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RESEARCH ARTICLE

Evaluation of Bioactive Substances in *Hibiscus tiliaceus* and its Potential as a Ruminant Feed Additive

Muhamad Bata* and Sri Rahayu

Department of Animal Production, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, P.O. Box: 0000-110, Indonesia

Abstract: ***Background:*** In this research, we investigated which bioactive substances are found in *Hibiscus tiliaceus* and whether they might make this plant a suitable additive to ruminant feed. The substances investigated include saponin, fumaric acid, and flavonoids. It is possible that these additives could minimise methane emission, and improve the growth performance of indigenous cattle.

Methods: We evaluated the nutrient contents of extracts of *H. tiliaceus*, including crude protein, crude fibre, amino acids, fumaric acid, and active compounds. We extracted large and small leaves and small flowers from *H. tiliaceus* using various types and concentrations of solvents. The solvents used were water, ethanol, ethyl acetate, and ethyl ether.

Results: We found more flavonoids and phenols in samples extracted with ethyl acetate than with the other solvents. In the case of the ethyl acetate solvent, the highest concentrations of both flavonoids and phenols were found in the small leaves rather than the large leaves. The fumaric acid content was higher in the small leaves than in the flower (48.18 vs. 35.47 ppm). The saponin content of the small leaves when dissolved in ethyl acetate and ethyl ether was higher than when water or ethanol were used as the solvent (24.6 and 32.0% *vs.* 3.0 and 1.2%, respectively). In the case of the small flowers, the highest saponin concentration was found in the extracts in water and ethanol (15.0 and 8.05% *vs.* 0.14 and 5.5%). We found higher concentrations of amino acids, protein, and low crude fibre in the small leaves. The fumaric acid content of the small leaves was higher than that of the small flowers. There were 24 organic compounds in the aqueous extracts of leaves. These mainly consisted of fatty acids and ester (31%), nitrogenous compounds (18.28%), and quinoline (23%).

Conclusion: The leaves and flowers of small varieties of *H. tiliaceus* can potentially be used as feed additives to manipulate rumen conditions, improve feed efficiency, and reduce methane emissions.

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1. INTRODUCTION

Approximately 80% of the world's rice is grown by small-scale farmers in developing countries, including countries in South East Asia. Rice straw is commonly used as animal feed [1]. However, feeding only rice straw to ruminants does not provide sufficient nutrients. This is because rice straw has a low nutritive value as it is a highly lignified material with low nitrogen and fermentable carbohydrate contents. Many methods have been developed to improve the utilisation of rice straw, and several recommendations have been made by researchers [2-5]. Our previous study showed that feeding local beef cattle 45-55% ammoniated rice straw-based diets ensiled with fermentable carbohydrates from cassava waste products and molasses resulted in an increase in crude fibre digestibility and a daily gain of 0.9 to 1.3 kg/d (unpublished data). However, the use of ammoniated rice straw-based diets causes an increase in ruminal methane emissions from 1.05 to 5.35 ML/d. Therefore, it decreases feed efficiency and contributes to global warming [6].

An approach to minimising the methane emissions from ruminants is to use additives containing fat, organic acids, and defaunating agents. The inclusion of fat, however, can reduce feed intake and fibre digestibility. Hence, fat has a negative effect on ruminant performance [7]. Therefore, the use of fat should be minimised to prevent negative effects on ruminant production. Organic acids such as fumaric acid and malic acid can be used to reduce methane production *in vitro* [8] and *in vivo* [9]. These are precursors to propionate formation in the rumen. Since the formation of propionate requires H_2, the potential for H_2 to react with CO_2 to form methane will be reduced [8]. Defaunating agents such as extracted plant materials containing saponin can be used as an additive

*Address correspondence to this author at the Department of Animal Production, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, P.O. Box: 0000-110, Indonesia; Tel/Fax: ++62-281-638-792; E-mails: muhamadbata@yahoo.com, m.bata@unsoed.ac.id

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to cattle feeds. These can reduce ruminal cilia protozoa and decrease ruminal methane production [10]. As both *Hibiscus tiliaceus* and *H. rosanensis* are in the same genera, the bioactive substances contained in *H. tiliaceus*, which include fumaric acid, malic acid, and saponin are the same as those which occur in *H. rosanensis*. The saponin contents of *H. rosanensis* can be used as a defaunating agent [11, 12]. In [13] the phenol, tannin, and flavonoid contents of *H. tiliaceus* wood extracts were determined. However, there is limited information available regarding the nutrient contents and active ingredients from different parts of *H. tiliaceus* extracted with different solvents.

The aim of this study was to determine whether it is feasible to improve tropical indigenous beef cattle in rural small holdings by using a locally available resource. We approached this question by determining whether the nutrient composition and bioactive agents of *H. tiliaceus* leaves and flowers would make them a good additive to ammoniated rice straw-based diets. The expected improvements would be indicated by a reduction in methane emissions, improved rumen fermentation products, feed efficiency, and production performance. The objectives of this study were to evaluate the nutrient composition and active compounds contained in large and small *H. tiliaceus* leaves and flowers extracted using a variety of solvents, namely: water, ethyl acetate, 95% ethanol, and ethyl ether.

2. MATERIALS AND METHODS

Preparation Step: Flour samples were produced from leaves and flowers of *H. tiliaceus*. The flours were used to produce leaf and flower extracts by dissolving them in a variety of solvents: water, 95% ethanol, ethyl acetate, and ethyl ether. Samples were extracted according to the method developed by Wettasinghe *et al*. [14] as follows: 12 g of *H. tiliaceus* flour was extracted with 200 mL of water, 95% ethanol, ethyl ether, or ethyl acetate. The solutions were homogenised using a magnetic stirrer at room temperature for 24 h. Subsequently, the solvents were filtered using Whatman No. 1 filter paper. The filtrate was dried using a vacuum rotary evaporator (Büchi rotary evaporator Model R-200) at a temperature of 40°C. In the case of the samples in ethanol, ethyl ether, and ethyl acetate, the concentrated extracts were put in a desiccator until they were free of solvent. The extract was stored in a refrigerator at a temperature of 4°C before analysis. The extracts in each solvent were analysed to determine the quantity of active compounds such as saponin, fumarate, phenols, and flavonoids.

The total phenolic content was measured using Folin-Ciocalteu's reagent. The solvents resulting from the extraction were centrifuged at 1000 × *g* for 15 min, and then the supernatant was transferred to a separatory funnel. Extract (100 µL) was added to 750 µL of Folin-Ciocalteu's reagent, which was diluted by a factor of 10 with distilled water. The resulting mixture was kept at a temperature of 22°C for 5 min. The solution was added to 750 µL of sodium bicarbonate (60 g/L) and left to stand for 90 min at a temperature of 22°C. Absorbance values of the test and standard ferulic acid solution as well as untreated reagent, were determined using a UV/Visible spectrophotometer with a wavelength of 575 nm [15].

We determined the flavonoid content using the method from Zhishen *et al*. as follows: Hibiscus extract (1 mL), either from the leaf or from the flowers, and catechin in concentrations of 20, 40, 60, 80, and 100 mg were added to 4 mL of deionised water. At the same time, 0.3 mL of 10% aluminium chloride ($AlCl_3$) was added to the mixture. After 6 min, the solution was added to 2 mL of 1 M sodium hydroxide (NaOH) and 2.4 mL of deionised water. This solution was homogenised and turned pink in colour. The absorbance of the test and standard solutions was determined in comparison to a blank reagent using UV/Visible spectrophotometer with a wavelength of 510 nm. The total flavonoid content was expressed in terms of mg/100 mg of dry sample weight equivalent of catechin [16]. The fumaric acid and saponin contents from these compounds were measured using a Beckman System Gold high performance liquid chromatography (HPLC) system (Beckman Instruments, Palo Alto, CA, USA). Proximate analysis to determine the nutrient composition of the *H. tiliaceus* leaves and flowers was conducted according to the guidelines published by the Association of Official Analytical Chemists (AOAC) [17]. The bioactive compounds were analysed using gas chromatography-mass spectrometry (GC-MS). We used the Shimadzu GC/MS (GC-17A) equipped with a ZB-1 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 m). We used an electron ionisation detection system with an ionisation energy of 70 eV. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperatures were set to 260 and 320°C, respectively. The oven temperature was programmed to increase from 60 to 320°C, initially at a rate of 3°C/min then to be held isothermally for 11 min, then finally to be raised 320°C at a rate of 10°C/min. The samples (1.0 L) diluted 1/100 (v/v) in methanol were injected manually in the split less mode. The relative percentage amount of each component was calculated by comparing its average peak area to the total area.

The total amino acid (TAA) content was determined via acid hydrolysis using a Beckman System Gold HPLC system (Beckman Instruments, Palo Alto, CA, USA). The freeze-dried samples (60 mg) were hydrolysed in 6 mL of 6 M hydrochloric acid (HCl) in screw-capped glass tubes. The tubes were flushed with nitrogen and then heated at 110°C for 24 h. The hydrolysates were dried using the rotary evaporation method at 40°C and then re-dissolved in a 1 M sodium acetic acid solution. The amino acids were separated using ion-exchange chromatography on a 20-cm Spherogel IEX High Performance sodium column (Beckman Instruments, Palo Alto, CA, USA) and were detected by measuring the absorbance at 570 nm (440 nm for proline) following post-column derivatisation with ninhydrin. We identified and quantified the detected amino acids using external standards for reference after using regression analysis to adjust the results. To make this comparison, we used an amino acid standard, which was a synthetic mixture of 17 stable amino acids from Beckman Instruments (Palo Alto, CA, USA).

3. RESULTS AND DISCUSSIONS

3.1. Nutrient Content

We used small and large *H. tiliaceus* leaves (Figs. **1A** and **1B**) and flowers (Fig. **1C**). The proximate contents are

Fig. (1A). Small *H. tiliaceus* leaves.

Fig. (1B). Large *H. tiliaceus* leaves.

Fig. (1C). Small *H. tiliaceus* flowers.

shown in Table **1**. The protein content of the small leaves was similar to that of the big leaves, while the crude fibre (CF) content of the big leaves was higher than that of the small leaves. The crude fibre contents of the small flowers and leaves were similar, but the crude protein (CP) content was lower in both the large and small leaves. All of the amino acids found in the small leaves and flowers are shown in Table **2**. Compared to the flowers, the leaves were rich in serine, leucine, valine, and arginine. This indicates that small leaves have a higher nutrient quality than the large leaves and small flowers. This conclusion is supported by field observations that ruminant animals prefer to eat small hibiscus leaves rather than large hibiscus leaves. Therefore, we focused more on the nutrient content of small leaves.

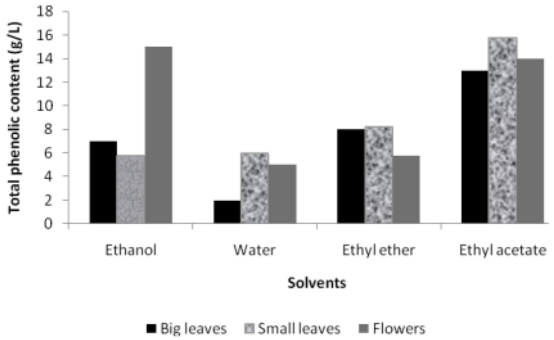

Fig. (2). Phenolic content of large and small *H. tiliaceus* leaves and flowers extracted using various solvents.

3.2. Active Compound Contents Based on the Solvent Used

Polyphenol compounds are a current area of active research because they are antioxidants and provide health benefits. Polyphenol compounds are found mainly in fruits and vegetables [18, 19]. The total phenolic (g/L) contents of the small leaves, large leaves, and small flowers from *H. tiliaceus* are shown in Fig. (**2**). There were variations in the total phenol content in both when the same solvent was used and when different solvents were used. The variation with different solvents is caused by the solvent polarity which determines the qualitative and quantitative properties of the extracted antioxidant compounds [20]. The polarities of water, ethanol, methanol, ethyl acetate, and ethyl ether are 10.2, 5.2, 12 4.4, and 2.8, respectively. The efficacy of the solvent extraction is affected by many factors such as the type of solvent, solvent concentration, time, temperature, pH, number of steps, liquid-to-solid ratio, and particle size of the plant material [21]. The highest yields are usually achieved with ethanol and methanol and their mixtures with water, although other solvents such as ethyl acetate or acetone have been widely used in the extraction of polyphenols from

Table 1. Nutrient content of *H. tiliaceus* leaves and flowers.

Ingredient	DM (%)	% DM				
		CP	EE	CF	Ash	NEF
Hibiscus tiliaceus leaves (large)	94.76	15.44	4.52	50.71	8.79	20.54
Hibiscus tiliaceus leaves (small)	95.50	15.74	4.60	20.61	8.23	50.82
Hibiscus tiliaceus flowers (small)	90.34	10.41	2.92	19.06	6.57	61.04

DM, dry matter; CP, crude protein; EE, ether extracts; CF, crude fiber; NFE, nitrogen free extracts

Table 2. Amino acid content of *H. tiliaceus* leaves and flowers.

No.	Amino Acid	*Hibiscus tiliaceus*	
		Small Leaves (%)	Flower (%)
1	Alanine	0.089	0.073
2	Arginine	0.091	0.070
3	Aspartat acid	0.126	0.123
4	Glutamat acid	0.246	0.247
5	Glycine	0.039	0.034
6	Histidine	0.030	0.022
7	Isoleucine	0.036	0.027
8	Leucine	0.124	0.102
9	Lysine	0.041	0.034
10	Methionine	0.038	0.026
11	Phenylalanine	0.065	0.057
12	Proline	0.064	0.042
13	Serine	0.181	0.147
14	Cystine	-	-
15	Threonine	0.037	0.027
16	Tyrosine	0.062	0.048
17	Valine	0.091	0.065

plants. Water and ethanol are the most widely used due to their low toxicity and high extraction yield. They also confer the advantage of being able to modulate the polarity of the solvent by using ethanol/water mixtures at different ratios [20]. However, there is no universal standardised set of optimum conditions for the extraction of phenolic compounds from different plant species [22]. The nature of the bioactive phenolic compounds and the presence of interfering substances were reported by [23] to be affected by several variables such as the extraction methods, type of solvent, pH, temperature, sample-solvent ratio, and extraction time.

Ethyl acetate tended to produce a higher total phenolic output than the other solvents we used for each plant part tested. The high polyphenol content of ethyl acetic extracts can be attributed to the fact that the bioactive compounds in the leaves and flowers of *H. tiliaceus* are soluble in a semipolar solvent. In this study, the ethyl acetate-extracted fraction of the different parts of *H. tiliaceus* contained a high total phenol content. This suggests that ethyl acetate is an effective solvent for the extraction of phenols from different parts of *H. tiliaceus*. This result agrees with other recent studies which showed that ethyl acetate is an effective solvent for the extraction of phenolic compounds from *Physalis ixocarpa* [24] and onion and citrus peel [48, 26]. Erol *et al.* reported that ethyl acetate fractions from fresh tea leaves and green tea had the highest level of polyphenol [27]. Hajaji *et al.* found that carob tree leaf fractions contained a mixture of phenolic compounds at different concentrations. These varied according to the polarity of the solvent used in the extraction process in the following order: ethyl acetate > ethyl ether > dichloromethane [28].

One of the phenol derivatives is a flavonoid often used to manipulate rumen fermentation. The total flavonoid contents of the different parts of *H. tiliaceus* are shown in Fig. (**3**). As with the total amounts of phenolic compounds, the flavonoid contents varied depending on which part of the plant they came from and which solvent was used during extraction. We obtained high yields from small leaves and flowers when

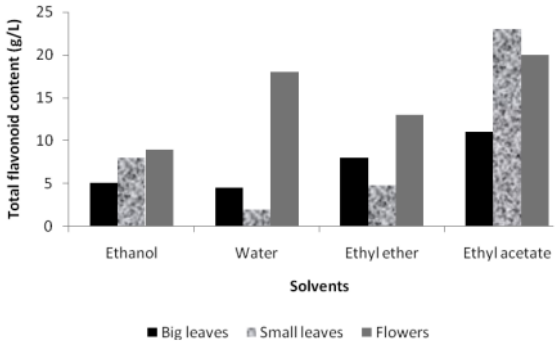

Fig. (3). Flavonoid content of *H. tiliaceus* leaves and flowers extracted using various solvents.

ethyl acetate was used as the solvent. When we used water and ethyl ether as the solvent, we found a high flavonoid concentration in the material extracted from small leaves and flowers. This variation was caused by several factors including the polarity of the solvent.

The high flavonoid content of small *H. tiliaceus* leaves and flowers indicates that this plant can be used as a ruminant feed additive to improve rumen fermentation efficiency. The effects of flavonoids on rumen fermentation have been reported in *in vitro* [29] and *in vivo* experiments [30]. When mixtures of plant flavonoids were tested in a continuous rumen culture system, flavonoids modified the fermentation conditions (pH, propionate proportion, and protein degradation), although the results were not homogeneous [31]. Supplementing the diet of ruminants with flavonoids may be an effective way to improve rumen fermentation and reduce the incidence of rumen acidosis, especially ruminants fed a high-concentrate diet [32]. This property of flavonoids can be explained by the fact that flavonoids can increase the number of lactate-consuming microorganisms (e.g. *Megasphaera elsdenii*) in the rumen.

Previous studies also used secondary substances from plants as a natural additive to manipulate the function of the rumen [33-35]. The use of plant extracts as natural additives, such as using dicarboxylic organic acids (fumaric acid and malaic acid) as methane inhibitors, has been demonstrated *in vitro* [8, 36] and *in vivo* [9, 37]. Our research found that the fumaric acid content of small *H. tiliaceus* leaves was higher than that of small *H. tiliaceus* flowers, as shown in Table **3**. This can be explained by the fact that the leaves are very active parts of the plant as they photosynthesise. Fumaric acid is one of the products of photosynthesis, resulting from the fixation of CO_2 during the photosynthetic process. Hence, this acid is predominantly found in photosynthetically active tissues. The concentration of fumaric acid increases with plant age and light intensity and is present in significant quantities in phloem exudates [38]. The highest fumaric acid content in extracts of small Hibiscus leaves was obtained when water was used as the solvent. When other solvents were used, the amount of fumaric acid decreased (Fig. **4**). This indicates that the fumaric acid contained in the leaves is more soluble in water than in ethanol, ethyl ether, or ethyl acetate.

The high levels of fumaric acid in small *H. tiliaceus* leaves and flowers indicate that these would be a good feed additive to improve rumen efficiency. The effects of adding fumaric acid to ruminant feed on *in vitro* rumen fermentation include increased contributions of propionate to the total volatile fatty acid (VFA) content, decreased methane production, improved fermentation efficiency, and an increase in the glucogenic VFA content compared to non-glucogenic content [39].

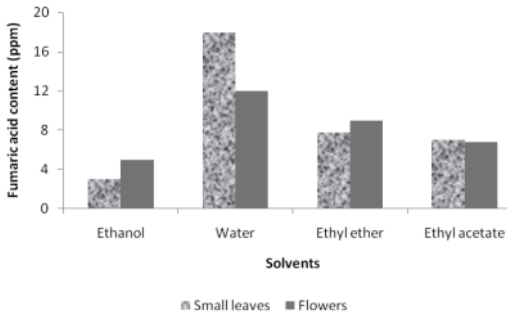

Fig. (4). Fumaric acid content of *H. tiliaceus* leaves and flowers extracted using various solvents.

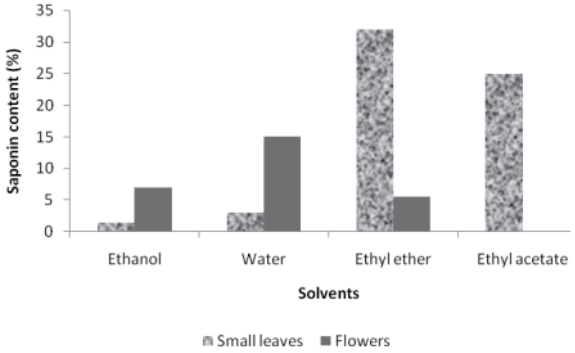

Fig. (5). Saponin content of *H. tiliaceus* leaves and flowers extracted using various solvents.

Saponins or saponosides are heterosides composed of two parts: a water-soluble glucosidic chain and a generally triterpenic or steroidic liposoluble structure. The triterpenes are subdivided into some 20 groups, depending on their particular structures. Saponins are freely soluble in both organic solvents and water [40]. Generalisations concerning the solubility of saponins are hazardous, but many are soluble in water and alcohol; some are soluble in ether, chloroform, benzene, ethyl acetate, or glacial acetic acid [41].

The saponin content (%) of the small *Hibiscus* leaves and flowers from different extractions are shown in Fig. (**5**). The saponin content of the small leaves extracted using ethyl ether and ethyl acetate was higher than when water and ethanol were used as the solvent. However, the saponin content of the samples extracted from the small flowers was lower than the saponin content of the samples from the small leaves. Mahmod *et al.* found that the saponin content of leaves from the *Verbascum thapsus* plant was higher than that of the flowers [42]. Zao *et al.* reported that the optimal conditions to extract all types of saponins from *Codonopsis lanceolata* occur when the solvent is 70% ethanol [43].

Table 3. Fumaric acid content of *H. tiliaceus* leaves and flowers.

No.	Part	Type of Analysis	Method	Result	Unit
1	Flowers	Fumaric acid	HPLC	35.47	ppm
2	Leaves	Fumaric acid	HPLC	48.18	ppm

Table 4. GC-MS analyzes of aquoues extracts of *Hibiscus tiliaceus* leaves.

RT (min)	Compounds	%	M Weight	M Formula
5.849	dl-Alanine ethyl ester	3.55	118.15	$C_5H_{12}NO_2$
59.789	Acetaldehyde	0.35	44.05	C_2H_4O
82.398	2-Ethylacridine	5.61	308.3	$C_{18}H_{20}N_4O$
106.602	7H-Dibenzo[b,g]carbazole, 7-methyl-	5.81	281.35	$C_{21}H_{15}N$
131.219	Acetamide, N,N-bis(2,4-dimethylphenyl)-	0.56	59.07	CH_3CONH_2
135.469	3,4-Dihydroxymandelic acid, ethyl ester 2-	27.5	184.15	$C_8H_8O_5$
145.445	Nonanone, 9-hydroxy-	0.96	142.24	$C_8H_{16}O$
151.526	1-Undecyne	3.66	154.29	$C_{11}H_{22}$
15.253	5-Dodecyne	0.62	138.25	$C_{10}H_{18}$
157.311	dl-.beta.-Ethyl-3-dimethylamino-3,4,6-trideoxyglucopyranoside	2.48	194.18	$C_7H_{14}O_6$
160.676	Quinoline, 4-(4-chlorophenoxy)-8-fluoro-2-trifluoromethyl-	23.14	341.687	$C_{16}H_8ClF_4NO$
16.227	2-Octanone	0.69	128.21	$C_8H_{16}O$
170.358	5-Hepten-2-one, 6-methyl-	1.49	126.19	$C_8H_{14}O$
178.504	Sulfonamide, N-cyclohexyl-N'-(5-methylisoxazol-3-yl)-	0.97	186.23	$C_7H_{10}N_2O_2S$
181.869	6,7-Benzo-phenothiazine-5,5-dioxide	4.15	365.03	$C_{20}H_{23}N_5O_2$
187.831	1-Anthracenamine	4.63	193.24	$C_{14}H_{11}N$
189.071	1-Hexanol, 2-ethyl-	2.43	130.22	$C_8H_{18}O$
192.613	Decanal	1.13	156.20	$C_{10}H_{20}O$
196.391	1,6-Octadien-3-ol, 3,7-dimethyl-	3.40	154.24	$C_{10}H_{18}O$
217.525	Piperonylamine	3.17	151.16	$C_8H_9NO_2$
218.647	Adenosine, 1,2-dihydro-2-oxo-	0.52	267.24	$C_{10}H_{13}N_5O_4$
222.011	2-Ethylacridine	1.40	207.27	$C_{15}H_{13}N$
227.797	Naphthalene	0.86	128.17	$C_{10}H_8$
271.068	3-Piperidinol	0.52	267.24	$C_6H_{13}NO$

RT, retention time; MW, molecular weight; MF, molecular formula.

Meanwhile, Kuang *et al*. found three new cycloartane-type triterpenoid saponins in the ethyl acetate soluble extract from the roots of *Cimicifuga simplex* [44]. The highest saponin content extracted from small *H. tiliaceus* flowers was obtained when a water solvent was used, followed by ethanol, then ethyl ether. These differences were caused by the chemical structure of saponin in leaves and flowers. The chemical structure of saponin contains both polar glycogenin and non-polar (sapogenin) groups [45]. As described previously, the polarity of the solution determines the quantity and quality of the extracted product.

The main organic compounds in aqueous extracts of small *H. tiliaceus* leaves (Table **4**) were fatty acids and ester (31%), nitrogenous compounds (18.28%), and quinoline (23%). Quinoline is an alkaloid with antiprotozoal [46] and antioxidant activity [47]. We found sulphonamide (0.97%) and piperonylamine (3.17%) in aqueous extracts of *H. tiliaceus* leaves. Sulphonamides are a class of synthetic antimicrobial drugs that interrupt the bacterial synthesis of folic acid which is essential for the synthesis of bacterial DNA [48]. Gutierrez *et al.* reported that sulphonamides clearly affect both the function and structural diversity of the soil microbial community. The soil microbial community is affected by sulphonamides even at relatively low concentrations [49].

4. CONCLUSION

The best solvent for total phenolic and total flavonoid extraction was ethyl acetate, while ethyl ether was the best solvent to extract saponin from small *H. tiliaceus* leaves.

Extraction using water as a solvent produced the highest concentration of fumaric acid. There were 24 organic compounds in the aqueous small leaf extracts. The leaves and flowers of the small variety of *H. tiliaceus* can potentially be used as additives for ruminant feed. This would enable us to manipulate the rumen conditions thereby improving feed efficiency and reducing methane emissions.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organisation regarding the material discussed in the manuscript. Funding for this research was provided by Jenderal Soedirman University, Purwokerto, Indonesia.

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