PRIMARY STUDY ON BIOLOGICAL ACTIVITIES AND CHEMICAL CONSTITUENTS OF *ANOECTOCHILUS ROXBURGHII* WALL. OF VIETNAM

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Abstract - The total methanol extract of whole plants of *Anoectochilus roxbughii* (*Orchidaceae*) from Kontum, Vietnam is evaluated to have antibacterial and antioxidant properties. The results indicate that at concentration of 200µg/ml, the extract shows weak activities. The methanol extract is filtered, combined, and concentrated under low pressure to give 16 g residue, which is fractionated by chromatography column eluting in turn with n-hexane, ethyl acetate, and methanol to obtain fractions-extracts after leaving solvents.Two compounds kinsenoside (**1**) and daucosterol (**2**), are isolated from the methanol and n-hexane fractions of this plant. Their structures are determined by mass, 1D and 2D NMR spectra. Especially, kinsenoside appears to have highly antihypertensive, hepatoprotective activities and inhibits the production of inflammatory mediators.

Key words - Anoectochilus roxbughii, kinsenoside; daucosterol; hepatoprotective activities;antibacterial activity; antioxidant activity.

1. Introduction

Anoectochilus roxbughii Wall., a herbal plant, belongs to genus *Anoectochilus*, family *Orchidaceae*. The plants are commonly used in folk medicine for treatment of some diseases such as cancer, hypertension, hepatitis, cough, rheumatism, bronchitis, backache, sore throat and also have neuro-protective, antidotal activities. Some reports of Chinese researchers showed the evidence of the presence of 3-hydroxy butanolide derivatives, flavonoids, phytosterols in this species [1,2].

In Vietnam, *Anoectochilus roxbughii* are rare herbs in danger of extinction due to over exploitation of local people to sell them to China. Vietnamese scientists initially succeeded in rapidly propagating this plant. However, so far there has been no publication on chemical and biological activity of it in Vietnam.

In this report, we present results of the evaluation of antibacterial and antioxidant activities, the isolation and theidentification of the structuresoftwo compounds: kinsenoside and daucosterol from the total methanol extract.

2. Experimental

2.1. Plant materials

The whole plants of *Anoectochilus roxbughii* were collected at Konkray, Kontum province,Vietnam in January 2014. The scientific name was identified by Dr Nguyen Van Du, Institute of Ecology and Biological Resources, VAST. The voucher specimen is preserved at Institute of Natural Product Chemistry, Vietnam Academy of Science and Technology.

2.2. General experimental procedures

Electrospray ionization mass spectra (ESI-MS) are performed on an AGILENT 1100 LC-MSD Trap spectrometer. The ^{1}H-NMR (500MHz) and ^{13}C-NMR (125MHz) spectra are recorded on a Bruker AM500 FT-NMR spectrometer and tetramethylsilane is used as an internal standard.

Column chromatography (CC) is performed using a silica gel (0.040 – 0.063mm) and YMC RP-18 resins (30 - 50µm). Thin layer chromatography (TLC) uses pre-coated silica gel 60 F_{254} and RP-18 F_{254S} plates and compounds are visualized by spraying with the solution of 10% H_2SO_4 in ethanol and heating for 1-3 minutes.

2.3. Isolation

The dried whole plants of *Anoectochilus roxbughii* (100 g) are powdered and extracted with methanol at 50^{o}C (3 times x 2 hours per time) on heated ultrasonic machine. The total methanol extract is filtered, combined, and concentrated under low pressure to give 16 g residue, then is fractionated by chromatography column eluting in turn with n-hexane (2.4 g), ethyl acetate (5.2 g) and methanol (8.4 g) fraction-extracts after leaving solvents. The methanol extract (8.4 g) is separated on silica gel CC eluting with chloroform: methanol: water (7:1:0.1, v:v:v) to obtain 6 fractions (E1→E6). The fraction E2 (1.5 g) is continuously purified on an YMC RP-18 column eluting with acetone: water (1:1, v:v) to obtain compound (**1**) (25 mg). The n-hexane extract (2.4 g) is separated on silica gel column and eluted with chloroform: methanol (10:1, v:v) to obtain 5 fractions (F1→F5). The fraction F2 (350 mg) is further separated on silica gel CC eluting with ethyl acetate: methanol (8:1, v:v) to give compound (**2**) (7.8 mg).

Kinsenoside (1): White powder.

^{1}H-NMR (500MHz, MeOD): δ (ppm) 2.75 (1H, d, *J* = 18.5 Hz, H-2), 2.90 (1H, dd, *J* = 18.0, 6.5 Hz, H'-2), 3.20 (1H, dd, *J* = 8.0, 9.0 Hz, H-2'), 3.30 (1H, m, H-4'), 3.33 (1H, m, H-3'), 3.38 (1H, m, H-5'), 3.68 (1H, m, H-6'), 3.88 (1H, dd, *J* = 12.0, 1.0 Hz, H'-6'), 4.40 (1H, d, *J* = 6.0 Hz, H-1'), 4.49 (2H, m, H-4), 4.74 (1H, m, H-3).

^{13}C-NMR (125MHz, MeOD): δ (ppm) 36.98 (C-2), 62.68 (C-6'), 71.44 (C-4'), 74.82 (C-2'), 75.25 (C-4), 76.02 (C-3), 77.92 (C-5'), 78.08 (C-3'), 103.65 (C-1'), 178.87 (C-1).

Daucosterol (2): White crystals, m.p. 289°C – 291°C. ESI-MS m/z: 577 $[M+H]^+$, $C_{35}H_{60}O_6$.

^{1}H -NMR (500MHz, DMSO-d_6): δ (ppm): 0.78 (3H, s, CH_3-18); 0.81 (3H, d, J =7.0 Hz, CH_3-27); 0.83 (3H, d, J = 7.0 Hz, CH_3-26); 0.84 (3H, t, J = 7.0 Hz, CH_3-29); 0.91 (3H, d, J = 6.5 Hz, CH_3-21); 0.97 (3H, s, CH_3-19); 3.00 (1H, m, H-2'); 3.04 (1H, m, H-4'); 3.08 (1H, m, H-5'); 3.14 (1H, m, H-3'); 3.43 (1H, m, H-6'a); 3,47 (1H, m, H-3); 3.67 (1H, m, H-6'b); 4.22 (1H, d, J = 7.0 Hz , H-1'); 5.33 (1H, br d, J = 5.0 Hz, H-6).

^{13}C-NMR (125MHz, DMSO-d_6): δ (ppm) 37.2 (C-1); 29.5 (C-2); 70.1 (C-3); 38.7 (C-4); 140.2 (C-5); 122.1 (C-6); 31.8 (C-7); 31.8 (C-8); 50.1 (C-9); 36.6 (C-10); 21.0 (C-11); 39.7 (C-12); 42.2 (C-13); 56.7 (C-14); 24.2 (C-15); 28.1 (C-16); 55.9 (C-17); 11.8 (C-18); 19.6 (C-19); 36.0 (C-20); 19.1 (C-21); 33.9 (C-22); 26.0 (C-23); 45.8 (C-24); 29.1 (C-25); 18.9 (C-26); 18.6 (C-27); 23.0 (C-28); 11.7 (C-29); 101.0 (C-1'); 75.6 (C-2'); 76.3 (C-3'); 73.4 (C-4'); 79.1 (C-5'); 61.8 (C-6').

2.4. Antibacterial and antioxidant procedures

2.4.1. Antibacterial study

Minimum inhibitory concentration (MIC) determination is performed by a serial dilution technique using 96-well microtiter plates according to the modern method of Vander Bergher & Vlietlinck (1991), and McKane, L. & Kandel (1996) at Experimental Biology Laboratory, Institute of Natural Products Chemistry. Micro-plates are incubated for 24h at 37°C for bacteria and 48h at 30°C for fungi and yeasts.

Bacterial strains: *E.coli, P.aeruginosa, B.subtillis, S.aureus, A.niger, F.oxysporum, S.cerevisiae, C.albicans.*

Positive controls: Streptomycin for Gram (+) bacteria, Tetracyclin for Gram (-) bacteria, Nystatin or Amphotericin B for fungi and yeasts. These agents are dissolved in DMSO 100% at 4 mM of Streptomycin, 10 mM of Tetracyclin, 4 mM of Nystatin.

Negative controls: bacteria without antibiotics and studied sample.

2.4.2. Antioxidant study

The DPPH essay method is based on the reduction of DPPH, a stable free radical (Brand-Williams *et al.* 1995, Shela *et al.* 2003). Investigated sample is dissolved in dimethyl sulfoxide (DMSO 100%) and DPPH is dissolved in ethanol 96%. Antioxidant activity of the sample is evaluated by the absorption of DPPH at λ = 515 nm recorded by ELISA machine after dropping DPPH into the investigated solution on 96-well microtiter plates.

3. Results and Discussion

We have carried out preliminary evaluation of antibacterial activity and anti-oxidation of methanol extract of *Anoectochilus roxbughii.* The result of antioxidant activity is shown in table 1.

Table 1. *Antioxidant activity of the Anoectochilus roxbughii methanol extract*

No	samples	Concentrations (µg/ml)	Scavenging capacity (SC,%)	SC50 (µg/ml)
	Positive control	50	98.00±0.4	13.35
	Negative control	-	0.0±0.0	-
1	AS/Me	200	17.10±0.1	-

The result shows that the methanol extract of *Anoectochilus roxbughii* at concentrations of 200µg/ml shows weak antioxidant activity. The extract also expresses weak antibacterial activity against 8 tested strains.

From the methanol extract by using chromatography column method with suitable solvents we obtain two compounds (**1**) and (**2**).Compound (**1**) is obtained as a white powder. Combining spectral signals from ^{1}H-NMR, ^{13}C-NMR, HSQC spectrum of compound (**1**) indicate this compound has 10 carbon signals, in which 6 signals belong to a glucose moiety with the anomeric carbon at δ_C 103.65 (C-1') and the other carbon signals at δ_C 74.82 (C-2'), 78.08 (C-3'), 71.44 (C-4'), 77.92 (C-5'), 62.68 (C-6'). The β-anomeric configuration of glucose is judged based on the large $^3J_{H\text{-}1'',H\text{-}2''}$ coupling constant of the anomeric proton (δ_H 4.40, d,*J* = 6.0 Hz). The 4 remaining carbon signals are assigned to a butyrolactone frame with δ_C 178.87 (C-1, C=O), 36.98 (C-2, CH_2), 76.02 (C-3, CH), 75.25 (C-4, CH_2). This is confirmed by H-2 (δ_H 2.75)/H-3 (δ_H 4.74), H-3 (δ_H 4.74)/H-4 (δ_H 4.49) cross-peaks in COSY spectra, and the interaction of H-3 with C=O group in HMBC spectra. On the other hand, an HMBC crosss-peak between H-3 (δ_H 4.74) and C1' (δ_C103.65) show that the *β*-glucopyranose unit is attached to the C-3 position of the aglycone. These spectral data of (**1**) is appropriate to kinsenoside in a reported paper [4]. Compound (**1**) is thus identifed as kinsenoside (3-O-β-D-glucopyranosyl-(3R)-hydroxybutanolide).

Table 2. *NMR spectra data of compound (1) compared to Kinsenoside [4]*

No	Compound (1)		Kinsenoside [4]	
	δ_H, ppm (*J*, Hz) (500MHz, MeOD)	δ_C, ppm(125MHz, MeOD)	δ_H, ppm (*J*, Hz) (400MHz, Pyridin)	δ_C, ppm (100MH, Pyridin)
1		178,87		175,9
2	2,75 (1H, d, 18,5 Hz) 2,90 (1H, dd, 18,0, 6,5 Hz)	36,98	2,85 (2H, m)	35,7
3	4,74 (1H, m)	76,02	4,87 (1H, m)	75,2

4	4,49 (2H, m)	75,25	4,71 (1H, dd, J= 10,2, 1,6 Hz) 4,43 (1H, dd, J= 10,2, 4,6 Hz)	74,8
1'	4,40 (1H, d, 6 Hz)	103,65	4,90 (1H, d, J= 7,9 Hz)	104,1
2'	3,20 (1H, dd, 8,0, 9,0 Hz)	74,82	3,08 (1H, dd, J = 7,6, 9,2 Hz)	74,7
3'	3,33 (1H, m)	78,08	4,24 (1H, m)	78,7
4'	3,30 (1H, m)	71,44	4,21 (1H, m)	71,4
5'	3,38 (1H, m)	77,92	3,95 (1H, m)	78,3
6'	3,68 (1H, m) 3,88 (1H, dd, 12,0, 1,0 Hz)	62,68	4,55 (1H, dd, J= 11,8, 2,3 Hz) 4,35 (1H, dd, J= 11,8, 5,6 Hz)	62,7

* Some little diversities between the signals may be due to the differences in measurement solvents and frequencies

Compound (**2**) was obtained as white crystals. Its basic ion peak at *m/z* 577 $[M+H]^+$ was observed on positive-ion ESI-MS analysis reveal the molecular formula to be $C_{35}H_{60}O_6$. The ^{1}H-NMR, ^{13}C-NMR and DEPT data of (**2**) reveals 6 methyl groups in aglycone moiety CH_3-18, CH_3-19, CH_3-21, CH_3-26, CH_3-27, CH_3-29 at δ_H 0.78, 0.97, 0.91, 0.83, 0.81, 0.84 ppm and δ_C 11.8, 19.6, 19.1, 18.9, 18.6, 11.7 ppm, a double bone (C-5, C-6) at δ_H 5.33 ppm and δ_C 140.2, 122.1 ppm. These signals are appropriate to spectral data of a known alycone β-sitosterol. The remaining signals are identified as glucose moiety with the anomeric carbon at δ_C 101.0 ppm and δ_H 4.22 ppm. From the above evidences and comparison with spectral data of daucosterol in literature, compound (**2**) is deduced to be daucosterol (sitosterol-3-O-β-D-glucopyranoside) [3].

4. Conclusion

The methanol extract of whole plants of *Anoectochilus roxbughii* (Orchidaceae) at concentration of 200µg/ml shows weak antibacterial and antioxidant activities. From the extract, two compounds whose structures are isolated and indentified such as kinsenoside (**1**), daucosterol (**2**). Their structures are determined on the basis of spectroscopic (1D and 2D NMR, ESI-MS) methods. Kinsenoside is the main constituent of Anoectochilus plants. It is also demonstrated to inhibit the production of inflammatory mediators and enhance anti-inflammatory cytokine generation, have significant hepatoprotective activity, vascular protective effect, anti-hyperliposis, and used as an antihypertensive drug [4,5,6,7]. This is our first announcement of the preliminary results on biological activities and chemical components of *Anoectochilus roxbughii* in Vietnam. And we are continuing to carry out follow-up intensive investigations of this plant .

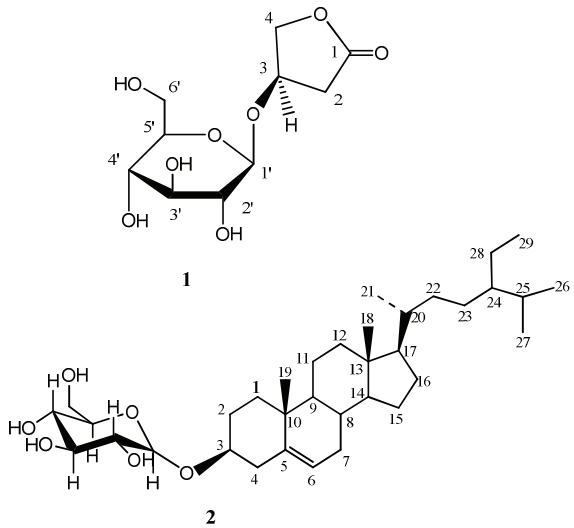

Figure 1. *Structure of two compounds (**1**), (**2**)*

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