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INVESTIGATION OF ANTI-*HELICOBACTER PYLORI* ACTIVITY AND CHEMICAL CONSTITUENTS OF *LUDWIGIA HYSSOPIFOLIA* AERIAL PARTS

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Abstract. *Helicobacter pylori* (*H. pylori*) is considered as the main cause of peptic ulcer and gastric carcinoma. Nowadays, due to the growing resistance to antimicrobials, there is a strong demand for finding new anti-*Helicobacter pylori* from natural resources. *Ludwigia hyssopifolia* was traditionally used so far for treatment of *H. pylori* infection in Mekong Delta region of Viet Nam. In this study, five extracts and fractions of the aerial parts of *Ludwigia hyssopifolia* (G. Don) Exellincluding *n*-hexane, chloroform, ethyl acetate, methanol, and ethanol ones were screened for anti-*H. pylori* activity by disk diffusion method. The preliminary results showed that all five extracts and fractions displayed a moderate activity in which the methanol one exhibited the strongest inhibitory activity with the inhibition zone (d = 17 mm) at the concentration of 0.5 mg/disk. Phytochemical study on the bioactive methanol extract led to the isolation of four known compounds as *β*-sitosterol (**1**), oleanolic acid (**2**), 3-*O*-*β*-*D*-glucopyranosyl-*β*-sitosterol (**3**), and ellagic acid (**4**). Their structures were elucidated by spectroscopic methods including 1D and 2D-NMR and in comparison with literature data. Ellagic acid and oleanolic acid seem to be major effective constituents for anti- *H. pylori* activity of the methanol extract.

Keywords: antimicrobial, disk diffusion, ellagic acid, *Helicobacter pylori*, *Ludwigia hyssopifolia*.

Classification numbers: 1.1.1, 1.1.6.

1. INTRODUCTION

Ludwigia hyssopifolia, belonging to Onagraceae family, is widely distributed in Mekong Delta region of Viet Nam. It was used as a traditional treatment for dysentery, infectious hepatitis, and edema [1]. Study on its chemical constituents showed that *L. hyssopifolia* contained piperine, palmitic acid, isovanillin, *β*-sitosterol, stigmasterol-3-*O*-*β*-D-glucopyranoside, gallic acid, ethyl gallate, oleanolic acid, 2,4,6-trihydroxybenzoic acid, ursolic acid, kaempferol, ginsenoside Rb_1, 6*β*,24-hydroxy tormnetic acid, xanthyletin, (+)-*trans*-decursidinol, *β*-sitosterol-*β*-D-glucopyranoside, 6*β*,23-hydroxy tormentic acid, 23-hydroxy tormentic acid, and 6*β*,23-hydroxy tormentic acid [1]. Investigation of its biological activities showed that the plant exhibited several activities including anticancer, antibacterial, antidiarrheal, anti-inflammatory, and anti-ulcer [2 - 4]. In Mekong Delta region, *L. hyssopifolia* has been traditionally used for treatment of *H. pylori* infection. *H. pylori* has been involved in several gastrointestinal diseases for examples gastritis, peptic ulcer disease, and gastric cancer [5]. Normally, multiple drug therapy has been applied in treatment of these infections. However, the rate of failure of such eradication therapy remained from 5 % to 20 % due to the increasing resistance to antibiotics used. Thus, screening for potential antimicrobial agents is an urgent demand. Many natural products known to exhibit antibacterial activity against *H. pylori* as well as several plants and metabolites isolated from plants were used to treat gastrointestinal disorders. In this study, the activity against *H. pylori* of different solvent extracts and fractions from *L. hyssopifolia* was evaluated and phytochemical investigation of the highest bioactive methanol extract was performed to establish experimental evidence supporting for traditional use of *L. hyssopifolia* in treatment of *H. pylori* infection.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial parts of *Ludwigia hyssopifolia* (G. Don) Exellwere collected in Can Tho city, Viet Nam in May 2019. The identification was conducted by Dr. Dang Minh Quan, School of Education, Can Tho University, Viet Nam. A voucher specimen with the code No LH-0519 was deposited in the herbarium of the Faculty of Science, Can Tho University of Medicine and Pharmacy, Can Tho, Viet Nam.

2.2. General experimental procedures

The NMR experiments were performed on a Bruker DMX 500 spectrometer. HRMS-ESI was carried out on a MICROMASS ZABspecTOF spectrometer for electrospray ionization. Vacuum liquid chromatography (VLC), column chromatography was performed on normal phase silica gel (40 - 63 *μ*m, Keselgel 60, Merck, Darmstadt, Germany). Thin-layer chromatography was performed on Kieselgel $60F_{254}$ plates (Merck, Darmstadt, Germany) and spots were visualized under UV light or sprayed with vanillin (0.5 g vanillin in 80 mL sulfuric acid and 20 mL ethanol), then heated. All solvents used were purchased from Chemsol, purity ≥ 99.0 %.

2.3. Anti-*helicobacter pylori* activity test

Disk diffusion method was performed as Do *et al.* [6]. Two reference drugs (amoxicillin and metronidazole) were used as positive control.

2.4. Extraction and isolation

Dried and powdered *L. hyssopifolia* aerial parts (2.0 kg) were successively and exhaustively extracted by ultrasonic-assisted maceration with methanol (16 L). The solvent wasremoved under reduced pressure to give 99 g of total methanol extract. The total methanol extract (2 × 20 g) was subjected to silica gel VLC (6 × 10 cm) using isocratic elution with increasing polarity of solvents (*n*-hexane, chloroform, ethyl acetate) to furnish *n*-hexane fraction (4.0 g), chloroform fraction (6.0 g) and ethyl acetate fraction (9.0 g), respectively. To obtain the ethanol extract, 100 g of material was macerated with 1 L ethanol, the solvent was removed under reduced pressure to give 3.5 g ethanol extract. All the extracts and fractions were stored at 4 ^{o}C for anti-*H. pylori* activity screening.

In order to isolate potent anti-*H. pylori* activity compounds of methanol extract, 5 g of the extract was subjected to a silica gel column and eluted with a solvent system of chloroform–methanol with increasing methanol ratios to obtain 9 fractions from Me1 to Me9. The fraction Me1 was re-chromatographed on silica gel, eluting with *n*-hexane–ethyl acetate (8:2) to give compound **1** (6 mg). The fraction Me7 was subjected to a silica gel column and eluted with dichloromethane–methanol with increasing methanol ratios to yield compounds **2** (7 mg), **3** (23 mg) and **4** (9 mg).

β-Sitosterol (**1**): white amorphous powder; ^{1}H NMR ($CDCl_3$, 500 MHz) $\delta_{H\ ppm}$ 5.35 (1H, *b rs*, H-6), 3.52 (1H, *m*, H-3), 0.68 (3H, *s*, H-18), 1.01 (3H, *s*, H-19), 0.93 (3H, *d*, 6.5, H-21), 0.82 (3H, *d*, 6.5, H-26), 0.84 (3H, *d*, 6.5, H-27), 0.85 (3H, *t*, 7.0, H-29); ^{13}C NMR ($CDCl_3$, 125 MHz) $\delta_{C\ ppm}$ 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.8 (C-5), 121.7 (C-6), 31.9 (C-7), 31.8 (C-8), 50.2 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.2 (C-13), 56.1 (C-14), 24.3 (C-15), 28.3 (C-16), 56.8 (C-17), 11.9 (C-18), 19.4 (C-19), 36.2 (C-20), 18.8 (C-21), 33.9 (C-22), 26.1 (C-23), 45.9 (C-24), 29.2 (C-25), 19.8 (C-26), 19.1 (C-27), 23.1 (C-28), 12.0 (C-29).

Oleanolic acid (**2**): white amorphous powder; ^{1}H NMR (pyridine-d_5, 500 MHz) $\delta_{H\ ppm}$5.47 (1H, *m*, H-12), 3.34 (1H, *dd*, 10.0, 6.5, H-3), 3.28 (1H, *dd*, 13.0, 3.5, H-18), 1.25 (3H, *s*, H-27); 1.20 (3H, *s*, H-23); 1.02 (3H, *s*, H-24); 1.01 (1H, *s*, H-30); 1.00 (3H, *s*, H-26); 0.95 (3H, *s*, H-29); 0.88 (3H, *s*, H-25); ^{13}C NMR (pyridine-d_5, 125 MHz)$\delta_{C\ ppm}$38.6 (C-1), 27.9 (C-2), 77.4 (C-3), 38.9 (C-4), 55.5 (C-5), 18.4 (C-6), 33.4 (C-7), 39.7 (C-8), 49.1 (C-9), 37.9 (C-10), 23.6 (C-11), 122.3 (C-12), 144.5 (C-13), 42.6 (C-14), 29.0 (C-15), 23.1 (C-16), 48.2 (C-17), 41.5 (C-18), 46.2 (C-19), 30.6 (C-20), 34.0 (C-21), 32.8 (C-22), 28.2 (C-23), 16.6 (C-24), 15.2 (C-25), 17.7 (C-26), 25.9 (C-27), 180.5 (C-28), 33.4 (C-29), 23.1 (C-30). ESI-MS *m/z* 457.3682 $[M+H]^+$ calcd. for $C_{30}H_{49}O_3$. Found 457.3679.

3-*O*-*β*-*D*-Glucopyranosyl-*β*-sitosterol (**3**): white amorphous powder; ^{1}H NMR ($CDCl_3$-CD_3OD, 500 MHz) $\delta_{H\ ppm}$ 5.37 (1H, *br s*, H-6), 0.64 (3H, *s*, H-18), 0.95 (3H, *s*, H-19), 0.89 (3H, *d*, 6.0, H-21), 0.80 (3H, *d*, 7.0, H-26), 0.82 (3H, *d*, 7.0, H-27), 0.98 (3H, *s*, H-29), 4.42 (1H, *d*, 7.5, H-1'), 3.86 (1H, *dd*, 12.0, 3.5 Hz, H-6'a), 3.78 (1H, *dd*, 12.0, 3.5 Hz, H-6'b); ^{13}C NMR ($CDCl_3$-MeOD, 125 MHz) $\delta_{C\ ppm}$37.2 (C-1), 29.1 (C-2), 76.4 (C-3), 39.7 (C-4), 140.2 (C-5), 122.1 (C-6), 31.9 (C-7), 31.8 (C-8), 50.1 (C-9), 36.7 (C-10), 21.1 (C-11), 38.7 (C-12), 42.3 (C-13), 56.7 (C-14), 24.2 (C-15), 26.0 (C-16), 56.2 (C-17), 11.8 (C-18), 19.2 (C-19), 36.1 (C-20), 18.9 (C-21), 33.9 (C-22), 24.22 (C-23), 45.8 (C-24), 28.2 (C-25), 18.7 (C-26), 19.7 (C-27), 23.0 (C-28), 11.9 (C-29), 101.1 (C-1′), 76.9 (C-2′), 73.5 (C-3′), 70.1 (C-4′), 76.9 (C-5′), 61.8 (C-6′).

Ellagic acid (**4**): yellow crystals; ^{1}H NMR (DMSO-d_6, 500 MHz) $\delta_{H\ ppm}$ 7.26 (2H, *s*, H-5, H-5'); ^{13}C NMR (DMSO-d_6, 125 MHz) $\delta_{C\ ppm}$ 112.4 (C-1, C-1′), 136.1 (C-2, C-2′), 140.0 (C-3, C-3′), 148.4 (C-4, C-4′), 110.9 (C-5, C-5′), 108.4 (C-6, C-6′), 160.9 (C-7, C-7′). ESI-MS *m/z* 303.0141 $[M+H]^+$ calcd. for $C_{14}H_7O_8$. Found 303.0147.

3. RESULTS AND DISCUSSION

The extracts and fractions of *L. hyssopifolia* aerial parts (*n*-hexane, chloroform, ethyl acetate, methanol, and ethanol) were screened primarily for anti-*H. pylori* activity at the concentration of 0.5 mg/disk. Moderate activity was exhibited by all five extracts and fractions with inhibition zone diameters (IZD) ranging from 11 to 17 mm (Table 1). The methanol extract displayed the highest antibacterial activity against *H. pylori* with 17 mm of IZD (Figure 1).

Do *et al.* evaluated anti *H. pylori* activity of 30 Vietnamese medicinal plants [7]. Ten plants showed potential anti-*H. pylori* with IZD range between 12 to 42 mm at the concentration of 4 mg/disk which was eight times higher than that of *L. hyssopifolia* in the present study [7]. Research conducted by Cogo *et al.* on anti-*H. pylori* activity of seven plants traditionally used for the treatment of gastrointestinal disorders in Brazil showed that the extracts produced IZD from 7 to 10 mm at 0.5 mg/disk [5]. In comparison to these previous studies, *L. hyssopifolia* extracts possessed significantly more potent against *H. pylori* [5]. The results firstly supported scientific evidence for traditional use of *L. hyssopifolia* intreatment of *H. pylori* infection. However, further study should be done to determine MIC values of methanol extract to compare better the degree of antimicrobial activity.

Table 1. Anti-*H. pylori* activity of extracts and fractions by disk diffusion test.

Samples/Drugs	Inhibition zone (mm)
n-Hexane[a]	11
Chloroform[a]	13
Ethyl acetate[a]	14
Methanol[a]	17
Ethanol[a]	12
Amoxicillin[b]	8
Metronidazole[b]	8

[a]0.5 mg/disk, [b]0.05 mg/disk

Figure 1. Inhibitory effect of *L. hyssopifolia* extracts and fractions. *(H: n-hexane fraction, C: chloroform fraction, EA: ethyl acetate fraction, M: methanol extract, E: ethanol extract).*

To obtain knowledge of metabolites which could be responsible for anti-*H. pylori* activity, phytochemical study on the bioactive methanol extract was performed. Four known compounds

were isolated and their chemical structures were determined to be *β*-sitosterol (**1**), oleanolic acid (**2**), 3-*O*-*β*-*D*-glucopyranosyl-*β*-sitosterol (**3**), and ellagic acid (**4**) (Figure 2) [8 - 10]. So far, oleanolic and ellagic acids have been reported as anti-*H. pylori* agents. Oleanolic acid, an active compound in ethyl acetate extract of *Forsythia suspense* strongly inhibited urease activity of *H. pylori*. This enzyme contributes to the ability of *H. pylori* to colonize the acidic environment of the stomach by providing an alkaline microenvironment. With urease inhibition activity, oleanolic acid is promised as a potential therapeutic candidate against *H. pylori* [11]. Besides, ellagic acid inhibited the growth of 55 *H. pylori* strains from clinical isolates from patients suffering from various gastroduodenal pathologies in India with MIC values ranged from 5 to 30 mg/L [12]. In fact, the presence of such compounds in the methanol extract of *L. hyssopifolia* could be involved in its higher anti-*H. pylori* activity.

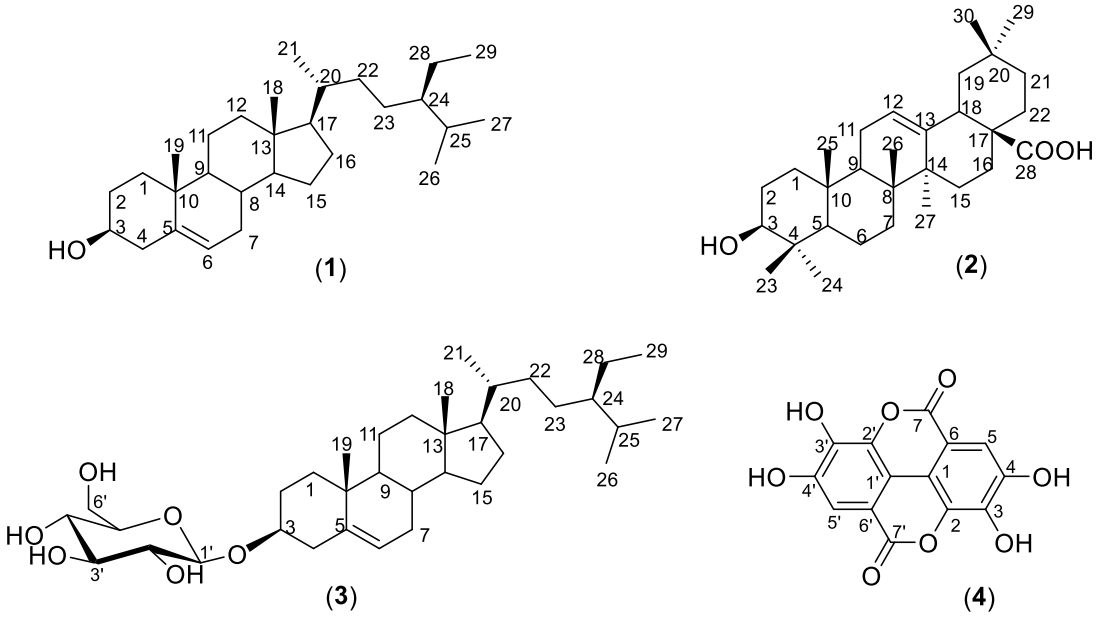

Figure 2. Structures of isolated compounds from *Ludwigia hyssopifolia*.

4. CONCLUSION

Increasing drug resistance in bacteria alarms for development of new drug sources, and traditional plants seem to be a promising source of new antibacterial compounds. This study firstly demonstrated activity against *H. pylori* of *Ludwigia hyssopifolia* (G. Don) Exell aerial parts collected in Mekong Delta, Viet Nam. Methanol extract showed the strongest anti-*H. pylori* activity and four known compounds as *β*-sitosterol (**1**), oleanolic acid (**2**), 3-*O*-*β*-*D*-glucopyranosyl-*β*-sitosterol (**3**), and ellagic acid (**4**) were isolated from such extract. Further phytochemical and biological in vestigations need to be continued to find more potential anti-*H. pylori* agents.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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