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Bioactivities of Extracts and Isolation of Compounds from *Tridax procumbens* L.

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Abstract. This study was conducted to investigate the bioactivities of extracts, isolation and identification the compounds from aerial parts of *Tridax procumbens* (*T. procumbens*). The standardized ethyl acetate extract from *T. procumbens* shown antibacterial activity against gram negative bacteria as *Escherichia coli* and *Pseudomonas aeruginosa*, while the petroleum ether extract did not shown antibacterial activity. Besides, antioxidant activity of ethyl acetate extract (IC_{50}=0.52 mg/mL) is higher than petroleum ether extract (IC_{50}=1.12 mg/mL). Additionally, the ethyl acetate extract resulted significant anti-inflammatory on mice paw edema at the concentration of 200 mg/kg. Moreover, ethyl acetate extract contains two isolated compounds, namely β-sitosterol-3-O-β-D-glucopyranoside (1) and 3′,5-dihydroxy-4′,3,6-trimethoxyflavone-7-O-β-D-glucopyranoside (2), which were identified by ^{1}H-NMR, ^{13}C-NMR, Mass spectrometry. The pharmacological activities of ethyl acetate extract and their compounds indicated that *T. procumbens* is promising resource for pharmaceutical industry.

Keywords: *Tridax procumbens*, antibacterial, anti-inflammatory, antioxidant, daucosterol, centaurein.

1. Introduction

Thousand years ago, people had already known about the usage of natural source as medicinal agents, nowadays, with rapid development of science and technology, there are a lot of modern drugs with less side effects which were derived from natural source. Every year, abundant new compounds were isolated from traditional medicine or herbal [1]. This also indicates that the isolated compounds from herbal plants play an very important role in pharmaceutical industry, however, a lot of herbal plants still have not been explored for their phytochemical constituents [2], [3]. This present study, *T. procumbens* was chosen to research. In addition, *T. procumbens* (Asteraceae family) is a common herb with a woody base and wild growth in many tropical countries. *T. procumbens* was well known in traditional medicine as abundant wild herbal which can treat various diseases, such as bronchial catarrh, dysentery, diaherrea, preventing hair loss [4]. Moreover, there were research indicated that *T. procumbens* had many important bioactivities including anti-inflammatory, antioxidant, hepatoprotective, wound healing, immunomodulatory, antimicrobial, antiseptic, hypotensive and bradycardiac effects [5]-[8]. Furthermore, some pharmaceutical chemistry reports shown that there were many isolated constituents from *T. procumbens*, including: saturated and unsaturated fatty acids [9], terpenoids, flavonoids, lipids, polysaccharides, such as β-sitosterol, puerarine, dexamethason, esculetin, oleanolic acid, lupeol, quercetin, isoquercetin, fumaric acid, centaureidin, and luteoline [10]. There are many reported about this plant, however, the important bioactivities and compounds was not focused as well as the interpretation of bioactivities and the nexus between bioactivities and their causal isolated compounds was not clarified. Therefore, the aims of this study is the evaluation

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of antibacterial, antioxidant and anti-inflammatory ability, isolation and identification the causal isolated compounds in order to interpret these bioactivities from *T. Procumbens*.

2. Material and Methods

2.1. Plant Material and Preparation of Plant Extract

Aerial parts of *T. procumbens* were collected from the campus of Can Tho University in August 2014. Aerial parts of *T. procumbens* which were free of microbial infection and uniform maturity were collected. Firstly, aerial parts were washed with water to remove the contaminating foreign particles. Then, aerial parts were cut into small pieces and dried under sunlight. After that, aerial parts were powdered using a heavy duty blender. The powder was extracted with ethanol according to the maceration method and the ethanol extract was filtered by filter paper. The filtrate was concentrated using a vacuum rotary evaporator at 45 ^{0}C to remove all traces of ethanol.

2.2. Fractionation of Extract

The ethanol extract was fractionated with petroleum ether, ethyl acetate, and methanol–water (50:50. *v/v*) solvents. The extracts were concentrated by vacuum rotary evaporator to yield petroleum ether residue (9.85 g), ethyl acetate residue (15.03 g) and methanol residue (25.15 g).

2.3. Bioactivity Assays

2.3.1. Antibacterial activity

The bacterium were used for antibacterial test include gram-positive and gram-negative which were *Bacillus faecalis* and *Escherichia coli*, *Pseudomonas aeruginosa*, respectively. They were collected from Microbial laboratory of Ho Chi Minh University of Medicine and Pharmacy.

2.3.2. Antioxidant activity

Free radical scavenging activities of extracts were determined by the widely used 1,1-Diphenyl-2-picrylhydrazyl (DPPH, Sigma) assay. The extracts were dissolved in methanol and the IC_{50} parameter was defined as the concentration of the substrates that cause 50% loss of DPPH activity [11].

2.3.3. Anti-inflammatory activity

The anti-inflammatory activity was investigated on carrageenan – induced inflammatory paw edema. Firstly, 0.025 mL of carrageenan 1% solution was injected into the footpad of the mice's paws to make the edema, then the initial mice's paw volume was measured with plethylsmometer. The first mice's paw edema measurement was at 3 hr after the injection and the next measurements were conducted daily during 5 days. The mice were distributed into 3 groups which were given tween solution (1% *w/w*), *T. procumbens* extracts (200 mg/kg of mice's body weight) in tween solution (1% *w/w*) and ibuprofen (10 mg/kg of mice's body weight) in tween solution (1% *w/w*) orally, respectively. The anti-inflammatory activity of *T. procumbens* extract was compared with that of 10 mg/kg ibuprofen. The percentage inhibition of the inflammation was calculated by the equation (1), where V_0 is the initial volume of mice's paw before injection, V_t is the daily average volume of mice's paw of the drug treated mice [12].

$$\text{Paw edema (\%)} = \frac{V_t - V_0}{V_0} \times 100 \quad (1)$$

2.4. Isolation of Compounds

The ethyl acetate extract (6.00 g) was subjected to column chromatography (CC) on silica gel (80 g), eluting with ethyl acetate in petroleum ether, up to 100% and then gradient of methanol up to 20%. Eluted fractions were evaluated by thin layer chromatography (TLC) and combined upon similar appearance, yielding 8 fractions (Ea1-Ea8). Fraction Ea5 was eluted with petroleum ether–ethyl acetate (30:70, *v/v*) and further purified by repeated CC on silica gel using dichloromethane and methanol gradient elution to yield compound (1) (40 mg). Fraction Ea8 was eluted with ethyl acetate–methanol (90:10, *v/v*) and further purified

by repeated CC on silica gel using ethyl acetate and methanol gradient elution to yield compound (2) (35 mg). Finally, compound (1) and (2) were identified by using ^{1}H-NMR, ^{13}C-NMR, Mass spectrometry [13].

2.5. Statistical Analysis

The observations were expressed as mean ± SE (standard error of mean). All statistical calculations were analysis using Kruskal – Wallis, then using Mann – Whitney by SPSS 22.0 application. The difference are significant when $p<0.05$.

3. Results and Discussion

3.1. Results of Bioactive Activities

The *in vitro* antibacterial activity of standardized petroleum ether and ethyl acetate extract were evaluated and shown in Table 1. Comparatively, ethyl acetate extract exposed the better activity against the gram-negative organism. The petroleum ether extract did not shown activity against the entire tested organism. In both cases, the extracts of aerial parts of *T. procumbens* do not have the strong activity against the tested bacteria. It seems possible that the antibacterial activity of the commercial antibiotic was better than the crude extracts from *T. procumbens* because of their high concentration and purity [4].

Table 1: Antibacterial activity of *T. procumbens* extracts.

Concentration (mg/mL)	Petroleum ether extract			Ethyl acetate extract		
	B.faecallis	*E.coli*	*P.aeruginosa*	*B.faecalis*	*E.coli*	*P.aeruginosa*
25	-	-	-	-	+	+
Std. Ampicillin 200	+	+	+	+	+	+

Std. Ampicillin 200: standard ampicillin with 200 mg/mL; +/-: yes/no activity.

Natural concentrated antioxidants markedly delayed the oxidation of the food components as well as biological system without any toxicity, however synthetic antioxidants caused liver damage and carcinogenicity [14]. This finding implies the important of the natural concentrated antioxidant, therefore the antioxidant activity of petroleum ether and ethyl acetate extracts was evaluated by DPPH free radical method. The result is shown in Fig. 1.

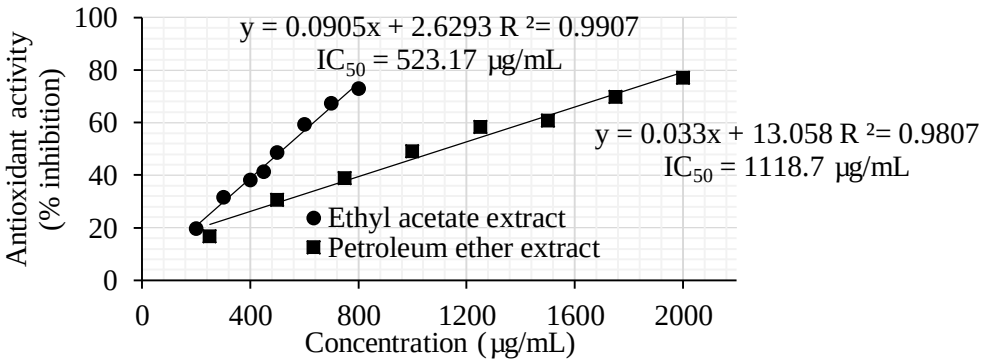

Fig. 1: Antioxidant activity of *T. procumbens* extracts

The result of DPPH radical scavenging activity indicated that the inhibition increased with the elevated concentration for both extracts. This result also indicated that ethyl acetate extract had higher antioxidant activity compared to petroleum ether extract (IC_{50}=1.12 mg/mL and 0.52 mg/mL, respectively). This finding is consistent with previous report of Jachak *et al.*, 2011 [8]. Additionally, phenolic compounds, including flavonoids, were considered as the most important antioxidative components in plant materials [15], therefore, the high antioxidant activity of ethyl acetate extract might due to the high concentration of phenolic compounds in ethyl acetate extract from *T. procumbens*.

The oxygen free radicals liberated from phagocytes are important in the inflammation process. While, the activation of transcription factor and nuclear factor induces the formation of inflammatory cytokines and COX-2 [16]. Additionally, during the inflammatory response, the increased free radical generation is one of the tissue damaging factors. Therefore, the ethyl acetate extract was studied in assay *in vivo* to investigate the effect of anti-inflammatory by carrageenan induced paw volume and the result is shown in Table 2. The

percentage volume of paw treated mice was started to reduce after 3 hr of treatment with ethyl acetate extract compared to controls. The mice paw edema was reduced gradually during 5 days of treatment and the reduction of edema in day 3, day 4, and day 5 of group ethyl acetate were significant and similar to group ibuprofen. This present finding is in agreement with previous reports that ethyl acetate extract performed the best anti-inflammatory activity amount different solvent extracts from *T. procumbens* [8], [17].

Table 2: Effect of *T. procumbens* ethyl acetate extract on carrageenan induced paw volume.

Treatment	Dose (mg/kg)	Percentage increase in paw volume induced by carrageenan					
		3h	Day 1	Day 2	Day 3	Day 4	Day 5
Controls		83.46 ±3.24	76.08 ±1.67	69.50 ±2.10	62.93 ±2.63	54.94 ±3.76	45.00 ±3.77
Ethyl acetate extract	200	84.00 ±3.37	69.41 ±4.44	62.92 ±4.34	48.00 ±5.23**	42.44 ±4.35*	36.95 ±4.26*
Ibuprofen	50	84.39 ±3.09	70.27 ±4.04	64.88 ±4.38	55.65 ±3.61*	43.12 ±4.03*	33.76 ±5.75*

Values are expressed in mean ±SEM; **: p<0.005, *: p<0.05.

3.2. Isolation of Compounds

Compound (**1**) and (**2**) were isolated from ethyl acetate extract and then identified by using ^{1}H-NMR, ^{13}C-NMR, Mass spectrometry.

Compound (**1)** named β-sitosterol-3-O-β-D-glucopyranoside (daucosterol) (Fig. 2). Elution with dichloromethane–methanol (90:10, *v/v*) gave a white crystal, recrystallized from chloroform–methanol (50:50, *v/v*) with mass of 40 mg (0.67% yield); R_f 0.4 chloroform–methanol (90:10, *v/v*);^{1}H NMR (500 MHz; $CDCl_3$&MeOD); δ 1.01 (3H, s, CH_3-27), 0.92 (3H, d, J = 8.5, CH_3-21), 0.85 (3H, d, J = 1.5, CH_3-18), 0.83 (3H, d, J = 4 Hz,CH3-26), 0.81 (3H, s, CH_3-19), 0.69 (3H, s, CH_3-29), δ 5.37 (1H, d, J = 4.5 Hz, H-6), 4.41 (1H, d, J = 8 Hz, H-1′), 3.84 (1H, dd, J = 2.5,12 Hz, H_a-6′), 3.77 (1H, dd, J = 4,12 Hz, H_b-6′), 3.59 (1H, m, H-3), 3.44 (1H, m, H-5′), 3.42 (1H,m, H-3′), 3.29 (1H, m, H-4′), 3.25 (1H, m, H-2′); ^{13}C NMR(125 MHz; $CDCl_3$&MeOD); δ 140.46 (C-5), 122.32 (C-6), 77.15 (C-3), 56.13 (C-14), 55.38 (C-17), 49.55 (C-9), 45.09 (C-24), 41.82 (C-13), 39.28 (C-4), 38.26 (C-12), 36.79 (C-1), 36.18 (C-10), 35.45 (C-20), 33.29 (C-22), 31.38 (C-7), 31.26(C-8), 29.23 (C-2), 28.64 (C-25), 27.76 (C-16), 25.36 (C-23), 23.83 (C-15), 22.56 (C-28), 20.56 (C-11), 19.69 (C-26), 19.07 (C-19), 18.89 (C-27), 18.58 (C-21), 11.75 (C-29) and 11.64 (C-18), 100.73 (C-1′), 77.67 (C-3′), 77.42 (C-5′), 75.97 (C-2′), 76.58 (C-4′), 61.96 (C-6′); ESI MS *m/z* (rel. int.%); 576[M^+] ($C_{35}H_{60}O_6$) (5), 411 (5.2), 397 (118), 383 (30), 255 (7.5), 135 (2.7).

Compound (**2)** named 3′,5-dihydroxy-4′,3,6-trimethoxyflavone-7-O-β-D-glucopyranoside (centaurein) and shown in Fig. 3. Elution with ethyl acetate–methanol (95:5, *v/v*) gave a yellow crystal, recrystallized from methanol with mass of 35 mg (0.58% yield); R_f 0.43 dichloromethane–methanol (85:15, *v/v*); ^{1}H NMR (500 MHz; DMSO-d_6); δ 3.77, 3.80, 3.87 (each 3 H, s, 3-OMe, 6-OMe, 4′-OMe, respectively), 6.95 (1H, s, H-8), 7.15 (1H, d, J=8.4Hz, H-5′), 7.57 (1H, d, J=2.4Hz, H-2′), 7.65 (1H, dd, J=8.4, 2.4Hz, H-6′), 3.22 (1H, dd, J=9.6, 8.8Hz, H-4″), 3.48 (1H, m, H-5″), 3.48 (1H, dd, J=8.8, 7.2 Hz, H-2″), 3.51 (1H, dd, J=12, 6 Hz, H_a-6″), 3.72 (1H, dd, J=12, 2 Hz, H_b-6″), 5.09 (1H, d, J=7.2Hz, H-1″); ^{13}C NMR (125 MHz; DMSO-d_6); δ 55.68 (3′-OMe), 59.77 (3-OMe), 60.32 (6′-OMe), 94.12 (C-8), 106.29 (C-10), 111.90 (C-2′), 115.17 (C-5′), 120.44 (C-6′), 122.21 (C-1′), 132.23 (C-6), 137.96 (C-3), 146.35 (C-3′), 150.37 (C-4′), 151.31 (C-5), 152.06 (C-9), 156.00 (C-7), 156.52 (C-2), 60.62 (C-6″), 69.57 (C-4″), 73.19 (C-2″), 76.67 (C-3″), 77.23 (C-5″), 100.11 (C-1″); ESI MS *m/z*(rel. int.%); 523[M+H]$^+$ ($C_{24}H_{26}O_{13}$) (100), 361 (21).

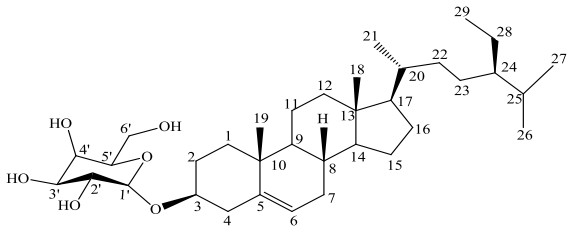

Fig. 2: β-Sitosterol-3-O-β-D-glucopyranoside

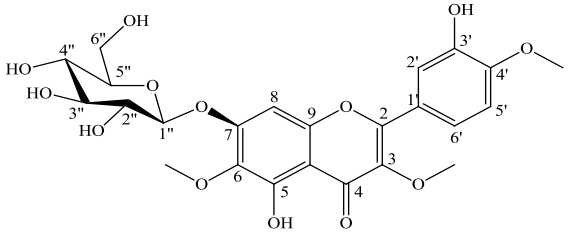

Fig. 3: 3′,5-Dihydroxy-4′,3,6-trimethoxyflavone-7-O- β-D-glucopyranoside

Daucosterol was reported by Shankar Subramaniam*et al.*, 2014 that gave the effect against gram-positive and gram-negative with small MIC [18] and was isolated as analgesic constituents [19]. Shulin Chang *et al.*, 2007 found that NK and T cells increased IFN-γ production in response to centaurein [16]. Additionally, Sanjay M. Jachak*et al.*, 2011 also found that centaurein resulted the significant anti-inflammatory in both assay *in vivo* and *in vitro* [8]. These findings of daucosterol and centaurein also revealed the root of bioactivities from *T. procumbens* ethyl acetate extract.

In conclusion, the standarlized ethyl acetate extract from*T. procumbens* aerial parts revealed the activity against gram-negative bacteria as *Escherichia coli* and *Pseudomonas aeruginosa* while the petroleum ether extract did not shown antibacterial activity. Besides, ethyl acetate extract exhibited higher antioxidant activity than petroleum ether extract. Additionally, the ethyl acetate extract resulted the significant anti-inflammatoryat the concentration of 200 mg/kg. Moreover, two compounds were isolated from ethyl acetate extract, namely β-sitosterol-3-O-β-D-glucopyranoside (**1**) and 3′,5-dihydroxy-4′,3,6-trimethoxyflavone-7-O-β-D-glucopyranoside (**2**). This present result implies that *T. Procumbens* is promising alternative resource for pharmaceutical industry.

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