Evaluating antibacterial efficacy of extracts from *Ludwigia hyssopifolia* against pathogenic bacteria

Uyen To Pham[1], Y Thi Nhu Hoang[1], Bao Le[1*]
[1]Ton Duc Thang University, Ho Chi Minh City, Vietnam
[*]Corresponding author: lebao@tdtu.edu.vn

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ABSTRACT

Ludwigia hyssopifolia extracts have historically been consumed as a healthful drink and rich in biomass. Therefore, the aims of this study were to determine the most effective fractionated extracts of *L. hyssopifolia* against pathogens. The whole plants of *L. hyssopifolia* were extracted various fractions with methanol, hexane, chloroform, ethyl acetate, and aqueous. The total phenolics and flavonoids was screened using quantitative approaches. The extracts tested for antibacterial activity against pathogens, including *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella Typhi,* and *Helicobacter pylori* through the agar diffusion technique and the microtiter broth dilution method. The largest yield was obtained from the aqueous fractional extract. The phytochemical composition discovered in ethyl acetate was more abundant in phenolics and flavonoid compounds. Fractionation enhanced the antibacterial effect of the extract. The extracts showed a broad spectrum of antibacterial activity against all the bacteria tested, particularly *H. pylori*. In parallel, ethyl acetate extract showed antibacterial activity. The current results could provide rational support for the traditional use of *L. hyssopifolia* to treat infectious diseases and provide data for developing natural products.

1. Introduction

Ludwigia hyssopifolia is a semi-terrestrial plant belonging to the Onagraceae family (Raven, 1974). The plants are found in moist lands and have been used to prepare dishes in Asian countries (Chauhan, Pame, & Johnson, 2011). *Ludwigia* sp. is an annual herbaceous plant (living for 01 year) and grows abundantly in the Central and Southern provinces, from Hue outward to the Mekong Delta provinces (Nguyen, Huynh, Pham, Phan, & Nguyen, 2020). *Ludwigia* leaves rich in phytochemical compounds, such as polyphenolics, terpenoids, and saponin with biological properties (Das, Kundu, Bachar, Uddin, & Kundu, 2007; Mangao, Arreola, Gabriel, & Salamanez, 2020; Rao, Merugu, & Atthapu, 2013; Shawky, Elgindi, & Hassan, 2023). The methanol extract of *L. hyssopifolia* exerts anti-diarrheal activity by reducing diarrhea episodes in mice (Shaphiullah et al., 2003). While the ethyl acetate extract of *L. hyssopifolia* with the alkaloid component 1-[5-(1,3-benzodioxol-5-yl)- 1-oxo-2,4-entadienyl] piperidine, commonly known as piperine showed potential antitumor and antibacterial activity (Das et al., 2007). Kundu, Das, Kundu, and Bachar (2014) published a model to determine the anti-inflammatory, analgesic, and diuretic activities of *L. hyssopifolia*. In Vietnam, *L. hyssopifolia* is used to treat colds, fever, pharyngitis, stomatitis, enteritis, diarrhea, sprue, dysentery, and is used externally to treat (Chi, 1997).

Antibacterial agents are one of the important and effective therapies for treating diseases caused by microbial infections (Belete, 2019). However, indiscriminate use of antibiotics leads to the emergence and spread of multidrug resistant (MDR) strains among bacterial pathogens, which significantly reduces or sometimes completely neutralizes the effectiveness of antibiotics (Jubair, Rajagopal, Chinnappan, Abdullah, & Fatima, 2021). Thus, under pressure from rapid MDR spreading globally, it is paramount to identify products with antibacterial properties in parallel with finding new treatment methods that do not depend on drugs. Following the recommendations proposed by the World Health Organization (WHO) on the use of medicinal plants to treat bacterial injection may exert better outcomes and duration of effect than commercial antibiotic drugs (Organization, 2019). Secondary metabolites produced in plants plays a major role in defense against herbivores and microbes (Taylor, 2013; Wink, Ashour, & El-Readi, 2012).

Ludwigia genus has been shown to possess broad spectrum antimicrobial activities. Thus, Oyedeji, Oziegbe, and Taiwo (2011) studied the effect of extracts of *L. abyssinica* and *L. decurrens* leaves on the inhibition growth of clinically important species of bacteria and fungi. They showed the highest Minimum Inhibitory Concentration (MIC) of n-butanol and ethyl acetate extracts on bacteria species. Yakob, Sulaiman, and Uyub (2012) study the MIC and Minimal Bactericidal Concentration (MBC) of twelve extracts of *L. octovalvis* on *Escherichia coli* O157:H7. Results showed that leaf extracts showed a strong effect on resistance *Escherichia coli* strain, whereas root extracts were against *Pseudomonas aeruginosa*. However, there is still no study that comprehensively demonstrates the influence of different extracts on the antibacterial ability of *L. hyssopifolia*.

L. hyssopifolia is widely used as a traditional medicine for treating various diseases. For that evidence, our current study aims to contribute to the knowledge of different extracts from *L. hyssopifolia*, to understand the relationship between its phytochemical compounds and *in vitro* antibacterial activity against the most common bacterial pathogens.

2. Materials and methods

2.1. Speciments and extract preparation

The plants were identified by taxonomists (Dr. Dang Van Son) of the Institute of Tropical Biology, Ho Chi Minh City, Vietnam. After authentication, fresh aerial parts of the matured plants were collected in bulk from the rice-cultivating area in Go Vap District, Ho Chi Minh City, Vietnam, in February 2022, washed, shaded dried, and then milled into coarse powder by a mechanical grinder. After grinding, the plant supernatant was extracted by sequential extraction procedure using methanol (MRM), hexane (HRM), chloroform (CRM), ethyl acetate (EARM) and aqueous (DRM). Each solvent extraction was done in the ratio of 1:25 (v/v), and then, the mixture was filtered through Whatman No. 42 filter paper, and the organic matter was re-extracted two additional times under the same conditions. Each extract was then evaporated under reduced pressure at 45°C to yield the following equation:

$$Percentage\ yield\ (\%) = \frac{Dry\ weight\ of\ extract}{Dry\ weight\ of\ plant\ material} \times 100 \quad (1)$$

The dried extract was stored in a desiccator till further study.

2.2. Quantification of total phenolics and total flavonoid

For the determination of the total phenolic (TPC) and flavonoid (TFC) contents, the extracts were solubilized in methanol or DMSO (1 mg/mL) and filtered through a 0.45μm filter. The $AlCl_3$ method was applied in our study to determine the total amount of flavonoid content. In brief, 500μL of a serially diluted sample solution (6.25 - 400 μg/mL) was diluted with 3ml of distilled water and then 300μL of 5% $NaNO_2$ was added and allowed to stand for 05min. Then, 600μL of 10% $AlCl_3$ was added. After 06min, 2mL of 1M NaOH was added, and the absorbance was measured at 510nm vs. the reagent blank. The Total Flavonoid Content (TFC) was then calculated using a standard calibration curve, prepared from quercetin, and expressed as mg of quercetin equivalent (QAE) per gram of extract. The Folin-Ciocalteu method was used to determine the total amount of phenolic content. In detail, 800μL of a serial diluted sample solution (6.25 - 400 μg/mL) was mixed with 100μL Folin-Ciocalteu reagent in a microtube. After 05 minutes of incubation, 100μL of 20% sodium carbonate was added, and the mixture was allowed to stand for 30 minutes protected from light. The absorption was measured at 725nm using a UV spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). The TPC was expressed in terms of mg of Gallic Acid Equivalent (GAE) per gram of extract.

2.3. Microbial strains and culture media

The five different human pathogenic bacterial strains were tested, including *Bacillus cereus* KACC 11240, *Staphylococcus aureus* KCCM 11335, *Escherichia coli* K99, *Salmonella* Typhi KACC 10764, and *Helicobacter pylori* 51932. These tested strains were subcultured and stored in Mueller Hinton Agar (MHA) media at 4°C until analysis.

2.4. Determination of zone of inhibition

To screen the antibacterial activity of extracts, the tested strain was inoculated in Muller Hinton Broth (MHB) to adjust the turbidity equal to 0.5 McFarland standard (1.5×10^8 CFU/mL). The MHA plate was then swabbed with standardized microbial culture broth. Five wells of 6mm were punched in the inoculated media using a sterile borer. Each well was filled with 50μL extracts from diluted extracts and Kanamycin 50 mg/mL as positive control. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 24 hours at 37°C. After incubation, plates were observed for the formation of a clear zone around the well, which corresponds to the antimicrobial activity of tested extracts. The diameter of inhibitory zones (in mm) formed around each well was measured in mm.

2.5. Determination of MIC and MBC

To verify the antibacterial activity of extracts, the broth microdilution method was used to determine the MIC according to Clinical and Laboratory Standards Institute (CLSI). A set of two-fold serial dilutions of extracts (100μL) and Mueller Hinton Broth (100μL) was placed in a microtiter plate to obtain various concentrations. The bacterial suspension (turbidity equal to 0.25 McFarland standard) was put into each well and mixed thoroughly. The plate was sealed and incubated aerobically at 37°C. Following 24h incubation, 0.01% sterile resazurin solution (20μL) was added for colour development. MIC is defined as the lowest extract concentration which could retard the multiplication of the tested bacteria. The MBC values of the extracts were recorded as the lowest concentration that showed no visible growth.

2.6. Statistical analysis

All the determinations were performed in triplicate. The statistical analysis was performed using GraphPad Prism 9. The significance level was chosen before performing the statistical analysis ($\alpha = 0.05$).

3. Results and discussion

3.1. Chemical profile

Liquid-liquid partition is a critical analytical technique in extraction/purification/ separation of the bioactive secondary metabolites, which are often present in low quantities in plant material. In this study, the yield of crude extract (MRM) of the *Ludwigia hyssopifolia* leaves was 9.45% w/w, which was calculated based on the weight of dried plant material (Table 1). Liquid-liquid partition for HRM, CRM, EARM, and DRM produced approximately 15.61%, 5.49%, 6.94%, and 30.23% yields, respectively (Table 1). The yield of the aqueous fraction (30.23%) was the highest among the four fractions, which was calculated based on the weight of the crude MRM extract used (Reddy et al., 2021).

Table 1

Extraction yields and obtains dry weights of *Ludwigia hyssopifolia* extract and its partitioned fractions

Extracts	Yields (%)
MRM	9.45
HRM	15.61
CRM	5.49
EARM	6.94
DRM	30.23

Note: Values are presented as means. MRM, crude methanol extracts; HRM, hexane extract; CRM, chloroform extract; EARM, ethyl acetate extract; DRM, aqueous extract

Based on the results obtained by colorimetric assays for evaluating the total contents of phenolic and flavonoid in the tested extracts. A noteworthy difference in total phenolic and flavonoid contents between five solvent extracts was observed in Table 2. Among the partition extracts, the EARM extract exhibited the highest level of total phenolics, with a value of 464.8mg GAE/g, followed by ethanol (305.3mg GAE/g), aqueous (183.4mg GAE/g), chloroform (171.4mg GAE/g), and n-hexane (40.7mg GAE/g). The EARM was also significantly ($p > 0.05$) richest in total flavonoid content (156.9mg RE/g) compared to other partition extracts. The total flavonoid content of the four fractions of the partitioned extract was different and decreased in the following order: CRM > MRM > DRM > HRM ($p > 0.05$). In recent reports, phytochemical screening showed that the members of the genus *Ludwigia* extracts contained phenolic compounds, flavonoids, condensed tannins, and alkaloid in different parts, which agrees with our result. Besides that, it was reported by Mangao et al. (2020) that the total phenol content in the aqueous extracts from the leaf of *L. hyssopifolia* was 26.66mg GAE/g, therefore lower than that showed in our study. Similarly, the total phenolic and flavonoid contents were reported by Praneetha, Reddy, and Kumar (2018) as 29.6mg GAE/g and 70.2mg RE/g in the methanol-based

lyophilized extract. Environmental differences such as geographical and climatic conditions may contribute to the differences between these studies.

Table 2

Total phenolic and flavonoid content of *Ludwigia hyssopifolia*

Extracts	TPC (mg GAE/g)	TFC (mg RE/g)
MRM	305.3 ± 0.96^{b}	28.3 ± 1.49^{c}
HRM	40.7 ± 0.61^{e}	4.8 ± 2.70^{e}
CRM	171.4 ± 0.83^{d}	35.0 ± 1.43^{b}
EARM	464.8 ± 0.52^{a}	156.9 ± 2.70^{a}
DRM	183.4 ± 0.83^{c}	15.2 ± 2.18^{d}

Note: Values are mean ± SD (n = 3), and with different superscripts in lowercase alphabets in the same column are significantly different ($p < 0.05$). TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalent; RE, rutin equivalent

3.2. Antibacterial activity

Figure 1 illustrates the anti-bacterial activity of different extracts of *L. hyssopifolia* in terms of zones of inhibition, as investigated under the agar well diffusion method against pathogens, which are tabulated in Table 3. Different extractives of *L. hyssopifolia* were screened against human pathogenic organisms to evaluate antibacterial activities by disc diffusion method. Among different fractions tested, ethyl acetate fraction of the *L. hyssopifolia* exhibited moderate inhibitory activity (4.5 - 15mm) followed by methanol fraction (5.5 - 12.5mm) whereas the hexane fraction showed little activity on the tested microorganisms. The most sensitivity was observed in *B. cereus* (15mm) and *H. pylori* (13.5mm) by ethyl acetate fraction of the *L. hyssopifolia*.

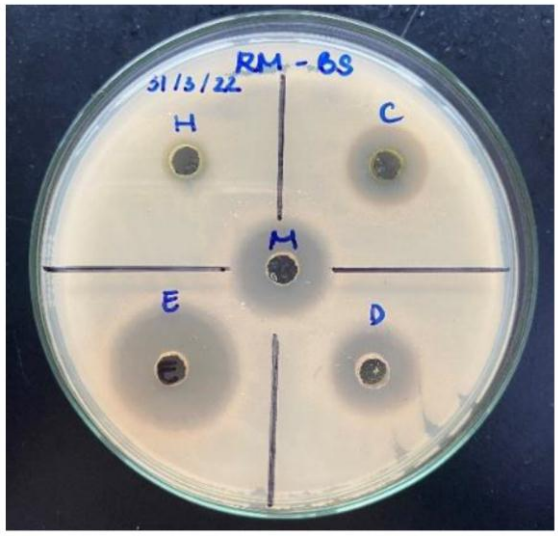

Figure 1. The inhibitory zone again *Bacillus cereus* in different extracts of *L. hyssopifolia*

Table 3

Inhibitory zones (mm) of tested strains after applying the extracts at different concentrations

Extracts	Concentration (µg/mL)	Strain				
		BC	**SA**	**EC**	**ST**	**HP**
MRM	500	10.00 ± 0.06	5.50 ± 0.06	6.50 ± 0.12	10.00 ± 0.06	12.50 ± 0.50
	250	9.50 ± 0.12	3.50 ± 0.50	4.50 ± 0.12	8.50 ± 0.06	11.00 ± 0.12
	125	6.50 ± 0.50	2.00 ± 0.50	2.00 ± 0.12	5.50 ± 0.12	9.50 ± 0.12
	62.5	5.00 ± 0.06	1.00 ± 0.50	1.50 ± 0.50	2.50 ± 0.50	5.50 ± 0.50
HRM	500	3.00 ± 0.06	4.50 ± 0.06	6.50 ± 0.06	4.00 ± 0.06	9.00 ± 0.12
	250	1.50 ± 0.12	4.50 ± 0.06	4.50 ± 0.12	2.50 ± 0. 12	6.00 ± 0.06
	125	1.50 ± 0.12	2.50 ± 0.12	2.50 ± 0.50	2.50 ± 0. 12	5.50 ± 0.06
	62.5	1.00 ± 0.06	1.00 ± 0.50	1.50 ± 0.50	1.00 ± 0.06	3.00 ± 0.06
CRM	500	9.00 ± 0.06	4.50 ± 0.06	4.50 ± 0.06	10.00 ± 0.06	9.50 ± 0.12
	250	7.50 ± 0. 06	4.00 ± 0.06	3.50 ± 0.12	9.50 ± 0.12	6.50 ± 0.50
	125	4.50 ± 0. 06	3.00 ± 0.12	1.00 ± 0.12	7.50 ± 0.12	5.00 ± 0.06
	62.5	3.00 ± 0.06	2.50 ± 0.50	1.00 ± 0.50	4.50 ± 0.06	3.00 ± 0.06
EARM	500	15.00 ± 0.12	5.50 ± 0.12	4.50 ± 0.06	13.00 ± 0.50	13.50 ± 0.50
	250	12.50 ± 0.06	4.50 ± 0.12	2.50 ± 0.06	11.50 ± 0.50	11.50 ± 0.12
	125	9.50 ± 0.06	3.00 ± 0.12	1.50 ± 0.50	9.50 ± 0.06	8.50 ± 0.12
	62.5	6.50 ± 0.50	1.50 ± 0.50	1.50 ± 0.12	6.50 ± 0.50	4.50 ± 0.50
DRM	500	11.00 ± 0.12	4.50 ± 0.12	5.50 ± 0.12	9.50 ± 0.06	9.50 ± 0.06
	250	8.50 ± 0.12	4.50 ± 0.12	4.50 ± 0.12	7.50 ± 0.50	5.50 ± 0.50
	125	6.00 ± 0.50	3.50 ± 0.06	3.00 ± 0.06	5.00 ± 0.12	4.00 ± 0.12
	62.5	4.00 ± 0.06	2.00 ± 0.50	2.50 ± 0.50	3.00 ± 0.06	4.00 ± 0.06

Note: Values are reported as the mean ± S.D. of three parallel measurements. BC, *Bacillus cereus* KACC 11240; SA, *Staphylococcus aureus* KCCM 11335; EC, *Escherichia coli* K99; ST, *Salmonella* Typhi KACC 10764; HP, *Helicobacter pylori* 51932

The MIC and MBC results are summarized in Table 4. In case of all studied pathogens, the MIC and MBC values are lowest for the ethyl acetate extract. The MIC and MBC values vary between 3.9 to 62.5 and 7.8 to over 500 µg/mL, respectively. The skin and the underlying soft tissue infections encompass a variety of pathological conditions and may lead to severe necrotizing infections by Gram-positive as well as Gram-negative bacteria (Sartelli et al., 2022). *Bacillus* spp. are frequently occurring strains classified as Gram-positive, while *Salmonella* is among the Gram-negative strains (Parija, 2023). In our study, a higher antibacterial activity of the *L. hyssopifolia* extract against bacteria of genus *Bacillus* and bacteria of genus *Salmonella*

than against *Helicobacter*, *Staphylococcus*, and *Escherichia* genera was found. Das et al. (2007) confirmed the antibacterial activity of ethyl acetate extract of *L. hyssopifolia* against *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus subtilis* strains. Moreover, n-butanol and ethyl acetate extract extracts from seeds of *L. abyssinica* A. Rich. and *L. decurrens* Walter showed antibacterial activity against *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, *B. subtilis*, *Klebsiella pneumoniae*, *Clostridium sporogenes*, and *Serratia marcescens* strains. According to Smida et al. (2018), the sensitivity of *Cutibacterium acnes* was also observed with *L. peploides* leaves crude extract. These results indicate that extracts of *Ludwigia* are active against the two groups of bacteria underlining their ethnomedicinal use for the treatment of various infectious diseases.

Table 4

The minimum inhibition concentration and minimum bactericidal concentration of *Ludwigia hyssopifolia*

Extracts	BC		SA		EC		ST		HP	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MRM	7.8	15.6	62.5	125	62.5	> 500	3.9	7.8	3.9	7.8
HRM	62.5	> 500	62.5	125	62.5	125	62.5	125	15.6	31.3
CRM	7.8	125	15.6	125	15.6	125	31.3	62.5	7.8	125
EARM	3.9	15.6	3.9	62.5	7.8	> 500	3.9	7.8	3.9	15.6
DRM	7.8	31.3	15.6	31.3	15.6	31.3	7.8	15.6	7.8	12.5

Note: Values are reported as the mean ± S.D. BC, *Bacillus cereus* KACC 11240; SA, *Staphylococcus aureus* KCCM 11335; EC, *Escherichia coli* K99; ST, *Salmonella* Typhi KACC 10764; HP, *Helicobacter pylori* 51932

4. Conclusions

The results revealed that ethyl acetate extract of *Ludwigia hyssopifolia* had a larger yield of total phenolics and flavonoids when compared to other extracts. Furthermore, the extracts showed various antibacterial activity against the tested bacterial strains, particularly against both the Gram-positive and Gram-negative strains, with the ethyl acetate extract being more effective overall. The ethyl acetate extract showed the best potential antibacterial activity and was also selected for further investigation.

The authors declare no conflict of interest.

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