter paper discs (6 mm) were

soaked with stock solution of the extract then placed over the seeded plates at equidistance. The plates were then inverted and incubated at 37 °C for 24 h. After the incubated period, the plates were observed for a clearance zone around the disks which indicates positive antibacterial activities of the respective plant extracts. The clear zones formed around each disk were measured in millimeter.

Conclusion

The bioassay guided extraction of the seeds of *P. lapathilium* revealed that extracts and the isolated compounds demonstrated significant antibacterial and antifungal activities with compound 2 showed highest zone of growth inhibition against *S. aureus* bacterial strain and *Trichoderma* spps fungal train, which are almost comparable to that of the reference drugs, gentamycin and clotrimazole respectively. The good activity of these extracts and the isolated compounds could give insight about the potentials of the bioactive compounds from this plant as lead structure in development of antimicrobial drugs.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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