

Phytochemicals, characterization and antimicrobial tests of red betel leaves on three solvent fractions as candidates for endometritis phytotherapy in Aceh cattle, Indonesia

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Abstract. Armansyah T, Siregar TN, Suhartono, Sutriana A. 2022. *Phytochemicals, characterization and antimicrobial tests of red betel leaves on three solvent fractions as candidates for endometritis phytotherapy in Aceh cattle. Biodiversitas 23: 2111-2117.* The use of antibiotics to treat endometritis in cattle carries with it some weaknesses in the form of toxicity risks and unexpected side effects. The use of medicinal plants as antimicrobials and immunomodulators has the potential to be an alternative therapy. Red betel leaves could be considered viable candidates for phytotherapy in cattle with endometritis. This study aimed to determine the differences in biological activity and antibacterial ability of red betel leaf through the use of three different solvents, namely n-hexane, ethyl acetate, and ethanol. The highest yield of betel leaf extract was obtained from the ethyl acetate solvent fraction consisting of flavonoids, terpenoids, steroids, tannins, phenolics, and saponins. The highest biological compounds in red betel leaf identified by GC-MS in n-hexane solvent, ethyl acetate, and ethanol were 5-isobutylidene-N, N-dimethylbarbituric acid, benzenamine, 4,4' - (1,2-ethenediyl) bis-, and 9-octadecadienoic acid, respectively. Among the three solvent fractions of red betel leaf extract, it was observed that ethyl acetate exhibited higher antibacterial activity than hexane and ethanol. Hence, it can be concluded that ethyl acetate extract of red betel leaves solvent has secondary metabolites and better antibacterial potential than ethanol and n-hexane solvents.

Keywords: Ethanol, ethyl acetate, n-hexane, red betel

INTRODUCTION

Endometritis is a disease that arises in cows, especially after calving (postpartum), and causes high economic losses for farmers. Not only does this disease lead to a heavy financial cost, it can also cause a disruption of postpartum ovarian activity, long calving intervals, decreased conception rates, and decreased milk production (Budiyanto et al. 2016), embryo mortality, implantation failure, and infertility (Ahmed and Elsheikh 2014; Ali and Ameen 2014). It has been found that various microorganisms cause uterine infections (Yilmaz et al. 2012), as well as many secondary infections (Ali and Ameen 2014). *Escherichia coli* and *Arcanobacterium pyogenes* were the most frequently isolated bacteria from the uterine lumen of infected cattle, followed by various anaerobes, including *Prevotella* sp., *Fusobacterium necrophorum*, and *Fusobacterium nucleatum* (Sheldon and Owens 2017). Other non-specific microorganisms associated with pathological conditions in the endometrium are *Corynebacterium pyogenes* and anaerobic gram-negative bacteria (Wang et al. 2018). *Bacillus*, *Streptococcus*, and *Enterococcus* bacteria, in addition to coagulase-negative *Staphylococci*, are also the bacteria

most frequently isolated from the uterus of infected cattle and have been described as potential or opportunistic pathogenic bacteria (Wagner et al. 2014; Carneiro et al. 2016). In Aceh cattle, it was reported that uterine infections were dominated by *E. coli* and *Pseudomonas sp.* (Rafika et al. 2020).

Various therapies in cattle with endometritis have been widely used, such as antibiotics, prostaglandins (Drillich et al. 2006; Mido et al. 2016; Scenzi 2016), and iugol (Ahmad et al. 2014; Ahmed and Elsheikh 2014; Alyasiri et al. 2015). Although antibiotics are effective in treating endometritis, the use of medicinal plants as antimicrobials and immunomodulators is becoming popular due to the toxicity and side effects of antibiotics usage (Bhardwaz et al. 2018). Extracts from various plants have antibacterial properties, which give them a role in controlling infection (Dwidjoseputro 1994 cited by Dima et al. 2016). Intermediate herbal antiseptics such as red betel leaves (*Piper crocatum*) can be used as a solution to replace commercial antiseptics and are considered to be safer to use. Red betel leaves extract can function as an antibacterial for Gram-positive and Gram-negative bacteria (Syahidah et al. 2017). The leaves is known to contain

essential oils, alkaloids, saponins, tannins and flavonoids that function as antibacterial compounds (Putra et al. 2017).

Red betel leaves juice has been shown to have antibacterial activity against *E. coli* and *Staphylococcus aureus* bacteria at an effective concentration of 10% (Indriati et al. 2012). With the ethanol fraction, it is known that the antibacterial compounds present in its extract are able to inhibit the growth of *B. subtilis* and *Pseudomonas aeruginosa* with weak activity, but are unable to inhibit *S. aureus* and *E. coli* bacteria (Puspita et al. 2018). The ethyl acetate fraction of the leaves has the greatest antibacterial activity compared to the hexane and methanol fractions (Gunarti and Utari 2018). Ethanol extract from the leaves has an inhibitory effect on the growth of *Staphylococcus aureus* ATCC 6538 at concentrations of 10%, 20%, 40%, 80%, and 100%, (Candrasari et al. 2011). The effectiveness of the extraction of a compound by a solvent is highly dependent on the solubility of the compound in the solvent. A compound will dissolve in a solvent with the same properties (Sudarmadji et al. 1997). Based on the data given, it is necessary to know the compound content of red betel leaves in three fractions, namely ethanol, hexane, and ethyl acetate, and their potential as an antibacterial agent.

MATERIALS AND METHODS

Sample preparation

The red betel leaves used in this research had been identified (determination test) at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Banda Aceh, Indonesia. The red betel leaves were cleaned and separated from the stems and then dried at room temperature. After drying, the leaves were blended until becoming smooth and weighed.

Extraction by multistage maceration method

Extraction was carried out using the multistage maceration method with a sample of red betel leaves simplicia powder. The solvents used were solvents with increasing polarity, namely n-hexane, ethyl acetate, and 96% ethanol, with each solvent used being as much as 100 mL (1:10). The simplicia powder was weighed as much as 10 g and put into a 250 ml Erlenmeyer flask. The first maceration of simplicia was soaked with 100 mL of n-hexane for 24 hours while being stirred occasionally (every 6 hours). After 24 hours, the residue was separated from the filtrate and the simplicia pulp was dried in an oven at 50°C. When the residue was dry, it was macerated again for 24 hours with 100 mL ethyl acetate while being stirred occasionally (every 6 hours). After that, the residue was separated from the filtrate. When maceration with ethyl acetate was completed, the residue was dried and then macerated with 96% ethanol solvent using the same procedure. The extract obtained from 96% ethanol was put into a 100 mL flask and filled to the limit and after 96% ethanol was added, then the extract was precipitated for 24 hours (modified Permadi et al. 2015).

Phytochemical screening

After a thick extract was obtained, phytochemical screening tests were carried out, including a flavonoid test, tannin test, saponin test, alkaloid test, and finally a steroid and triterpenoid test according to the instructions (Madike et al. 2017; Nurasikin et al. 2020).

GC-MS Analysis

The thick extract of leaves was taken at as much as 10 µL and dissolved with 240 µL methanol, and then injected into the GC-MS system (Fitriani et al. 2020). Identification of compounds was carried out using the Wiley/NIST Library software (Shimadzu QP 5000).

Antibacterial Potential Test

Pure cultures of *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *P. aeruginosa* (ATCC 27853) were propagated on a Nutrient Broth (NB) media and then incubated for 24 hours in an incubator. Bacterial cultures on the NB media were mixed with 0.9% NaCl and the turbidity level was standardized with Mc Farland 0.5 (Savitri et al. 2018). Bacterial cultures whose turbidity levels had been standardized were swabbed evenly on the surface of a Mueller Hinton Agar (MHA) media and left for 5 minutes. Paper discs that had been soaked for 15 minutes in macerated leaves extract with different solvents were made in concentrations of 0%, 10%, 20%, 40%, 60% each for the solvent n-hexane, ethyl acetate, 96% ethanol, and the antibiotic chloramphenicol C30 No. CT0013B catalog (positive control) was placed on the surface of the MHA media and slightly pressed to adhere. The MHA medium was incubated at 37°C for 24 hours. The diameter of the inhibition zone formed was measured with a caliper (Priyambodo and Zainal 2019).

RESULTS AND DISCUSSION

The results of the identification of herbarium samples (at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Banda Aceh, Indonesia) are presented in Table 1. Identification is important to determine and ensure the accuracy of the characteristics of the leaves studied.

Table 1. Identification of red betel leaves herbarium samples

Regnum/Kingdom	Plantae
Sub Regnum/Sub Kingdom	Tracheobionta
Super Divisio/Super Division	Spermatophyta
Divisio/Division	Magnoliophyta
Classis/Class	Magnoliopsida
Sub Classis/Sub Class	Magnoliidae
Ordo/Order	Piperales
Familia/Family	Piperaceae
Genus/Genus	<i>Piper</i> L.
Species/Species	<i>Piper crocatum</i> Ruiz & Pav.

The results of phytochemical examination with three solvent fractions showed different secondary metabolites. The highest number of metabolites yield obtained was with the ethyl acetate solvent fraction with secondary metabolites consisting of flavonoids, terpenoids, steroids, tannins, phenolics, and saponins. In the ethanol solvent fraction, secondary metabolites of flavonoids, steroids, and phenolics were obtained, while in the n-hexane solvent fraction, only one secondary metabolite was obtained, namely steroids, as presented in Table 2.

Flavonoids and phenolics were found in the ethyl acetate and ethanol fractions of leaves extract. This is because flavonoids are polar so they require polar or semi-polar solvents. Ethanol and ethyl acetate are polar and semi-polar solvents. Flavonoids have broad biological activities including antimicrobial and anti-inflammatory properties (Hodek et al. 2002); thus, they have the potential to be used in endometritis therapy. The presence of these secondary metabolites indicates that red betel leaves can produce a pharmacological effect and has the potential to be used as an antibacterial agent. According to Katuuk et al. (2019), differences in the phytochemical content of a plant are influenced by internal and external factors. Internal factors include the plant's genes, while external factors include pH, temperature, humidity, light, altitude and nutrient content in the soil.

Flavonoids are phenolic compounds that work by damaging the permeability of bacterial cell walls and inhibiting its motility (Zubaidah et al. 2021). Tannins act as an antibacterial by inhibiting DNA topoisomerase, the reverse transcriptase enzyme, so that bacterial cells are not formed. In addition, they also stop the activity of bacterial cell adhesin and enzymes and interfere with protein transport (Ngajow et al. 2013). Alkaloids work as an antibacterial agent by interfering with the peptidoglycan constituent components of bacterial cells, which results in the incomplete formation of cell walls and eventual death of the bacterial cells (Tjandra et al. 2020). Steroids act as an antibacterial agent by interacting with bacterial cell phospholipid membranes which are permeable to lipophilic compounds, thus causing decreased membrane integrity and changes to a cell's membrane morphology resulting in fragile cells and lysis (Rijayanti et al. 2014). Terpenoids work as an antibacterial agent by reacting with the porin (transmembrane protein) on the outer membrane of the bacterial cell wall, causing the porin to become damaged that can result in bacterial cells being unable to retain crucial nutrients, then leading to cell death (Rahmitasari et al. 2020). Phenol has an antibacterial mechanism of action in two concentrations. At low concentrations, it destroys the cytoplasmic membrane of the bacteria and causes leakage in the cell nucleus while at high concentrations, it coagulates with cellular proteins within the bacteria (Novita 2016).

Unidentified alkaloid compounds in leaves and in some fractions may be due to several different components. Alkaloid compounds are generally semi-polar and they were reported to be effectively soluble in non-polar (n-hexane) and semi-polar solvents (ethyl acetate) (Dewi et al.

2014). However, different results were obtained in this study. In addition, the presence of unidentified alkaloid compounds may also be due to the influence of environmental factors that affect the number of secondary metabolites in plants, namely the altitude where they grow, soil pH, sunlight intensity, and air humidity.

The results obtained in this study are different from the research conducted by Safithri et al. (2008) and Weni (2014) who stated that the leaves extract contains flavonoid compounds, alkaloids, and tannins in 70% ethanol crude extract. Antimicrobial compounds include alcohol, phenolic compounds, chlorine, iodine, and ethylene oxide (Pelczar and Chan 1988). Flavonoids, phenolic compounds, hydroquinones, and tannins belong to the class of phenolic compounds. Flavonoids are water soluble and act as natural defense factors. Ethanol 70% is better at extracting flavonoid compounds (Harborne 1987).

The results of the analysis (Table 2) show that the leaves extract contains steroid compounds. Steroids can inhibit the growth of Gram-positive bacteria (Zhu et al. 2001). In the ethyl acetate fraction, the leaves contains tannins. Tannins also have potential as an antibacterial compound. They are polyphenolic compounds that are soluble in water, glycerol, methanol, hydroalcoholic, and propylene glycol but insoluble in benzene, chloroform, ether, petroleum ether, and carbon disulfide. The mechanism of inhibition of tannin compounds as an antibacterial agent is that they react with cell membranes, which results in inactivation of essential enzymes, and the destruction or inactivation of the function of genetic material (Harborne 1987). Phytochemical compounds are secondary metabolites included in bioactive compounds and have an important role in the research of drugs produced from plants. Testing phytochemical compounds is carried out qualitatively and needs to be done in order to prove secondary metabolites that act as antibacterial agents. The principle used to extract compounds in medicinal plants is *like dissolve like*, meaning that the polarity of a compound will determine the solvent used to extract the compound.

Table 2. Phytochemical test results of red betel leaves extract on three solvent fractions

Secondary metabolite compounds	Test results		
	n-hexane	Ethyl acetate	Ethanol
Flavonoids	-	+	+
Terpenoids	-	+	-
Steroids	+	+	+
Tannins	-	+	-
Phenolic	-	+	+
Saponins	-	+	-
Alkaloids			
- Dragendroff	-	-	-
- Meyer	-	-	-
- Wagner	-	-	-

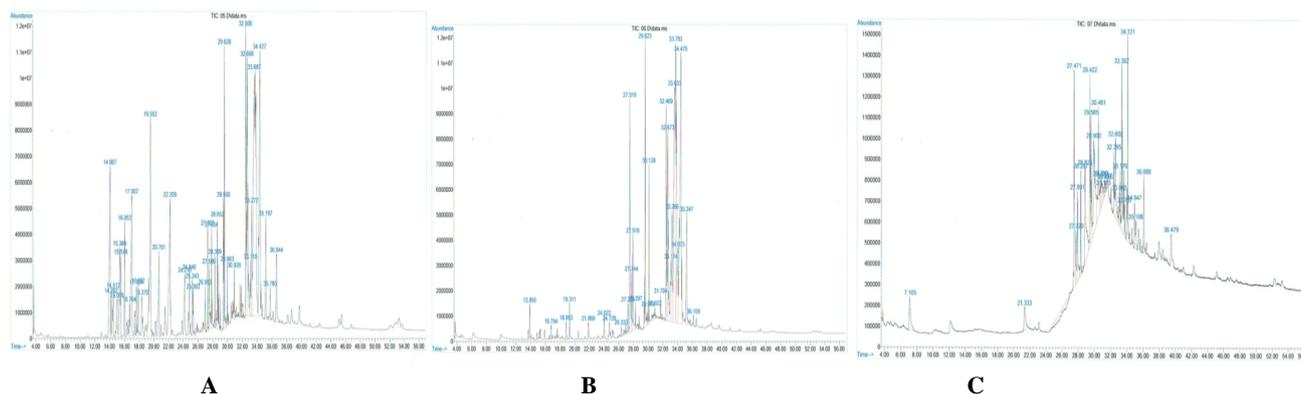


Figure 1. GC-MS chromatogram of the compound in 25 red betel leaves n-hexane fraction (A), 15 leaves ethyl-acetate fraction (B), and 24 leaves ethanol fraction (C)

Table 3. Biological activity of the main compounds of red betel leaves extract identified by GC-MS

Fraction	Compound	Biological Activity
n-hexane	5-isobutylidene-n,n-dimethylbarbituric acid	-
	Benzenamine, 4,4' - (1,2- ethenediyl) bis-	Anti-inflammatory, antioxidant (Chawla et al. 2014)
Ethyl acetate	Benzenamine, 4,4' - (1,2- ethenediyl) bis-	Anti-inflammatory, antioxidant (Chawla et al. 2014)
	5-isobutylidene-n,n-dimethylbarbituric acid	-
Ethanol	9-Octadecenoic acid	Antioxidant and anti-inflammatory (Rajeswari and Rani 2015)
	1- Methylphenazine 5-oxide	-

Plant profile character test

To determine the content of the compounds, present in the leaves extract fractions, the fractions were tested through phytochemical screening and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis. This was performed to compare the results of qualitative phytochemical screening with the results of quantitative tests using GC-MS. Analysis with GC-MS has been widely used to identify hundreds of compounds present in plant cells that cannot be carried out by ordinary phytochemical screening because such screening is limited to identifying groups of compounds (Hamuel 2012).

Identification of compound components isolated from the n-hexane, ethyl acetate, and ethanol fractions in the leaves was carried out by comparing the fragmentation patterns of the mass spectrum and the reference compound. The data from the analyses of the components of the isolated plant compounds were obtained using the WILLEY9THN 08.L data bank. The principle of GC-MS is that the components in the mixture are separated by gas chromatography, and each component can be made up of a mass spectrum with greater accuracy. The result of the separation by gas chromatography is a chromatogram, while the result of the mass spectrometry examination of each compound is called as 'spectrum' (Nurhaen et al. 2016). GC-MS results are shown in the form of peaks in the GC-MS spectrum and are presented in Figure 1.

In gas chromatography systems, compounds that have a low boiling point will come out first to the detector because a lower point causes the compound to evaporate more easily so that the retention time is faster. The retention time of each compound is determined by the boiling point of the compound. The difference in retention time of the compounds in some of these plants could be due to the interaction of the compound with the stationary phase which in this case is the column used in the gas chromatography system. The resulting chromatogram is formed based on the total number of ions formed from each component of the chemical compound contained in a sample. The greater the percentage of a component in the sample, the higher the peak produced, and vice versa (Harianingsih et al. 2017).

From the results of research on the leaves extract using the ultrasonic wave extraction method, it is obvious that more than 50 compounds can be extracted. The profile characterization of red betel leaves with three solvents is presented in Table 3.

Figure 1 shows that the GC-MS analysis of the leaves extract produced 25 peaks in the n-hexane fraction, 15 peaks in the ethyl acetate fraction, and 24 peaks in the ethanol fraction, meaning that there were 25, 15, and 24 compounds present in each fraction of n-hexane, ethyl acetate, and ethanol. The percentages of the main compound components produced from the three fractions and their biological activities are presented in Table 3.

Table 4. Antibacterial effectiveness of red betel leaves extract in three different solvents

Extraction solvent	Concentration (mg/mL)	Inhibition zone value (mm)		
		<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
N-hexane	0	0.0±0.0	0.0±0.0	0.0±0.0
	10%	0.0±0.0	0.0±0.0	0.0±0.0
	20%	0.0±0.0	0.0±0.0	0.0±0.0
	40%	0.0±0.0	0.0±0.0	0.0±0.0
	60%	0.0±0.0	0.0±0.0	0.0±0.0
Chloramphenicol		27.24±0.58	23.74 ± 6.45	0.0±0.0
Ethyl acetate	0	0.0±0.0	0.0±0.0	0.0±0.0
	10%	8.94±0.52	0.0±0.0	0.0±0.0
	20%	11.76±1.71	8.20±0.55	0.0±0.0
	40%	13.32 ±1.16	11.04 ±2.25	9.29±2.19
	60%	16.32±1.83	10.56±1.19	12.33±2.99
Chloramphenicol		27.11±0.27	26.30±6.35	0.0±0.0
Ethanol	0	0.0±0.0	0.0±0.0	0.0±0.0
	10%	0.0±0.0	0.0±0.0	0.0±0.0
	20%	0.0±0.0	0.0±0.0	0.0±0.0
	40%	0.0±0.0	8.90±2.64	0.0±0.0
	60%	0.0±0.0	11.28±3.68	0.0±0.0
Chloramphenicol		27.15±0.24	21.73±0.99	0.0±0.0

Antibacterial inhibition test

Antibacterial activity testing was carried out using gram negative bacteria *E. coli* and *P. aeruginosa* and gram positive *S. aureus*, as the pathogenic microorganisms that can cause endometritis in Aceh cattle. The method used in testing the antibacterial activity was disc diffusion. This method was chosen because the process is simpler and the results are quite thorough. The basis for this observation and method is to determine whether or not a clear zone is formed, which indicates the growth or non-growth of bacteria around the cylinder that has been moistened by each extract fraction. The wider the inhibition zone formed, the higher the effectiveness as an antibacterial agent the compound (Puspita et al. 2018; Priyambodo and Zainal 2019). Inhibition against bacteria by the leaves extract is presented in Table 4.

According to the results, it appears that red betel leaves extract has no antibacterial activity against *E. coli* and *P. aeruginosa* except for the ethyl acetate fraction. These results are in accordance with the report by Puspita et al. (2018) that the leaves extracted by the maceration and reflux method with ethanol solvent did not show activity against *E. coli*. This study also found that in the ethanol fraction, the leaves did not show any antibacterial activity. Hermawan (2007) also reported that the ability of leaves extract against *E. coli* was relatively weak. The diameter of the inhibition shown did not meet the specified minimum standard, which was 12-24 mm. In this study, the ethanol fraction of red betel leaves showed antibacterial activity at concentration more than 40%.

Although all fractions represented low or no inhibition among the three fractions it was seen that ethyl acetate had

higher antibacterial activity than hexane and ethanol. Similar results were reported by Gunarti and Utari (2018) that red betel leaves in the ethyl acetate fraction had higher antibacterial activity than the hexane and methanol fractions. The data above show that the diameter of the ethyl acetate extract was smaller than those of the positive controls (Chloramphenicol). These data indicate that the ethyl acetate extract is less effective as an antibacterial at concentrations below 60%, but if the concentration of the extract is increased it is likely to increase its antibacterial activity because there is a tendency to increase concentrations increase the inhibition zone formed. Therefore, it was concluded that red betel leaves extract with ethyl acetate solvent had highest number of metabolites and better antibacterial potential than ethanol and n-hexane solvents.

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