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EXTRACTING PHYTOCOMPOUNDS FROM *Mucuna pruriens* LEAVES AS POTENTIAL RUMINANT FEED ADDITIVES USING DIFFERENT SOLVENTS

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ABSTRACT: Some secondary metabolites of plants could serve as ruminant feed additives. They primarily preserve protein from rumen breakdown, reduce rumen protozoa population, and decrease methane gas production. The current study aimed to identify the phytocompounds content of extracted *Mucuna pruriens* leaves using the Microwave-assisted extraction method using three different solvents of methanol 70% (EM), aquadest (EA), and combinations of EM and EA (EK). The phytocompounds were identified by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Some phytocompounds identified in the *Mucuna pruriens* substances from GC-MS curve proportion area of EM were 10.35% inositol, 3.1% quinazoline, 4.72% anthraquinone, 3.76% Coptisine, 2.06% isoquinoline, 2.18% D-gluconic acid, 2.83% D-Fructose, 3.91% D-glucose, and 4.59% butanedioic acid. The phytocompounds for EK were 17.22% inositol, 6.36% Niclosamide, 1.4% Acetamide, 1.32% Aniline, 55.97% 4-Amino-2-(4-methoxyphenyl)-5,6,7,8-tetrahydrofuro[2,3-b] quinoline-3-carbonitrile, 17.22% inositol. Furthermore, 22.73% inositol, 6.55%, ribonoic acid, 5.58%, silanol, 21.27% butanodioic acid, 2.88% Fluoroquinoxaline, 5,31%, glycerol, 1,64%, D- gluconic acid were found in the EA. The EA had high inositol content, the EK had high quinoline content, and the EM showed moderate results for all phytobiotics. The total phenolics, flavonoids, tannins, and saponins content significantly differed among the three solvents. The EA yielded the highest concentrations of total phenolics, flavonoids, and tannins, but the lowest concentration of total saponins. In contrast, the EM yielded the lowest total phenolics, flavonoids, and tannins content, but the highest total saponins content. Meanwhile, the EK yielded modest results for all phytocompounds, with values between EA and EM. In conclusion, the methanolic extract of Mucuna pruriens substance had the highest phytocompounds and bioactive potential as ruminant feed additives.

Keywords: Feed additives, Gas chromatography, *Mucuna pruriens*, Phytocompounds, Solvent, Secondary metabolites

INTRODUCTION

Some plants produce bioactive compounds in the form of secondary metabolites. The secondary metabolites, such as saponin (Unnawong et al., 2021), tannins (Patra and Saxena, 2011), flavonoids (Gohlke et al., 2013), and polyphenols, are known to function as rumen modifiers. Studies have indicated that the secondary compounds possess antimicrobial properties against bacteria, fungi, and protozoa, decrease acetate and ammonia concentration, acetate to propionate ratio, and methane production, and increase propionate *in vitro* (Klevenhusen et al., 2011). *Mucuna pruriens*, also known as cowhage or velvet bean, contains active compounds commonly used as components for traditional medicine. For example, seeds of *Mucuna pruriens* contain fairly high L-Dopa, commonly used for managing and treating Parkinson's disease (Lieu et al., 2010) and as antidepressants in cases of depressive neurosis (Rana and Galani, 2014). In animal feeding, the seeds can also be used as a substitute for protein sources (Yantika et al., 2016).

Mucuna pruriens can improve soil fertility and is adapted to the humid or subhumid tropics and commonly used in maize and cotton rotations systems intercropping (Peters et al., 2001), It is also called abonera (fertilized field) and play a role in soil structure improvement, soil protection against erosion, and weed control (Buckles et al., 1998). *Mucuna pruriens* is a multi-purpose legume, particularly for smallholder farmers. In Indonesia, especially in Wonogiri, Central Java, *Mucuna pruriens* seeds are commonly used as raw materials for tempeh making. The product is popularly called koro benguk tempeh, which is a dish. Some Small and Medium Enterprises (UMKM) further process the tempeh to produce tempeh benguk chips (Winami and Dharmawan, 2017). With their nutrient contents, especially antioxidant potential, *Mucuna pruriens* can be effectively used to formulate food products for reducing factors associated with non-transmissible chronic diseases and aimed at meeting general nutritional needs (Encalada and Campos, 2021) and could affect fertility (Daramola et al., 2015).

The development of *Mucuna pruriens* cultivation provides higher access to forage. Besides, being a source of animal feed in the form of fresh or hay, *Mucuna* leaves can also be used for their secondary metabolite content. For this reason, it is necessary to identify metabolites that can be maximally used for livestock productivity, especially the content of compounds that have antimicrobial properties, so that they can maximize the nutrients provided.

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Therefore, the current study aimed to identify the phytocompounds of *Mucuna pruriens* leaves extract with different solvent and their potential for ruminant feed additives.

MATERIALS AND METHODS

Ethical approval

The current study was conducted in accordance with the Animal Care and Use Committee, University of Brawijaya Malang, East Java Indonesia, with ethical clearance number 141-KEP-UB-2022.

Collection of *Mucuna pruriens* leaves

Fresh leaves of *Mucuna pruriens* were collected from farms located in Wajak, Malang, East Java, Indonesia, in October 2021. The leaves were aerated under the shades, dried in an oven at 60°C, milled into a fine powder using a mechanical grinder, and stored in air-tight plastic for further analysis.

The extraction of *Mucuna pruriens* leaves

For extraction, microwave-assisted extraction (MAE) method was used with three different solvents, including methanol 70% (EM), aquadest (EA), and a combination of EA and EM by ratio 1:1 (EK). The MAE was performed using a modification of microwave sharp type R21D0SIN with the power 450 W, voltage 220-240 volt/50 Hz, and the dimension of 52cm × 40.7cm × 32cm in length, width, and height, respectively. The close vessel unit and temperature marker were used to ensure the temperature does not exceed 40°C (Yuan et al., 2018) with modification. As much as 33 g of *Mucuna pruriens* leaves powder was placed in a 250 ml round flask, and 200 ml of solvent based on each treatment (EM, EA, and EK) was added and homogenized. The homogenized samples were extracted in the microwave for 15 minutes with a temperature not exceeding 40°C. The chiller pump was used to flow the coolant liquid in and out the condenser then the flask was attached to a condenser. The extract was then filtered with filter paper (Whatman grade 1). The filtrate was extracted again with 200 ml solvent based on each treatment (EM, EA, and EK). After the extraction process, the volume of filtrate liquid was reduced from the original volume with three times evaporation using MAE for 15 minutes per evaporation process.

Compounds Identification using Gas Chromatography-Mass Spectrometry analysis

Gas Chromatography-Mass Spectrometry (GC-MS) was used as a qualitative identification method for extracting *Mucuna pruriens* leaves following a study by Karimi and Jaafar (2011) with some modifications. In the next step, 1 µL of samples without derivatization was injected into the GC-MS system (Thermo Scientific ISQ LT equipped with TriPlus RSH autosampler Waltham, MA, USA) with splitless mode. The A TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm) was installed in the GC. The temperature was set at 50°C for two minutes, then increased at 5°C per minute, and reached up to 150°C for 10 minutes, and then it was again increased at 10°C per minute and reached up to 200°C and held for 5 minutes. Finally increase was 15°C per minute to reach 320°C and held for 5 minutes. During the compound separation carrier, hydrogen gas was generated by a hydrogen generator (Thermo Scientific Waltham, MA, USA) at a flow rate of 1 mL per minute. The injector and transfer line were set at 200°C and 320°C, respectively. The result of mass spectra obtained from the respective extracts matched the mass spectra library of the National Institute of Standard and Technology NIST version 2.2 (Yusnawan et al., 2021). The compounds that exceeded 1% level were listed.

Qualitative analysis of phytobiotics

The total phenolic contents of the *Mucuna pruriens* leaf extracts were analyzed using the Folin-Ciocalteu reagent (Singleton et al., 1999; Xu and Chang, 2007). The 100 µL of the hydrophilic extract was mixed with 2.9 mL of deionized water, 0.5 mL of Folin-Ciocalteu reagent, and 2.0 mL of 20% Na_2CO_3 solution, then stood for 90 minutes. The measurement of the absorbance was conducted using 760 nm versus reagent blank. The total phenolic content was expressed as gallic acid equivalent.

Total flavonoid content was analyzed using the aluminum chloride method (Heimler et al., 2005; Xu and Chang, 2007). The sample extract of 1 mL was placed in a 10-mL volumetric flask containing 4 mL of distilled water, 0.3 mL of 5% $NaNO_2$, and 0.3 mL of 10% $AlCl_3$, then remained for 6 minutes at room temperature. The 2 milliliters of 1 M (mol) NaOH were added, and the solution was diluted to 10 mL with distilled water. The absorbance measurement was conducted using 510 nm of the solution versus a reagent blank. The total flavonoid was expressed as catechin equivalents (CE) per gram of sample (mg CE/g sample (Heimler et al., 2005; Xu and Chang, 2007).

The total tannin contents of the *Mucuna pruriens* leaf extracts were estimated using the Folin Denish reagent (Galvao et al., 2018). Then, 2 grams of the sample extract were placed in a 500 mL boiling flask, 350 mL of distilled water was added, and refluxed for about 3 hours. Next, filtered 2 mL samples were placed in a 100 mL volumetric flask containing 2 mL of Folin Denish reagent and 5 mL Na_2CO_3. The mixture was allowed to stand for 40 minutes at room temperature. The absorbance measurement was conducted using 725 nm of the solution versus a reagent blank. The tannin content was expressed as gallic acid equivalent.

The total saponin contents of the *Mucuna pruriens* leaf extracts was analyzed based on Hiai Oura and Nakajima (1976) and the standard using Quillaja Bark (Sigma Aldrich Chemie, Steinheim, Germany). The sample extract was added

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to 0.2 mL vanillin, 0.25 mL ethanol, and 2.5 mL 72% H_2SO_4. The solution was vortexed and heated in a water bath (Watson Victor Ltd. Bw6t, Watson Victor Limited, New Zealand) at 60°C for 10 minutes. The measurement of absorbance was performed using 544 nm. Quillaja Bark equivalent was used to express the total phenolic content in *Mucuna pruriens* leaves extract.

Statistical analysis

Qualitative data of phytobiotics contained in the *Mucuna pruriens* leaf extracts were descriptively analyzed. In contrast, the quantitative ones were analyzed using SPSS software version 25. The analysis of variance (ANOVA) for a completely Randomized design with 3 treatments and 6 replications was performed on the obtained data. Duncan's Multiple Range Test was conducted to compare the mean values between treatments to determine if there was a significant effect of the treatments on phytobiotics content ($p<0.05$).

RESULTS AND DISCUSSION

Identification phytocompound in the *Mucuna pruriens* leaves extract

The identified active compounds or phytobiotics in the *Mucuna pruriens* leaves extracts and their proportion curve area (%) are presented in Table 1. As can be seen, *Mucuna pruriens* substance from EM extractions resulted in the highest number of compounds (24 compounds), followed by EA (16 compounds), and EK (8 compounds). However, EK showed a slightly lower value (90.86%) than the EM (92.89%) based on the total curve area, while the EA indicated the lowest value (84.66%). This means that EM can result in the most diverse compounds with the highest proportion of 10.35% for inositol, while EK leads to the most concentrated compound with the highest proportion of 55.77% for 4-Amino-2-(4-methoxyphenyl)-5,6,7,8-tetrahydrofuro[2,3-b] quinoline-3-carbonitrile. The second highest proportion was 17.22% for inositol, which was also found as the highest proportion compound in EM and EA solvent, with the proportion curve area of 10.35% and 22.73%, respectively. The inositol reduces plasma glucose level and can be found in the *Mucuna pruriens* seeds as d-chiro-inositol with two galacto-derivatives, O-α-d-galactopyranosil-(1→2)-d-*chiro*-inositol (FP1) and O-α-d-galactopyranosil-(1→6)-O-α-d-galactopyranosil-(1→2)-D-*chiro*-inositol (FP2). The level of 7 mg d-*chiro*-inositol has been used as an antidiabetic agent (Lampairello et al., 2012).

Niclosamide in ruminants reduces the occurrence of *Paramphistomum* infection by decreasing the number of eggs and reducing oxidative stress, as well as improving the biochemical and hematological profile of livestock (Shaheen et al., 2013). In addition, quinoline is an active substance that is commonly used as an antiprotozoal (Fournet et al., 1994). It acts as an antioxidant agent (Korrichi et al., 2009) and antiparasitic (Kadri et al., 2014).

The GC-MS analysis of *Mucuna pruriens* substance using 70% methanol solvent showed 3.1% quinazoline, 4.72% anthraquinone, 3.76% Coptisine, 2.06% isoquinoline, and several types of esters, sugar compounds, and fatty acids (Table 1). Quinazoline is an active compound as a component for the treatment of carcinoma cancer (Ardiles et al., 2020). Another broad range of quinazoline moieties are reported to have medicinal activities like antifungal, antiviral, antidiabetic, anti-inflammatory, antibacterial, and antioxidant (Karan et al., 2020). Coptisine could inhibit urease activity (Li et al., 2018). Thus, ruminants benefit from microbial fermentation (He et al., 2022), through which coptisine improves urea-N utilization efficiency for production and can decrease environmental nitrogen emission. Coptisine is an isoquinoline alkaloid (Wu et al., 2019). Anthraquinone in ruminant feed reduces ruminal sulfide production, reduces methane production, increases propionate, and decreases acetate (Kung et al., 2003). Anthraquinone contained in *Cassia Alata L* in the study of Yusiati et al. (2010) has a high potency as a methanogenesis inhibitor, thus, recommended as a feed additive to increase rumen microbial protein supply.

The research by Anosike et al. (2019) in *Mucuna pruriens* leaves using methanol extract indicated high antioxidant activity. Agbafor and Nwachukwu (2011) showed that *Mucuna pruriens* leaf extract inhibited 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH), indicating their antioxidant activity. Antioxidant supplementation in animal feed would enhance the cow's health in a sensitive stage, such as the transition period, and could add value to the final product (milk or meat) that benefits the consumer's health (Castillo et al., 2013).

According to Table 2, the addition of three different solvents to *Mucuna pruriens* extract resulted in significant content of total phenolic, flavonoid, tannin, and saponin contents ($p<0.05$). The extraction of *Mucuna pruriens* phytocompounds using EA showed the highest results for total phenolic, flavonoid, and tannin but the lowest for total saponin content. On the contrary, extraction of *Mucuna pruriens* leaves using EM solvent revealed the lowest results for total phenolic, flavonoid, and tannin but the highest for total saponin content. Meanwhile, the combination of aqueous and methanol solvent showed moderate results for all phytobiotics, and the values were in between EA and EM results. The saponin content of the *Mucuna pruriens* leaf extracts is lower than *Isesbania sesban* by about 10% (Tatiya et al., 2013). According to Susanti and Marhaeniyanto (2014), the saponin contents in some plant leaves are *Hibiscus rosa-sinensis* (5.89%), *Erythrina variegata L* (3.42%), *Gliricidia sepium* (8.23%), *Calliandra calothyrsus* (8.33%), *Moringa oleifera* (7.19%), *Leucaena leucocephala* (4.54%), *Swietenia macrophylla* (4.31%), *Artocarpus heterophyllus* (5.79%), *Paraserianthes faicataria* (15.04%), and *Samanea saman* (3.98%). Nevertheless, the *Mucuna pruriens* leaf extract can potentially be hepatoprotective and nephroprotective for treating liver and kidney disease in rats (Ogunmoyole et al., 2021). The incidence of liver abscesses in feedlot cattle is between 10 and 20% due to polymicrobial infections with

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gram-negative bacteria (Amachawadi and Nagaraja, 2016). The *Mucuna pruriens* had antimicrobial activity against Gram-negative bacteria (Mastan et al., 2009) and their hepatoprotective potential should protect the liver from tissue damage.

Table 1 - The compounds and curve area proportion of *Mucuna pruriens* substances using three different solvents

Identified compounds	EM (%)	EA (%)	EK (%)
(3E)-1-methyl-3-[5-(4-methylphenyl)-1,3-oxazolidin-2-ylidene]-1,3-dihydro-2H-indol-2-one	2.07	0	0
(4-Bromophenyl) bis(2,4-dibromophenyl) amine	0	1.41	0
,3-bis[(trimethylsilyl)oxy]-butanedioic acid, bis(trimethylsilyl)ester	4.59	21.27	0
(Z)-1-[Quinazolin-4(3H)-ylidene] propan-2-one	1.06	0	0
1-[3-methyl-1-(phenylsulfonyl)-thiopheno[2,3-b] carbazole]-3,5-[bis(3-methyl-2-thiophenyl)] benzene	0	1.79	0
1-cyclododecyl-3-(4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3] dioxolo[4,5-G]isoquinolin-5-YL)-propan-2-ol	2.06	0	0
1-Methoxy-3-pentyl-6,6a,7,8-tetrahydro-6,6-dimethyl-9H-dibenzo[b,d]pyran-9-one	0	3.05	0
1-Phenyl-2-naphthol	1.48	0	0
2,3-Butanediol 2TMS PK A	0	0	5.32
2-Aziridinecarbonitrile,1-ethyl-3-phenyl-, cis-	3.04	0	0
2-Ethylthio-10-hydroxy-9-methoxy-1,4-anthraquinone	4.72	0	0
2-Fluoroquinoxaline	0	2.88	0
2-Isopropyl-4-methylpyridine-3,5-dicarbonitril	0	3.19	0
2-n-Butyl-2-[(p-hydroxymethyl)benzenesulfonyl]-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole	2.74	0	0
4-Amino-2-(4-methoxyphenyl)-5,6,7,8-tetrahydrofuro[2,3-b] quinoline-3-carbonitrile	0	0	55.97
4H,6H-Furo[3',2':3,4] naphtho[1,8-cd]pyran-4,6-dione,8,9-dihydro-3,7-dihydroxy-1,8,8,9-tetramethyl-, (S)-	4.07	0	0
5-(4-Chlorophenyl)-3,4-dihydro-2-methyl-4-oxothieno[2,3-d]-pyrimidine-6-carboxamide	1.73	0	0
5-Dimethylamino-1-ethoxy-5-ethylsulganyl-3-phenyl-1,3-cyclopentadiene	3.06	0	0
Acetamide, 2,2,2-trifluoro-	0	0	1.40
Aniline	0	0	1.32
Butanedioic acid, 2TMS derivative	0	1.80	0
Coptisine	3.76	0	0
D-Fructose, O-Methyloxim, pentakis-O-(Trimethylsilyl)-	2.83	0	0
D-Gluconic acid, 6TMS derivative	10.30	1.64	0
di(isityl)di[phosphino]silane	0	1.06	0
exo-2-oxyisoxazolidino[1,2-b]-1,3-dioxacyclopentane	0	3.72	0
Galaccititol TMS	7.06	0	0
Glycerol, 3TMS derivative	0	5.31	0
Indolo[2,1-b] quinazolin-6(12H)-one	2.04	0	0
Inositol,O,O,O,O,O,O-TMS	10.35	22.73	17.22
L-(-)-Fucose, tetrakis(trimethylsilyl)ether, methyloxime (syn)	0	1.60	0
Methyl galactoside (1S,2R,3S,4R,5R)-,4TMS derivative	4.20	0	0
N-(2'-Thiazolyl)-2,5-dimethylpyrrole-3-carbaldehyde	5.42	0	0
n-Hexadecanoic acid	0	0	1.39
Niclosamide	0	0	6.36
N-n-Butyl-2,2-bis-tert-butylacetamide	0	0	1.88
pentakis(trimethylsilyl)glucose-O-methoxime	2.59	0	0
Ribonic acid,2,3,4,5-tetrakis-O-(trimethylsilyl)-,trimethylsilyl ester	1.83	6.55	0
Silanol, trimethyl-, phosphate (3:1)	0	5.58	0
Sucrose, 8TMS derivative	2.10	0	0
Tartaric acid, 4TMS derivative	4.05	0	0
Trimethylsilyl2,3,4-tris[(Trimethylsilyl)oxy]butanoate	0	1.08	0
Xylitol, 5TMS derivative	4.80	0	0
Number of identified compounds	24	16	8
Total curve area	92.89	84.66	90.86

**Curve area proportion 0% means did not identify; EK: Combinations of aqueous and methanolic solvent, EM: Methanolic solvent, EA: Aqueous solvent

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Table 2 - The total phenolic, flavonoid, tannin, and saponin of the *Mucuna pruriens* substances using three different solvents in Indonesian

Treatment	Total phenolic (mg GAE/g)	Total flavonoid (mg CATE/g)	Total tannin (mg GAE)	Total saponin (mg QBE/g)
EA	14.55 ± 0.70^{b}	5.91 ± 0.29^{c}	0.84 ± 0.03^{c}	9.685 ± 0.18^{a}
EM	12.68 ± 0.30^{a}	3.83 ± 0.16^{a}	0.69 ± 0.02^{a}	12.41 ± 0.21^{c}
EK	13.88 ± 0.38^{b}	4.89 ± 0.25^{b}	0.74 ± 0.02^{b}	10.65 ± 0.35^{b}

^{abc}Values with different superscripts in the same column are significantly different ($p < 0.05$); EA: Aqueous solvent, EM: Methanol solvent, EK: Aqueous and methanolic combination solvent, GAE: Gallic acid equivalent, CATE: Catechin equivalent, QBE: Quillaja bark equivalent

The highest total saponin content of extracted *Mucuna pruriens* leaves using methanol solvent (12.41 mg QBE/g) indicated the potential of this extract as an additive for ruminants. The potential of saponin as an additive in ruminants has been widely studied with several benefits. As reported by Wina and Muetzel (2020), saponin plays a role in the defaunation effect by lysing protozoa cell membranes and then decreasing the protozoa. Since cholesterol is one thing avoided in meat consumption because it affects human health, saponins positively decrease blood cholesterol concentrations (Aazami et al., 2013). The administration of tea saponins showed changes in the ruminal bacterial microbiota and response metabolites (Wang et al., 2019). Another research showed that condensed tannins and saponins could reduce the proportion of methane during rumen fermentation using sugar cane top (Widiawati and Puastuti, 2016). The use of saponin also affected the rumen environment, and then could modulate the microbial community and ruminal metabolites, reducing ammonia concentrations without causing adverse effects on pH, microbial protein, and cellulase activity (Wang et al., 2019). Administration of 1% tannins and 0.6% saponins could increase microbial protein supply to the host by up to 30% and reduce methane production by up to 11% (Newbold et al., 2015) due to decreasing rumen protozoa (Wahyuni et al., 2014). An *in vivo* meta-analysis by Ridla et al. (2021) shows that dietary saponins in a low level increase dry matter, organic matter, and acid detergent. There the recommended amount is the maximum of 0.5% DM.

CONCLUSION

The extraction of the *Mucuna pruriens* leaf using methanol solvent (EM) showed the highly identified phytocompounds with the highest saponins contents (12.41 mg QBE/g). The *Mucuna pruriens* phytocompounds have the potential to reduce the occurrence of *Paramphistomum* infections, antiprotozoal, antioxidant agent, antiparasitic, antifungal, antiviral, anti-inflammatory, antibacterial, reduces methane production, increase propionate, and decreases acetate. Therefore, they can be used as feed additives for livestock productions.

DECLARATIONS

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Data availability and materials
Data will be available on request.

Ethical consideration
Consent to publication and misconduct, plagiarism, data fabrication and double submission of the manuscript, and redundancy and other ethical issues were checked by the authors.

Authors' contributions
T. Muhartatik S. Chuzaemi, M.H. Natsir, Marjuki performed conceptualization, formal analysis, investigation, methodology, validation, writing original draft. Marjuki performed the supervision, review and editing of the manuscript for important academic content. All authors checked and approved the final revised article.

Conflict to interest
The authors have not declared any conflict of interest.

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