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Phytochemical studies and antibacterial activity of the aerial parts of *Physospermum verticillatum*

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Abstract

In this study, we focused on the isolation and identification of the main compounds, triterpene, sterol, phenolic and fatty acid from the aerial parts of *Physospermum verticillatum*, which has not been previously reported. Dichloromethane, ethyl acetate and *n*-butanol extracts led to the isolation of ß-amyrin (**1**), 3,11-dihydroxy-12-oleanene (**2**), pentadecanoic acid (**3**), spina-sterol (**4**), spinasterol-3-O-ß-D-glucopyranoside (**5**) and saikochromic acid (**6**). These compounds were determined by means of the combined systems with high resolution tandem mass spectrometry (HPLC-TOF/MS), 1D NMR (^{1}H NMR, ^{13}C NMR) and 2D NMR(COSY, HSQC and HMBC). Further, the extracts demonstrated significant antibacterial activity.

Introduction

The genus Physospermum belongs to the Apiaceae family includes 16 species, two of these growing in Algeria. *Physospermum verticillatum* and *Physospermum cornubiense* are used in flavor and sweet foods (Quezel, 1956; Quezel and Santa., 1963).

The literature reviews show that there are only a few papers about the phytochemical investigation of *P. verticillatum*. It has reported that three triterpene saponins isolated and identified from the roots of *P. verticillatum* and were investigated *in vitro* for their cytotoxic activity (Tundis et al., 2009). In another study, seven flavonoids are isolated from the genus Physospermum (Bencheriet et al., 2012). Recently, special attention is giving to the combination of saponins and other anti-cancer drugs as an efficient treatment against cancer cell line. This study concerns the preparation and characterization of microspheres based on a mixture of three triterpene saponins from *P. verticillatum*, as carrier for the specific release of gemcitabine (Trombino et al., 2016).

This study investigates the secondary metabolites of the aerial parts of *P. verticillatum*, as well as its *in vitro* antibacterial activity. The presence of these secondary metabolites in the aerial parts of this species is described for the first time.

Materials and Methods

Plant material

The aerial parts of *P. verticillatum* were collected during the flowering stage from Setif Region, East of Algeria in May, 2013 and was identified by Dr. H. Laouer from the Department of Biology and Plant Ecology, University of Setif, Algeria. A voucher specimen was deposited in the herbarium of our laboratory (ChifaDZUMCAPPV-00027).

Extraction and isolation of compounds

Air dried aerial parts (1 kg) of *P. verticillatum* were extracted three times with boiling 70% methanol. The hydromethanolic extract was evaporated to dryness and the residue was dissolved in boiling water. Then filtered, the filtrate was concentrated in a vacuum with a rotary evaporator to afford 240.8 g of methanol extract. The last residue was extracted with dichloromethane, ethyl acetate and *n*-butanol (3 times) successively to give 3.5 g of dichloromethane extract, 4.3 g of ethyl acetate extract and 40 g of *n*-butanol extracts.

The dichloromethane extract (3.5 g) was subjected to silica gel (100 g silica gel 60, 40-63 µm) column chromatography starting the elution with a mixture of cyclohexane/dichloromethane (from 100:0 to 0:100), then with a mixture of dichloromethane/ethyl acetate (from 100:0 to 0:100) and finally with ethyl acetate/methanol (from 100:0 to 50:50). The elutes were monitored using TLC and viewed under UV light (254 and 365 nm) and by spraying with 1% vanillin 2% sulfuric acid/ethanol reagent followed by heating at 100°C. The fractions [20-25] eluted with 30/70 cyclohexane/dichloromethane (30 mg) showed one spot contaminated by the chlorophyll, which was washed with acetone to give a white crystals: ß-amyrin (**1**) (22 mg) (Figure 1). Combined fractions [27-31] eluted with 10/90 dichloromethane/ethyl acetate (34 mg) afforded one spot polluted by chlorophyll; the fraction was purified by acetone to furnish white crystals: 3,11 dihydroxy-olean-12-ene (**2**) (28 mg). The fractions [41-45] eluted with 90/10 and 80/20 dichloromethane/ethyl acetate (500 mg) from the first column showed a mixture of two spots; the fraction was rechromatographed again to yield a white crystal pentadecanoic acid (**3**) (18 mg) and white crystals: Spinasterol (3ß, 5α, 22E) stigmas-ta-7,22-dien-3ol) (**4**) (21 mg). Spinasterol-3-O-ß-D-glucopyranoside (**5**) was precipitated as an anamorphous white powder from the ethyl acetate extract. However, saikochromic acid (**6**) was obtained from the *n*-butanol extract as a precipitate in the form of amorphous yellow powder.

Determination of antibacterial activity

Biological material

All of the bacteria, standard strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and clinical strains (*E. coli*, *Staphylococcus aureus, P. aeruginosa, Proteus mirabilis, Klebsiella pneumonia, Serratia* sp, *Enterobacter* sp), were obtained from the Bacteriology Laboratory of Constantine University Hospital.

Biological test

The crude dichloromethane, ethyl acetate and *n*-butanol extracts of aerial parts of *P. verticillatum* were investigated for their antibacterial activity using the disk diffusion method (Sivasothy et al., 2013). The bacterial strains were first cultured on Mueller-Hinton agar for 24 hours at 37°C. A sterile filter disk (6 mm diameter with Whatman paper No. 3) was placed on the infusion agar seeded with bacteria and impregnated with 40 µL of the extract suspended in ethanol 60%. For all concentration (2, 1, 0.5, 0.25 mg/mL) after staying the

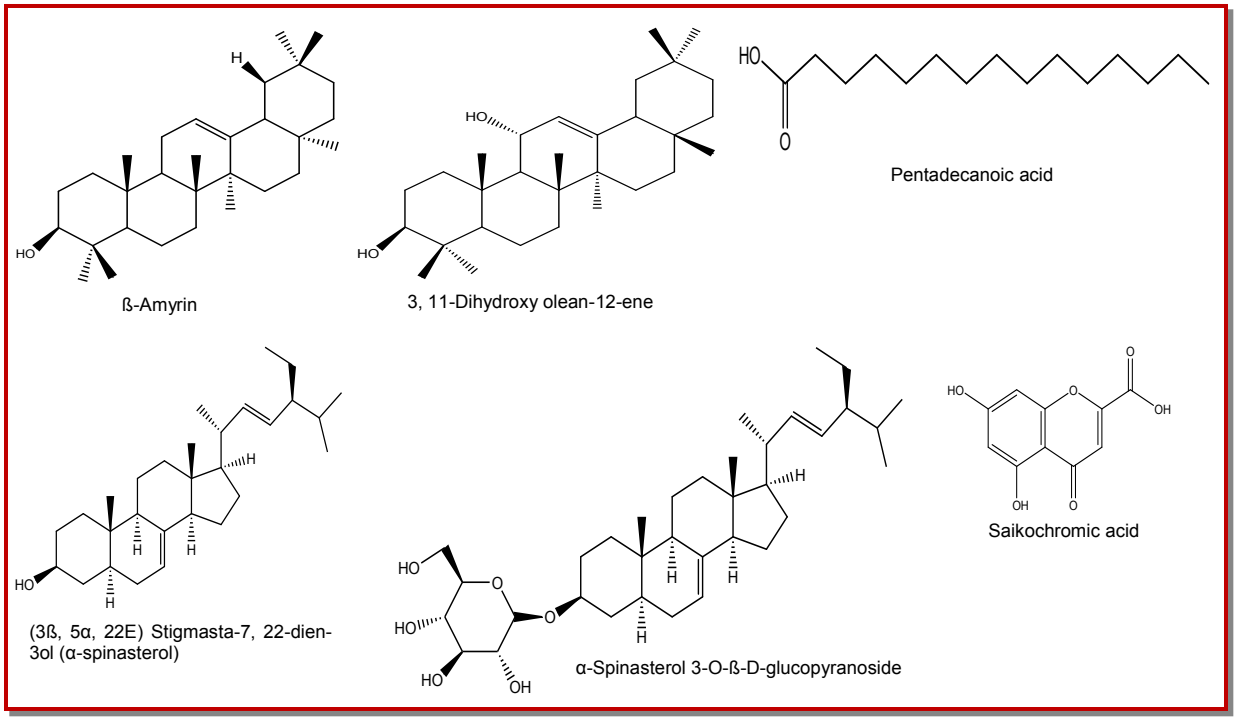

Figure 1: Structures of compounds isolated from *Physospermum verticillatum*

petri dish at 4°C for 1 hour, these were incubated at 37° C for 24 hours. The diameter of inhibition zone was measured in millimeters and all the tests were performed in triplicate. The antibacterial potential of the extracts was assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial activity were expressed as means ± SD. The control treatment (ethanol 60%) had no inhibitory effect on any of the tested microorganisms.

Results

Identification of compounds

Compound (**1**): $C_{30}H_{50}O$, white crystals, M=426,39 g/mol. ^{1}H-NMR (600 MHz, $CDCl_3$, δ/ppm, J/Hz):3.22 (^{1}H,dd, J=10.8, 4.4, H3)0.74(^{1}H,d, J=11.4, H5), 5.18 (^{1}H,s, H12),1.76(2H,td, J=13.8, 4.2, H16), 2.00(^{1}H,m, H19),0.79 (3H,s, H25),O.83(3H,s, H23),0.87(3H,s, H29), 0.87(3H, s, H30), 0.93(3H,s, H24),0.96(3H,s, H26),0.99(3H,s, H28), 1.13(3H,s, H27). ^{13}C-NMR(600 MHz, CDCl3, δ/ppm): (C1)38.76, (C2)27.22, (C3)79.00, (C4)38.58, (C5)55.16, (C6)18.37; (C7)32.45, (C8)39.78, (C9)47.62, (C10)36.93, (C11)23.52, (C12)121.70, (C13)145.16, (C14) 41.70, (C15) 26.93, (C16)26.15, (C17)32.64, (C18)47.21, (C19) 46.81, (C20)31.07, (C21)34.72, (C22)37.13, (C23)28.09, (C24) 15.49, (C25)15.58, (C26)16.80, (C27)25.99, (C28)28.39, (C29)33.33, (C30)23.69.EI.MS(70e.v):M/Z:426.4, 218 (100), 203(44), 189(17). It was identified as ß-amyrine (Vázquez et al., 2012; Fingolo et al., 2013).

Compound **(2)**: $C_{30}H_{50}O_2$, white crystals, M=442.72 g/mol. ^{1}H-NMR (600 MHz, $CDCl_3$, δ/ppm, J/Hz): 3.24 (^{1}H,m, H3), 0.78(^{1}H,d, J=11,4, H5), 4.19(^{1}H, m, H11), 5.24(1H12,s,H12), 2.05(1H,m, H18) 1.98(2H,t, J=13.8, H21),1.22(3H,s, H23), 0.84(3H,s, H24), 1.01(3H,s, 3H25), 1.01(3H,s, 3H26), 1.06(3H,s, 3H27),O.81(3H, s, H28), 0.89 (3H,s, 3H29), 0.89(3H,s, 3H30).13C-NMR(600 MHz, $CDCl_3$, δ/ppm): (C1)40.43, (C2)27.34, (C3)78.74, (C4) 38.04, (C5)55.20, (C6)18.45; (C7)33.10, (C8)43.44, (C9) 56.49, (C10)38.06, (C11)67.58, (C12)125.37, (C13)149.51, (C14) 41.80, (C15)26.72, (C16)26.26, (C17)32.28, (C18) 46.53, (C19) 46.52, (C20)31.08, (C21)34.66, (C22)36.97, (C23)28.11, (C24) 15.49, (C25)16.88, (C26)18.07, (C27) 26.22, (C28)28.47, (C29)33.23, (C30)23.59. EI.MS(70e.v): M/Z:442(20), 424(81),406(17), 255(39), 234(100),191(80), 95(45). It was identified as 3α, 11ß- dihydroxy-olean-12-ene (Wang and Li, 2016; Yuan et al., 1994).

Compound (**3**): $C_{15}H_{30}O_2$, white crystals, M=242.402 g/mol. ^{1}H-NMR (600 MHz, $CDCl_3$, δ/ppm, J/Hz):0.88 (3H,t, J =6.6, CH3), 1.26 (22H,s, H4-H14), 1.63(2H,t, J =7.2, H3), 2.35(2H,t, J =7.2, H2), 7.26(1H,s, OH). 13C-NMR (600 MHz, CDCl3, δ/ppm):(C1)179.28, (C2)33.89, (C3)24.67, (C4 to C12)29.04-29.66, (C13)31.91,(C14)22.67, (C15)14.09. It was identified as pentadecanoic acid (Pandey et al., 2006).

Compound (**4**): $C_{29}H_{48}O$, white crystals, M=412 g/mol.1H-NMR (600 MHz, CDCl3, δ/ppm,J/Hz):3.59(1H, m, H3), 5.15(1H, m, H7), 0.55(3H, s, H18), 0.80(3H, s, H19), 1.03(3H, d, J =5.4, H21), 5.15(1H, m, H22), 5.03 (1H, m, H23), 0.85(3H, d, J =6, H26)0.80(3H, s, H27), 0.80(3H, s, H29).13C-NMR(600 MHz, CDCl3, δ/ppm): (C1)37.14, (C2)31.48, (C3)71.05, (C4)38.00, (C5)40.26, (C6)29.63, (C7)117.45, (C8)139.56, (C9)49.45, (C10)34.21, (C11)21.54, (C12)39.46, (C13)43.28, (C14) 55.12, (C15) 23.00, (C16)28.48, (C17)55.90, (C18)12.04, (C19)13.03, (C20)40.80, (C21)21.36, (C22)138.15, (C23)129.44, (C24) 51.24, (C25)31.86, (C26)21.06, (C27)18.98, (C28)25.38, (C29)12.22. EI.MS(70e.v):M/Z:412(35),300(16) 271(100), 255(41), 81(28). It was identified as (3ß, 5α, 22E) stigmasta-7, 22-dien-3-ol (α-spinasterol) (Léa et al., 2014; Consolacion and Kathleen, 2005).

Compound (**5**): $C_{35}H_{56}O_6$, white amorphous powder, M=574.8 g/mol. ^{1}H-NMR (600 MHz, DMSO-d6, δ/ppm, J/Hz):0.50(3H, s,H18), 0.72(3H, s, H19), 0.76(6H, s, H26 and H29), 0.81(3H, d, J =6, H27), 0.98(3H, d, J =6.6, H21), 1.93(1H, d, J =11.4, H5),3.39(1H, m, H'6a), 3.53 (1H, m, H3), 3.62(1H, m, H'6b), 4.2(1H, d, J =7.8, H'1), 5.02(1H, dd, J =9, 15, H23), 5.10(1H, s, H7), 5.14(1H, dd,j=9, 15, H22). 13C-NMR(600 MHz, DMSO-d6, δ/ppm):(C1)37.00, (C2)29.54, (C3)76.75, (C4)34.40, (C5) 40.52, (C6)29.67, (C7)117.66, (C8)139.47, (C9)49.11, (C10) 34.40, (C11)21.70, (C12)39.70,(C13)43.28, (C14) 54.93, (C15)22.99, (C16)28.57, (C17)55.66, (C18)12.32, (C19) 13.24, (C20)40.74, (C21)21.45, (C22)138.37, (C23)129.43, (C24) 51.05, (C25)31.77, (C26)19.30, (C27)21.42, (C28) 25.33, (C29)12.57, (C1')101.30, (C2')73.94, (C3')77.20, (C4')70.55, (C5')77.15, (C6')61.57 was identified as spinasterol 3-O-ß-D-glucopyranoside (Fahee et al., 2013; Hye et al., 2012).

Compound (**6**): $C_{10}H_6O_6$, yellow amorphous powder, M=222.151 g/mol. ^{1}H-NMR (600 MHz, DMSO-d6, δ/ppm, J/Hz):6.16(1H, s, H6), 6.46(1H, s, H8), 6.51(1H, s, H3), 12.9(OH5, s), 11.10(OH7, s). 13C-NMR (600 MHz, DMSO-d6, δ /ppm): (C2)160.87, (C3)108.62, (C4)183.74, (C5)161.86, (C6)99.14; (C7)164.94, (C8)94.50, (C9)158.12, (C10)104.88, (C11)163.84.EI.MS (170e.v):M/Z:222.97[M]+. It was identified as saikochromic acid (Liang et al., 2000).

Antimicrobial activity of all extracts

The antibacterial activity of the aerial parts of *P. verticillatum* extracts assessed on 10 bacterial strains is reported in Table I. The extracts were found to be a moderate antibacterial agent according to the disk diffusion method. The negative control sample did not affect the growth of testing bacteria (data not shown). All the fractions showed very similar antibacterial activity, dose depending ranging from 10 mm to 17.5 mm.

The dichloromethane, ethyl acetate and n-butanol extracts of *P. verticillatum*, tested for antibacterial activity against two gram positive and eight gram

Table I

Antibacterial activity of extracts

Microorganism	Dichloromethane extract (mg/mL)				Ethyl acetate extract (mg/mL)				*n*-Butanol extract (mg/mL)				
	0.25	0.5	1	2	0.25	0.5	1	2	0.25	0.5	1	2	Control
E. coli ATCC 25922	13.3 (0.6)	14.3 (0.6)	14.8 (0.3)	16.5 (0.5)	13.7 (1.2)	14.5 (1.0)	15.2 (0.8)	16.5 (0.5)	15.8 (0.6)	16.3 (0.6)	16.8 (1.4)	17.5 (1.3)	-
E. coli	12.0 (1.0)	13.5 (0.9)	14.3 (0.8)	15.3 (0.8)	12.0 (0.5)	12.8 (0.3)	13.5 (0.5)	14.3 (0.8)	12.5 (1.5)	14.5 (1.5)	15.3 (1.0)	16.3 (0.6)	-
S. aureus ATCC 29213	11.5 (1.3)	13.2 (1.7)	14.2 (1.3)	15.3 (0.6)	11.8 (0.8)	12.5 (0.5)	13.2 (0.3)	13.8 (0.8)	11.7 (0.6)	13.2 (0.8)	13.7 (0.8)	14.7 (0.6)	-
S. aureus	10.0 (1.0)	11.7 (1.5)	13.3 (0.8)	14.3 (0.3)	10.7 (0.6)	11.5 (0.5)	12.0 (0.5)	13.0 (0.5)	10.2 (0.8)	11.3 (1.3)	11.7 (1.5)	12.7 (1.5)	-
P. aeruginosa ATCC 27853	13.0 (0.9)	14.0 (1.5)	15.5 (0.5)	16.3 (0.6)	13.3 (0.3)	14.2 (0.3)	15.5 (0.9)	16.5 (0.9)	13.5 (1.3)	14.8 (1.0)	15.2 (0.8)	16.3 (0.6)	-
P. aeruginosa	11.2 (1.0)	12.5 (0.5)	13.2 (0.3)	14.5 (0.9)	12.8 (0.8)	13.5 (0.5)	14.2 (0.8)	15.0 (1.0)	12.5 (0.5)	13.7 (0.3)	14.5 (0.5)	15.3 (0.6)	-
P. mirabilis	12.7 (1.0)	13.7 (0.3)	14.5 (0.5)	15.3 (1.0)	13.5 (0.0)	14.3 (0.6)	15.3 (0.6)	16.7 (0.3)	12.3 (0.6)	13.5 (0.0)	14.0 (0.0)	14.5 (0.9)	-
K. pneumoniae	14.3 (1.2)	14.8 (1.0)	16.0 (1.0)	17.0 (1.0)	14.7 (1.2)	15.5 (0.6)	16.0 (0.5)	17.0 (0.0)	13.5 (0.5)	14.5 (0.5)	15.0 (0.5)	15.8 (0.3)	-
Serratia sp	13.7 (0.8)	14.2 (0.3)	14.8 (0.3)	16.3 (0.3)	14.2 (0.8)	15.2 (0.6)	15.8 (0.8)	17.3 (0.3)	13.2 (0.8)	14.3 (0.3)	14.8 (0.3)	15.7 (0.6)	-
Enterobacter sp	14.0 (1.0)	14.5 (0.9)	15.7 (0.8)	17.0 (0.9)	14.0 (1.7)	15.0 (1.3)	16.7 (0.8)	17.0 (1.0)	13.8 (0.6)	14.7 (0.3)	15.2 (0.3)	16.3 (0.6)	-

Data are means (SD)

negative at 0.2, 0.5, 1, and 2 mg/mL; The CH2Cl2 extract has the best activities against *Enterobacter* sp , *K. pneumonia* and *E. coli* ATCC 25922 with a high concentration of 2 mg/mL which gave the inhibition diameters respectively 17 ± 1.0 mm, 17 ± 0.9 mm and 16 ± 0.5 mm. The others strains showed an inhibition zone varied from 14.3 to 16.3 mm. While ethyl acetate extract exhibited a good activities against *Serratia* sp (17.3 ± 0.3 mm), *K. pneumonia* (17 ± 0.0 mm) and *Enterobacter* sp (17 ± 1.0 mm) with a high concentration of 2 mg/mL and the other strains showed an inhibition zone varied from 13 to 16.7 mm. However, The *n*-butanol extract present the best activities against *E. coli* ATCC 25922, *E. coli*, *P. aeruginosa* ATCC 27853 and *Enterbacter* sp (17.5 ± 1.3, 16.3 ± 0.6, 16.3 ± 0.6, 16.33 ± 0.6 mm inhibition zone respectively) with a high concentration of 2 mg/mL, whereas the other strains have an inhibition zone varied from 12.7 to 15.8 mm. All the crude extracts displayed lower antibacterial activity against the two gram positive bacteria (*S. aureus* ATCC 29213 and *S. aureus*).

Discussion

The phytochimical studies of *P. verticillatum* revealed the presence of phenolic compounds, terpenoids and steroids, these compounds synthesized in the secondary metabolism of the plants are known by their active substance; for that reason the antibacterial assay was done for extracts of *P. verticillatum*. The results confirmed that dichloromethane, ethyl acetate and *n*-butanol extracts demonstrated antibacterial activities against all tested bacteria. A maximum zone of inhibition was found against gram negative however the least activity was seen against gram positive. All the extracts showed the maximum antibacterial activity with a high concentration of 2 mg/mL, this indicates that the active principles which inhibit the growth of susceptible bacteria may dissolve better in all the solvents used. After the obtained results, we can say that the extracts have a large inhibits activity in the different classes of the microorganisms tested.

As far as our literature survey could as certainly, we could reach no reports on the antibacterial activity of *P. verticillatum* in the literature. However, three triterpenes saponins from *P. verticillatum* were investigated *in vitro* for their citotoxic activity against a panel of seven different cancer cell lines including, ACHN, C32, Caco-2, COR-L23, A375, A549 and Hoh-7D12 (Tundis et al., 2009). The microsphere prepared from a mixture of the same three natural triterpene saponins used as anti-cancer agent; reducing the systemic drug toxicity, allowing the reduction of the dose number, increasing the drug half-life and eliminating the problems related to the fast clearance of gencitabine administration (Trombino et al., 2016).

Conclusion

Six compounds were isolated the first time from the extracts obtained from the aerial parts of *P. verticillatum*. The structures of the isolated compounds were identified as: ß-amyrine, 3,11-dihydroxy-12-oleanene, pentadecanoic acid, spinasterol, spinasterol-3-O-ß-D-glucopyranoside and saikochromic acid on the basis of spectroscopic and by comparing their physical proprieties reported in the literature. The present results of antibacterial activity showed that the plant extracts have great potential antimicrobial compounds against microorganisms.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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