The Journal of Phytopharmacology 2020; 9(3): 169-174
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2020; 9(3): 169-174
May- June
Received: 12-03-2020
Accepted: 12-05-2020
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doi: 10.31254/phyto.2020.9303

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Anti-inflammatory activity of chemical constituents from *Echinops gracilis* (Asteraceae)

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ABSTRACT

The present work concerns the anti-inflammatory activity of chemical constituents isolated from the roots of *Echinops gracilis*. The anti-inflammatory activity of the ethyl acetate extract as well as compounds 1, 2, and 3 were evaluated using inhibition of protein denaturation and antiproteinase methods. Structural elucidation was achieved through analysis of NMR spectra (^{1}H and ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC) and literature survey. Seven compounds were identified as erythrinasinate (1), vogelate (2), ferulic acid (3), *p*-coumaric acid (4), ursolic acid (5), oleanolic acid (6), and quercetin (7). All the isolated compounds were reported for the first time from this plant. Ethyl acetate extract showed potent inhibitory activity against protein denaturation (IC_{50} = 125.54 µg/mL). Erythrinasinate (1) and vogelate (2) showed a significant anti-inflammatory activity with an IC_{50} value of 469.43 and 413.71 µg/mL, respectively. The results obtained from the ethyl acetate extract can justify the use of *E. gracilis* roots in traditional medicine for the treatment of rheumatism.

Keywords: *Echinops gracilis*, Chemical constituents, Protein denaturation, Antiproteinase, *In vitro* anti-inflammatory activity.

INTRODUCTION

Strong correlation has been observed between heart or stroke, autoimmune disorder (rheumatoid, arthritis, lupus…) and chronic inflammation. Complications can occur when the inflammation progression delayed. Such situation needs to be kept under control. The search of biologically active constituents from Cameroonian medicinal plants have been of interest to Cameroonian research teams [1]. The genus *Echinops* belongs to family Asteraceae and comprises over 120 species spread in temperate areas of Europe, Central Asia and tropical Africa [2]. *Echinops gracilis* is a medicinal plant used by traditional healers to treat rheumatism [3]. Previous chemical investigation on the genus *Echinops* established the presence of thiophenes [4-5], acetylenic thiophenes [6], sesquiterpene hydrocarbons [7], triterpenes [5], sesquiterpene lactones [8], alkaloids [5], lignans [9], flavonoids [5], and hydroxycinnamates [10]. In the present state of our literature investigation, no phytochemical study has been undertaken on the species *E. gracilis*. Our goal in this study was to evaluate *in vitro* anti-inflammatory activity of ethyl acetate extract and isolated constituents from the roots of *E. gracilis*

MATERIALS AND METHODS

General experimental procedures

Electrospray ionization mass spectrometry (ESIMS) was recorded on QSTARXL of AB Sciex Company. Melting points of the isolated compounds were determined using an Electrothermal IA9000 Series digital melting point apparatus (Bibby scientific, Great Britain). UV and visible spectra were recorded in MeOH at 25 °C using a Kontron Uvikon spectrophotometer. The IR spectra were measured on a PerkinElmer 1750 FTIR spectrometer. The NMR spectra were measured on Bruker 500 MHz NMR Avance II spectrometers equipped with cryoprobe. Chemical shifts were recorded in δ (ppm) and the coupling constants (J) are in hertz relative to the internal standard tetramethylsilane (TMS). Silica gel 60 F_{254} (70-230; Merck; Darmstadt, Germany) was used as stationary phase for column chromatography. Precoated silica gel Kieselgel 60 F_{254} plates (0.25 mm thick) were used for TLC to monitor the purity of isolates and spots detected by spraying with 50% H_2SO_4 followed by heating at 100 °C. All solvents were distilled before using.

Plant Material

The roots of *E. gracilis* were collected at Melong, Littoral Region of Cameroon in Febuary 2011. Authentication was done through comparison with a voucher specimen (No 1550 SRF Cam) by an ethnobotanist technician, Mr Victor Nana at the Cameroon National Herbarium, Yaoundé.

Extraction and isolation

The roots of *E. gracilis* (3 kg) was air dried, chopped and pulverized into fine powder The ground plant was then submitted to a liquid extraction for 72h at room temperature with ethyl acetate (EtOAc) (15 L) as solvent and then filtered. The filtrate was concentrated in vacuo using a rotatory evaporator and combined to yield 40 g of the crude extract. 33.8 g of this extract were submitted to open column chromatography (CC) over silica gel, eluted with the mixture *n*-hexane (*n*-Hex)-EtOAc of increasing polarity (from 100:0 to 0:100) and EtOAc-MeOH (from 95:5 to 90:10). A total of Eighty eight fractions of 250 mL each were collected, concentrated *in vacuo* in a rotator evaporator and their compound profiles monitored using TLC plates. Similar fractions were combined giving a total of five major fractions [A (6 g, *n*-Hex/EtOAc (95:5, v/v), B (1 g, *n*-Hex/EtOAc (75:25), v/v), C (3.0 g, *n*-Hex/EtOAc (65:35, v/v), D (10 g, *n*-Hex/EtOAc (1:1, v/v), E (7.0 g, CH_2Cl_2/MeOH (98:12), v/v)].

Fraction A (6 g, *n*-Hex/EtOAc (95:5, v/v) was a complex mixture and was not investigated. Compound 1 (12 mg) crystallized from fraction B (1 g, *n*-Hex/EtOAc (75:25), v/v). Fraction C (3.0 g, *n*-Hex/EtOAc (65:35, v/v) was submitted to open column chromatography over silica gel with a gradient of *n*-Hex/EtOAc to give compounds 2 (20 mg). Fraction D (10 g) was subjected to column chromatography on Sephadex LH-20 using CH_2Cl_2/MeOH (9:1) and give three subsfractions (D1, D2 and D3). Subfractions D2 (0.5 g) and D3 (1.0 g) were combined and further chromatographed on silica gel column, eluting with mixtures of *n*-Hex/EtOAc (100:0, 95:5, 90:0, and 80:20) to yield a mixture of compounds 5 (6 mg) and 6 (6 mg). Fraction E (7.0 g, CH_2Cl_2/MeOH (98:12), v/v)] was separated by column chromatography over silica gel using mixture of *n*-Hex/EtOAc increasing polarity to give compounds 3 (10 mg) and 4 (9 mg), respectively. The mixture crystallized from subfraction E (52.5 mg) was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) to give compound 7 (10 mg).

Anti-inflammatory assays

Inhibition of protein denaturation

The anti-inflammatory activity of ethylacetate extract as well as compounds 1, 2, 3 and Diclofenac sodium (Standard) was studied by using inhibition of protein denaturation technique as earlier described by Padmanabhan *et al*. [11]. The reaction mixture consisted of extracts (50,100, 200, 500 and 1000 µg/mL), 5% aqueous solution of bovine serum albumin fraction and PBS solution (pH 6.4) of the reaction mixture. The sample extracts were incubated at 27 ºC for 15 min and then heated to 70 ºC for 10 min, after cooling; the turbidity was measured at 660 nm. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition =

((Abs Control –Abs Sample) / Abs control)*100.

Antiproteinase action

The test was performed according to the modified method of Sakat *et al*. [12]. The reaction mixture (2 mL) contained 0.06 mg trypsin, 1 mL of Tris HCl buffer (1M, pH 7.4) and 1 mL test sample of different concentrations (100 – 500 µg/ml). The mixture was incubated at 37 °C for 5 min and then 1 mL of 0.8% (W/V) casein was added. The mixture was incubated for an additional 20 min. 2 mL of 70% perchloric acid was added to stop the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase activity was calculated as below.

Percentage inhibition =

((Abs control – Abs sample) / Abs control)*100

Statistical analysis

Experiments were performed in triplicates and all data were expressed as mean ± standard deviation (S.D). Differences between IC_{50} values were analyzed for statistical significance using ANOVA and compared using the Fisher's least significant difference (LSD) at 5% confidence interval.

RESULTS AND DISCUSSION

Isolation of compounds

The ethyl acetate extract from *E. gracilis* were fractionated by silica gel column chromatography followed by repeat column chromatography and Sephadex LH-20 led to the isolation of seven compounds identified as erythrinasinate (**1**) [13], vogelate (**2**) [13], ferulic acid (**3**) [14], p-coumaric acid (**4**) [15], ursolic acid (**5**) [16], oleanolic acid (**6**) [16], and quercetin (**7**) [17] (Figure 1). The structures of isolated compounds were elucidated and identified by comparing their ESI mass spectra, 1D and 2D NMR spectroscopic data reported in the literature. To the best of our knowledge, all the isolated compounds were reported for the first time from *E. gracilis*.

Chemical structures of the isolated compounds

Compound 1: white needle crystal, m.p. 78 - 80 °C; *m/z* 586 $[M]^+$; ^1H NMR (500 MHz, $CDCl_3$): See Table 1; ^{13}C NMR (125 MHz, $CDCl_3$): See Table 1. From the comparison of these data with those reported in the literature by Ali *et al.* [13]. The substance was identified as erythrissinate (Figure 1)

Table 1: ^{13}C NMR (125 MHz; $CDCl_3$) and ^1H NMR (500 MHz; $CDCl_3$) data of Erythrinasinate (1) and Vogelate (2) (δ in ppm; *J* in Hz)

	1		**2**	
N°	δ_C	δ_H **(nH, m, *J* in Hz)**	δ_C	δ_H **(nH, m, *J* in Hz)**
1	167.4	-	168.7	-
2	109.2	6.29 (1H, d, *J* = 16)	110.4	6.30 (1H, d, *J* = 16)
3	144.6	7.62 (1H, d, *J* = 16.0)	145.6	7.61 (1H, d, *J* = 16.0)
1'	127.0	-	126.8	-
2'	115.6	7.03 (1H, br.s)	115.9	7.07 (1H, br.s)
3'	146.7		148.2	
4'	147.9	-	149.2	-
5'	114.7	6.92 (1H, d, *J* = 8.4)	115.0	6.87 (1H, d, *J* = 8.4)
6'	123.0	7.09 (1H, d, *J* = 8.4)	123.1	7.08 (1H, d, *J* = 8.4)
1"	64.6	4.18 (2H, t, *J* = 6.4)	64.7	4.19 (2H, t, *J* = 6.4)
2"	28.8	1.69 (2H, m)	28.7	1.71 (2H, m)
3"	26.0	1.28 (br.s)	26.2	1.55 (br.s)
4"	29.7		29.8	
5" to 25"	29.2		29.5	
26"	31.9		32.1	
27"	22.7		25.7	
28"	14.1	0.88 (3H, t, *J* = 6.0)	62.3	3.58 (2H, t, *J* = 6.0)
OCH_3	55.9	3.96 (3H, s)	56.7	3.92 (3H, s)

Compound 2: white needle crystal, m.p. 124 - 125 °C; *m/z* 602 $[M]^+$; ^1H NMR ($CDCl_3$): See Table 1, ^{13}C NMR ($CDCl_3$): See Table 1. From the comparison of these data with those reported in the literature by Ali *et al*. [13]. The substance was identified as vogelate (Figure 1)

Compound 3: colourless needles, m.p. 210-212 °C, *m/z* 194 $[M]^+$; ^1H NMR (500 MHz (DMSO-d_6): See Table 2. ^{13}C NMR (125 MHz, DMSO-d_6): See Table 2. From the comparison of these data with those reported in the literature by El-moaty *et al*. [14]. The substance was identified as ferulic acid (Figure 1).

Compound 4: colourless needles, m.p. 218-220 °C, *m/z* 164 $[M]^+$; ^1H NMR (500 MHz, MeOD): See Table 2. ^{13}C NMR (125 MHz, MeOD): See Table 2. From the comparison of these data with those reported in the literature by Filho *et al*. [15]. The substance was identified as *p*-coumaric acid (Figure 1).

Table 2: ^{13}C NMR data of Ferulic acid (3) and *P*-coumaric acid (4) (125 MHz; DMSO-d_6 and MeOD resp.) and ^1H NMR data of compounds 3 and 4 ((500 MHz; DMSO-d_6 and MeOD resp., δ in ppm; *J* in Hz)

	3			**4**	
N°	**δ_C**	**δ_H (nH, m, *J* in Hz)**	**N°**	**δ_C**	**δ_H (nH, m, *J* in Hz)**
1	168.4		1	168.4	
2	115.0	6.34 (1H, d, *J* = 15)	2	115.8	6.27 (1H, d, *J* = 15.0)
3	144.3	7.50 (1H, d, *J* = 15)	3	144.6	7.48 (1H, d, *J* = 15.0)
4	123.8		4	125.7	
5	127.6	6.79 (1H, d, *J* =9)	5; 9	130.5	6.79 (2H, d, *J* = 8.0)
6	116.1		6; 8	116.2	6.78 (2H, d, *J* = 8.0)
7	148.3	-	7	160.8	-
8	121.0	7.08 (1H, d, *J* = 2)			
9	123.0	7.08 (1H, dd, *J* = 8 and 2)			
OH	-	8.90 (s)	OH	-	8.90 (s)
OCH_3	56.1	3.81 (3H, s)		-	-

Compound **5**: white needles, m.p. 269-271 °C; *m/z* 456 $[M]^+$; ^1H NMR (500 MHz, $CDCl_3$): See Table 3. ^{13}C NMR (125 MHz, $CDCl_3$): See Table 3. From the comparison of these data with those reported in the literature by Begum *et al*. [16]. The substance was identified as ursolic acid (Figure 1).

Table 3: ^{13}C NMR (125 MHz; CDCl3) and ^1H NMR (500 MHz; CDCl3) data of Ursolic acid (5) and Oleanolic acid (6) (δ in ppm; *J* in Hz)

	5		**6**	
N°	**δ_C**	**δ_H (nH, m, *J* in Hz)**	**δ_C**	**δ_H (nH, m, *J* in Hz)**
1	38.0	1.00 [a] (1H, m, *J* = 2.8, 13.0)	38.9	0.98 [a] (1H, m)
		1.53 [e] (1H, m, *J* = 2.8, 3.4, 13.0)		1.64 [e] (1H, m)
2	27.0	1.80 [a] (1H, m, *J* = 9.0, 2.8, 13.0)	27.3	1.61 [a] (1H, m)
		1.80 [e] (1H, m, *J* = 6.0, 3.4, 13.0)		1.63 [e] (1H, m)
3	76.8	3.14 [e] (1H, dd, *J* = 10.0, 4.8)	79.2	3.22 [e] (1H, dd, *J* = 10.0, 4.8)
4	38.2	-	38.5	-
5	54.8	0.68 [e] (1H, dd, *J* = 12.0, 2.0)	55.3	0.75 (1H, dd, *J* = 12.0, 2.0)
6	18.0	1.28 [a] (1H, m, *J* = 12.0, 10.0)	18.5	1.38 [a] (1H, m, *J* = 12.0, 2.0)
		1.48 [e] (1H, m, *J* = 2.0, 3.0, 9.0		1.56 [e] (1H, m, *J* = 2.0, 3.0, 9.0)
7	32.7	1.26 [a] (1H, m, *J* = 2.0, 3.0, 9.0)	32.6	1.30 [a] (1H, m, *J* = 2.0, 3.0, 9.0)
		1.45 [e] (1H, m, *J* = 9.0)		1.55 [e] (1H, m, *J* = 9.0)
8	38.5	-	39.4	-
9	47.0	1.46 (1H, m, *J* = 6.0, 12.0)	47.8	1.56 (1H, dd, *J* = 6.0, 12.0)
10	36.5	-	37.2	-
11	22.	1.83 [a] (1H, m, *J* = 4.0, 11.0, 12.0)	23.1	1.61 [a] (1H, m, *J* = 4.0, 11.0, 12.0)
		1.86 [e] (1H, m, *J* = 4.0, 6.0, 11.0)		1.64 [e] (1H, m, *J* = 4.0, 6.0, 11.0)
12	124.6	5.21 (1H, t, *J* = 4.0)	122.8	5.28 (1H, m, *J* = 4.0, H-12
13	138.2	-	143.8	-
14	41.6	-	41.7	-
15	27.5	1.80 [e] (1H, m, *J* = 4.0, 3.0, 14.0)	27.8	1.08 [a] (1H, m, *J* = 4.0, 13.6, 14.0)
				1.11 [e] (1H, m, *J* = 4.0, 3.0, 14.0)

16	23.8	1.53 [a] (1H, m, *J* = 4.0, 13.0, 13.6)	23.5	1.88 [a] (1H, m, *J* = 4.0, 13.0, 13.6)
		1.92 [e] (1H, m, *J* = 4.0, 13.0, 3.0)		1.88 [e] (1H, m, *J* = 4.0, 13.0, 3.0)
17	46.8	-	46.7	-
18	52.4	2.10[a] (1H, dd, *J* = 2.0, 11.0),	41.1	2.82 [a] (1H, dd, *J* = 11.0)
19	38.4	1.31 [a] (1H, dd, *J* = 6.6, 11.3	46.0	1.15 [a] (1H, m, *J* = 11.0)
20	38.4	1.31 (1H, m, *J* = 2.0, 6.3)	30.8	-
21	30.2	1.27 [a] (1H, m, J=2.2, 14.0)	33.9	1.22 [a] (1H, m, *J* = 2.2, 14.0, 13.0,)
		1.43 [e] (1H, m, *J* = 13.0, 1.4, 3.0, 13.0)		1.35[e] (1H, m, *J* = 1.3, 2.9, 13.0)
22	36.3	1.51[a] (1H, m, *J* = 1.4, 1.4)	32.7	1.43 [a] (1H, m, *J* = 1.3, 14.0, 13.0)
		1.58[e](1H, m, *J* = 13.0, 2.2, 3.0, 13.0)		1.78 [e] (1H, m, *J* = 2.1, 3.0, 13.0)
23	28.2	0.89 (3H, s)	28.2	0.99 (3H, s)
24	16.1	0.67 (3H, s)	15.7	0.77 (3H, s)
25	15.2	0.86 (3H, s)	15.5	0.92 (3H, s)
26	16.8	0.74 (3H, s)	17.3	0.75 (3H, s)
27	23.4	1.04 (3H, s)	26.1	1.14 (3H, s)
28	178.3	-	183.3	-
29	18.0	0.81 (3H, d, *J* = 6.6)	33.2	0.91 (3H, s)
30	21.1	0.91 (3H, d, *J* = 6.3)	23.7	0.93 (3H, s)

[a] - Proton in axial position
[e] - Proton in equatorial position

Compound 6: white needles; m.p. 301-303 °C; *m/z* 456 $[M]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): See Table 3. ^{13}C NMR (125 MHz, $CDCl_3$): See Table 3. From the comparison of these data with those reported in the literature by Begum *et al.* [16]. The substance was identified as oleanolic acid (Figure 1).

Compound 7: yellow needles, m.p. 313-315 °C; *m/z* 302 $[M]^+$; ^{1}H NMR (500 MHz; DMSO): See Table 4. ^{13}C NMR (125 MHz; DMSO): See Table 4. From the comparison of these data with those reported in the literature by Kadeem *et al.* [17]. The substance was identified as quercetin (Figure 1).

Table 4: ^{13}C NMR (125 MHz; DMSO) and ^{1}H NMR (500 MHz; DMSO) data of Quercetin (7) (δ in ppm; J in Hz)

	7	
N°	**δ_C**	**δ_H (nH, m, *J* in Hz)**
2	145.1	-
3	135.8	
4	175.9	-
5	160.8	-
6	98.3	6.19 (1H, d, *J* = 2.0)
7	164.0	-
8	93.41	6.41 (1H, d, *J* = 2.0)
9	156.2	-
10	103.1	-
1'	122.0	-
2'	115.10	7.69 (1H, d, *J* = 2.2)
3'	146.9	-
4'	147.8	-
5'	115.7	6.89 (1H, d, *J* = 8.5)
6'	120.1	7.55 (1H, d, *J* = 8.5, 2.2)
5-OH	-	12.98 (1H, s)

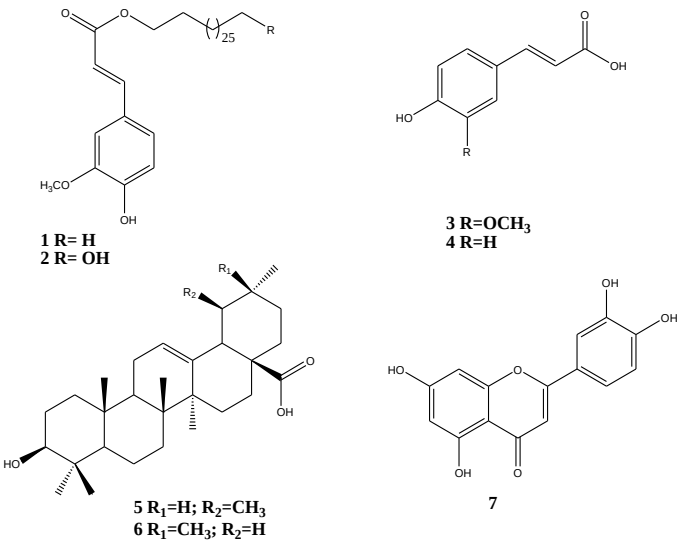

Figure 1: Chemical structures of the isolated compounds (1-7) from *E. gracilis*

Anti-inflammatory activity

The ethyl acetate extract as well as compounds 1-3 were screened for their anti-inflammatory activities through antiproteinase action and protein denaturation studied. The ethyl acetate extract of *E. gracilis* displayed an inhibitory potency with IC_{50} values of 125.54 µg/mL and it was effective in inhibiting heat induced albumin denaturation (Table 5). Maximum inhibition of 78% with IC_{50} (463.5 µg/mL) was observed at 1000 µg/mL with compound 1 compared to Diclofenac sodium, a standard anti-inflammatory drug, which showed the maximum inhibition of 91% with IC_{50} (183.7 µg/mL) at the same concentration (Table 6). This is the first report on anti-inflammatory activity of compounds 1 and 2.

Table 5: Effect of ethyl acetate extract of *E. gracilis* roots on albumin denaturation

Concentration (µg/mL)	Protein denaturation (BSA)	Antiproteinase inhibitory action	Standard
50	31.23 ± 1.58d	5.40 ± 0.84e	0.00 ± 0.00d
100	42.81 ± 4.80c	18.48 ± 0.19d	26.37 ± 8.76c
200	66.97 ± 2.34b	38.56 ± 5.33c	69.01 ± 2.00b
500	74.07 ± 1.10a	54.25 ±3.58b	87.15 ± 1.03a
1000	80.21 ± 0.64a	72.50 ± 8.07a	91.66 ± 0.08a
$F_{(4, 10)}$	204.35***	101.47***	293.70***
IC_{50} (µg/mL) (CI)	125.54 (106.04-145.98)	391.48 (338.28-460.23)	183.75 (147.41-227.11)

Each value represents mean ± SD. Values in column followed by the same letter did not differ significantly according to Tukey test (P = 0.05). ***P < 0.001. Each value represents the mean of 3 replicates. IC_{50}: 50% inhibitory concentration; SD = standards deviation. CI = Confident interval

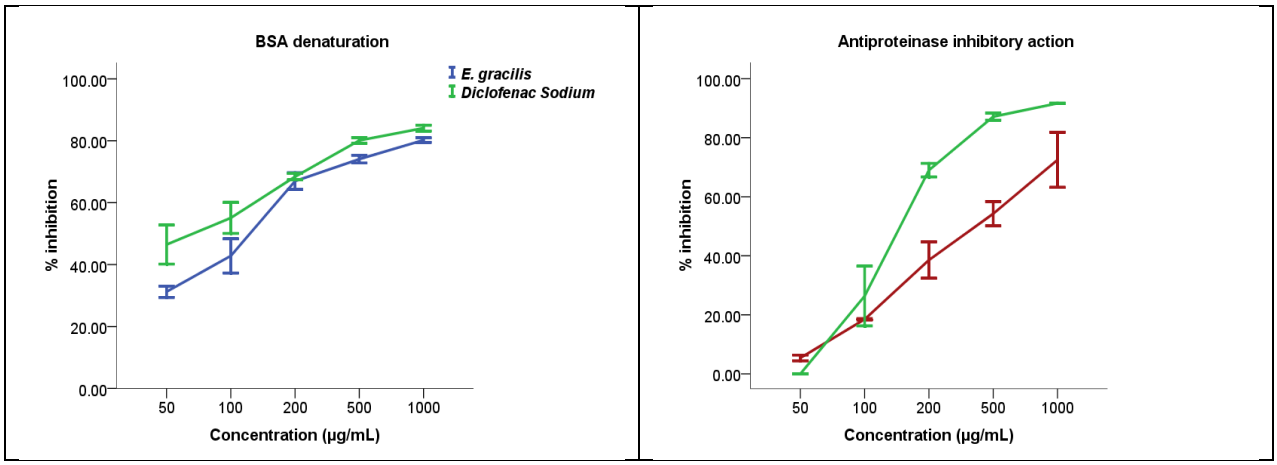

Figure 2: Inhibition percentage of ethyl acetate extract of E. gracilis roots following BSA denaturation and antiproteinase action methods

Table 6: Effect of compounds 1, 2 and 3 on albumin denaturation and proteinase inhibitory

Test methods	Concentration (µg/mL)	1	2	3	Diclofenac Sodium
BSA denaturation	50	1.35±1.35d	8.08±0.67e	23.38±1.39d	0.0±0.0d
	100	5.67±0.71d	12.54±0.61d	25.75±1.31cd	26.37±5.06c
	200	22.06±5.22c	29.16±1.06c	27.99±0.72bc	69.01±2.00b
	500	51.23±0.83b	59.43±1.55b	29.39±1.38b	87.15±1.03a
	1000	78.33±1.46a	71.86±0.58a	33.73±0.93a	91.66±0.08a
	$F_{(5, 14)}$	2546.95***	490.77***	32.81***	293.70***
	IC_{50} (µg/mL)	463.49	413.71	29209.00	183.75
	(CI)	(426.65-505.97)	(372.44-463.37)	(2962.6-5.77E14)	(147.41-227.11)
Antiproteinase inhibitory action	50	nd	nd	nd	0.0±0.0d
	100	nd	nd	nd	26.37±5.06c
	200	nd	nd	nd	69.01±2.00b
	500	nd	nd	nd	87.15±1.03a
	1000	nd	nd	nd	91.66±0.08a
	$F_{(5, 14)}$	-	-	-	293.70***
	IC_{50} (µg/mL) (CI)	-	-	-	183.75 (147.41-227.11)

Each value represents mean ± SD. Values in column followed by the same letter did not differ significantly according to Tukey test (P = 0.05).
***P < 0.001. Each value represents the mean of 3 replicates. IC_{50}: 50% inhibitory concentration, SD = standard deviation. nd= not determined, CI=Confident interval

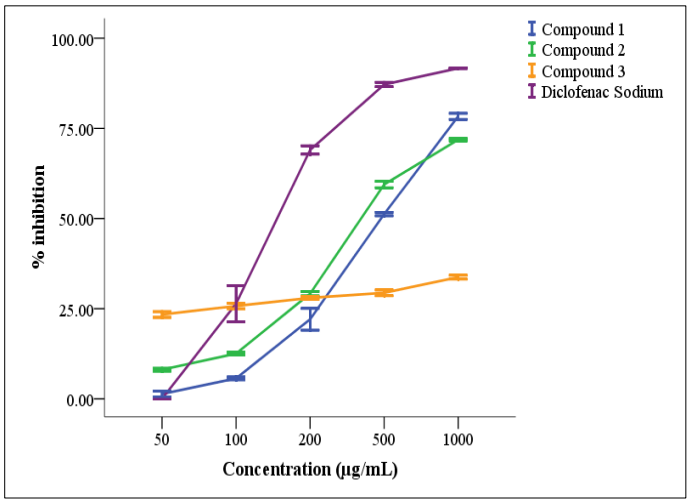

Figure 3: Inhibition percentage of compounds 1, 2 and 3 using BSA denaturation methods

CONCLUSION

The present phytochemical study of ethyl acetate extract of *E. gracilis* roots led to the isolation of seven known compounds **1-7**. Ethyl acetate extract showed potent inhibitory activity against protein denaturation with an IC_{50} value of 125.54 µg/mL. The results give a scientific evidence of the use of the roots of *E. gracilis* in the treatment of rheumatism.

Acknowledgments

The authors are grateful to TWAS-ICCBS Sandwich Postgraduate fellowship offered to AJP (FR No 3240275059) at the International Center for Chemical and Biological Sciences (ICCBS) of the University of Karachi, Karachi-75270, Pakistan. We also gratefully acknowledge the Fonds National de la Recherche Scientifique (FNRS), Belgium for a fellowship to A.T.T. by the Belgian National Fund for Scientific Research (FNRS).

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HOW TO CITE THIS ARTICLE
Lah FCW, Tchamgoue AD, Abdou JP, Kowa TK, Wabo HK, Tchinda AT *et al*. Anti-inflammatory activity of chemical constituents from *Echinops gracilis* (Asteraceae). J Phytopharmacol 2020; 9(3):169-174.