

The combination of *Luvunga sarmentosa* (Bl.) and *Eurycoma longifolia* Jackhydro-alcoholic extract as a source of antioxidant and analgesic agent

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ABSTRACT: The combination of *Luvunga sarmentosa* and *Eurycoma longifolia* is widely prescribed in traditional medicine by the local Dayak tribe in Central Kalimantan, Indonesia. The study aimed to evaluate the effect of antioxidants and analgesics from the combination of hydro-alcoholic extract of *L. sarmentosa* and *E. longifolia* stem. The phytochemical investigation was performed using an established method described in the literature. The total phenolic content was determined by the Folin Ciocalteu, whereas the flavonoid was determined by aluminum chloride. The in vitro antioxidant activity was determined using DPPH free radical scavenging assay. The analgesic effect was determined by acetic acid-induced writhing and hot plate tests in male mice. The result shows that combination extract (CE, IC₅₀ 162.5±0.21 µg/mL) had higher antioxidant activity than *L. sarmentosa* extract (LSE, IC₅₀ 200.20±0.23 µg/mL) and *E. longifolia* extract (ELE, IC₅₀ 293.45±0.32 µg/mL). All extracts showed significant dose-dependent analgesic activity against acetic acid-induced writhing and hot plate pain models. A higher dose of CE, LSE, and ELE (550 mg/kg) produced significant inhibition of writhing by 67.60±2.76, 56.45±2.34, and 68.64±1.65%, respectively. The maximum possible analgesia of CE, LSE, and ELE in the hot plate test at 550 mg/kg BW dose was 25.32%, 16.04%, and 26.72%. CE has higher analgesic activity than LSE and ELE. The extracts are more effective for peripheral than central analgesia. Its mechanism may be related to inhibiting the inflammatory mediator's release. The high antioxidant and analgesic activity at CE is relevant to the higher total flavones and phenols compared to LSE and ELE.

KEYWORDS: Antioxidant; analgesic; *Luvunga sarmentosa*; *Eurycomalongifolia*; medicine.

1. INTRODUCTION

The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) causes damage to macromolecules such as nucleic acids, proteins, enzymes, and lipids in several organs. ROS and RNS lead to disorders such as aging, atherosclerosis, malaria, diabetes, liver injury, Alzheimer, epilepsy, Parkinson, carcinogenesis, and other pathological events[1–4]. ROS is also responsible for pain induction and plays an important role as a signaling molecule and a mediator of inflammation[5]. Antioxidants effectively protect the body against damage by reactive oxygen species, which can serve as a medicine for several diseases. Currently, the search for antioxidants is mainly done by plants. The different antioxidative compounds have been isolated from various medicinal plant species [6].

Medicinal plants contain many bioactive molecules, such as alkaloids, phenolic acids, terpenes, tannins, lignans, and flavonoids[7]. Flavonoids and other phenolic compounds are well-known as the largest phytochemical molecules with antioxidant and bioactive agents, they are widely distributed in the plant [8].The majority of phenolic compounds of plant are phenolics acids, flavonoids, tannins, proanthocyanidins, stilbenes, and lignans[9].These compounds have biological activities, including hepatoprotective, anti-

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inflammatory, analgesic, antipyretic, anti-cancer, anti-arthritis, antimicrobial, cardioprotective, immune system promoting, and skin protection from UV radiation[8,10,11].

Luvunga sarmentosa (Bl.) Kurz, which belongs to the family Rutaceae, is a shrub growing in the rainforest of Vietnam. *L. Sarmentosa* has been used as a traditional medicine to treat toothache, limb pain, and rheumatism[12]. Phytochemical screening of *L. Sarmentosa* stem bark has identified some triterpenes, coumarins, and acridone alkaloids[13]. Its leaves contain apotirucallane triterpenoids named luvungins A-G, 1 α -acetoxyluvungin A, coumarins ostruthin, 8-geranyl-7-hydroxycoumarin, triterpenes friedelin, flindissone, melianone, niloticin, limonin, new apotirucallane triterpenoids (3-epi-skimmiaepin A and 21,23-epoxy-7R,21-dihydroxyapotirucalla-14,24-dien-3-one), and 3-epi-flindissol (new tirucallane triterpene)[14,15].

Eurycoma longifolia Jack (Simaroubaceae family) is a wild shrub widely distributed in Indonesia, Malaysia, Vietnam, Cambodia, Myanmar, Laos, and Thailand[16]. Traditionally, the plant has been used to cure lumbago, indigestion, fever, jaundice, cachexia, dropsy, diarrhea, increased energy, increased strength, and cancer. *E. longifolia* is one of the most popular folk medicines for its aphrodisiac effects and treatment of malaria[17-19]. This plant is a rich source of various bioactive compounds, including alkaloid, quassinoid, polyphenols, flavonoid, polysaccharide, triterpenoid. The major compound of *E. Longifolia* was quassinoids with various types like eurycumanone, eurycomanol, eurycomadilactone and eurylactone[18-21].

Luvunga sarmentosa (Bl.) Kurz and *Eurycoma longifolia* Jack are superior plants used by the general public for traditional medicine. These plants are often used in a mixed form to make potions by the local Dayak tribe in Central Kalimantan, Indonesia. The potion can increase stamina, sexual arousal, and male fertility [22]. They are sold in many traditional markets in Central Kalimantan. A mixture of these two plants (stem) was consumed three times a day by boiling or brewed with hot water and consumed once a day while warm [22,23]. The people of Kalimantan claim that the administration of *L. sarmentosa* and *E. longifolia* in a combined form gives a better effect than a single plant. Therefore, for treatment, these two plants are always mixed. The use of mixed plants by the general public possibly aimed to obtain a synergistic effect. Based on the literature, treatment with combination therapy can increase the pharmacologic effect and neutralize the potential toxicity and side effects of each of the individual plants [24]. Many studies have been reported to determine the pharmacology activities of *L. sarmentosa* and *E. longifolia* extracts. However, no reports indicate their antioxidant and analgesic effects in this plant combination. Hence, this study aimed to evaluate the effect of antioxidants in vitro and analgesics in vivo from the combination of hydro-alcoholic extract of *L. sarmentosa* (Bl.) Kurz and *E. longifolia* Jack stem.

2. RESULTS

2.1. Extraction and phytochemical screening

The stem of *Luvunga sarmentosa* (LSE), *Eurycoma longifolia* (ELE), and a combination of them (CE) were extracted using 70% ethanol. These extractions yielded 3.6%, 3.9%, and 3.3% w/w dry matter. The phytochemical analysis of the extracts revealed the presence of various pharmacologically active constituents such as triterpenoids, flavonoids, tannins, and coumarin (Table 1).

Table 1. Phytochemical constituents of the extracts

Serial no.	Constituent	Test results		
		<i>L. sarmentosa</i> extract (LSE)	<i>E. longifolia</i> extract(ELE)	Combination extract (CE)
1	Alkaloid	-	-	-
2	Flavonoids	+	+	+
3	Triterpenoids	+	+	+
4	Steroids	-	-	-
5	Tannins	+	+	+
6	Saponins	-	-	-
7	Carotenoid	-	-	-
8	Coumarin	+	+	+

+ : Present, - : Absent

2.2. Quantification of total phenolic content

The total phenolic contents of the LSE, ELE, and CE were calculated based on a standard curve of gallic acid with an equation, $y = 6.4421x + 0.0811$, $R^2 = 0.9965$ (Figure 1), and it was found to be 48.50; 45.10; 50.45 mg GAE in per gram of dry extract, respectively.

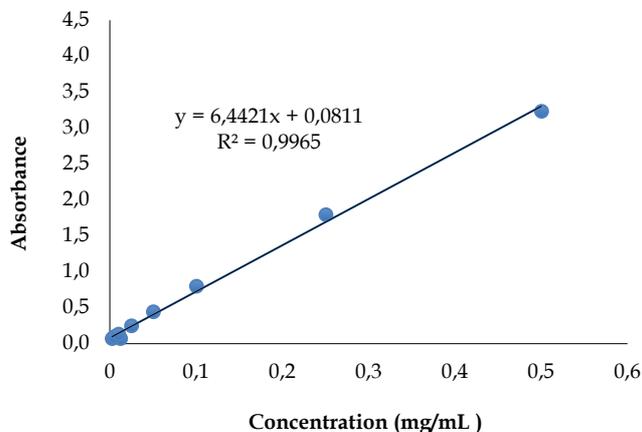


Figure 1. Total phenolic content determination of extract as a gallic acid standard calibration curve

2.3. Quantification of total flavonoid content

The total flavonoid contents of the LSE, ELE, and CE were calculated based on a standard curve of quercetin ($y = 1.6203x + 0.01829$, $R^2 = 0.9893$), and the values were found to be 40.64; 38.90; 43.56 mg QE per gram of dry extract, respectively (Figure 2).

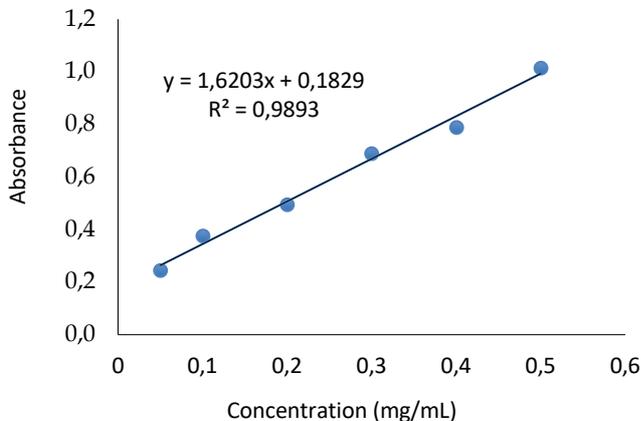


Figure 2. Total flavonoids content determination of extract as quercetin standard calibration curve

2.4. Antioxidant activity

Antioxidant activity was performed by DPPH-free radical scavenging assay where the IC_{50} value of LSE, ELE, and CE were 200.20 ± 0.23 ; 293.45 ± 0.32 ; 162.5 ± 0.21 $\mu\text{g/mL}$, respectively. The standard (ascorbic acid) showed a value of 8.0 ± 0.1 $\mu\text{g/mL}$ (Figure 3).

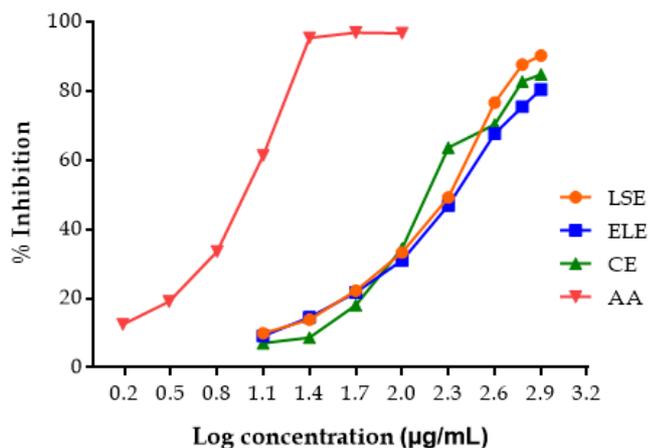


Figure 3. DPPH scavenging activity of *L. sarmentosa* extract (LSE), *E. longifolia* extract (ELE), combination extract (CE), and ascorbic acid (AA)

2.5. Analgesic activity

Acetic acid-induced writhing test

Treatment with extracts induced a dose-dependent inhibition of writhes in acetic acid-injected mice (Table 2). The extracts showed a significant reduction in writhes number compared to the negative control. The highest inhibition of extracts (LSE, ELE, and CE) was observed on a 550 mg/kg BW dose with the % inhibition of 67.60±2.76, 56.45±2.34, and 68.64±1.65%, respectively. While positive control (diclofenac sodium) inhibition was 74.74±2.02%. CE has higher % writhing inhibition compared to ELE and LSE.

Table 2. Effect of extracts on acetic acid-induced writhing in mice

Groups	Dose (mg/kg)	No. of writhes	% Writhing inhibition
Negative control		95.67±2.85	
Positive control	40	24.17±0.79****	74.74±2.02
<i>L. sarmentosa</i> extract (LSE)	50	68.83±206****	33.28±1.44
	300	48.50±3.16****	49.30±1.56
	550	31.00±3.33****	67.60±2.76
<i>E. longifolia</i> extract (ELE)	50	68.00±1188****	28.92±1.29
	300	55.00±2.84****	42.51±1.15
	550	41.67±2.45****	56.45±2.34
Combination extract (CE)	50	61.33±1.36****	35.89±1.43
	300	50.17±3.15****	47.56±3.29
	550	30.00±1.58****	68.64±1.65

Values are expressed as mean±SEM, Number of mice (n)=5, ****p <0.00001 compared to negative control

Hot plate test

The results of the analgesic effect of the extracts (LSE, ELE, and CE) using the hot plate method are presented in table 3 and figure 4. The treatment of mice with extracts and positive control increased the latency time in the hot plate test from 30 to 120 min compared to baseline values (0 min). The extracts and negative control observed a significant difference in the latency time to thermal pain during the 120 min observation.

Figure 4 illustrates the analgesic effect of extracts and positive control using MPA. Extracts and positive control elicited significant analgesic activity within 30 min following administration, as evidenced by the gradual increase throughout the observation period. At the peak of activity (90 min), the positive control showed an MPA of 55.58% and then declined. Similarly, with extracts, the peak of activity on 90 min declined after that. The highest increase in reaction time of three extracts (LSE, ELE, and CE) were observed on a 550 mg/kg BW dose (90 min) with an MPA of 25.32%, 16.04%, and 26.72%, respectively. The CE has a higher MPA value compared to LSE and ELE.

Table 3. Effect of extract and standard in increasing latency time on the hot plate model

Groups	Dose (mg/kg)	Reaction time				
		0 min	30 min	60 min	90 min	120 min
Negative control		4.26±0.36	4.55±0.34	4.87±0.27	5.28±0.21	5.13±0.39
Positive control	40	4.84±0.31	7.05±0.28***	9.16±0.71****	13.27±0.69****	11.58±0.72****
<i>L. sarmentosa</i> extract (LSE)	50	4.96±0.32	6.02±0.46	7.18±0.41**	7.67±0.54***	6.71±0.36
	300	4.95±0.29	6.88±0.23**	7.17±0.34**	7.47±0.27**	7.37±0.22**
	550	4.66±0.34	6.95±0.76**	7.67±0.64***	8.53±0.75****	7.72±0.64***
<i>E. longifolia</i> extract (ELE)	50	4.71±0.31	5.68±0.27	6.52±0.21	6.65±0.37	6.40±0.61
	300	4.95±0.25	6.18±0.20*	6.74±0.30*	7.22±0.30*	6.40±0.32
	550	4.95±0.31	6.63±0.81**	7.15±0.81**	7.37±0.34**	6.83±0.23*
Combination extract (CE)	50	4.89±0.40	6.12±0.34	6.86±0.35*	7.58±0.34**	6.94±0.32*
	300	4.95±0.27	6.34±0.37*	6.91±0.14*	7.69±0.15***	7.25±0.18**
	550	4.93±0.53	7.71±0.53****	8.98±0.56****	8.98±0.57****	8.11±0.51****

Data are presented as mean±SEM. The number of mice (n)=5. P<0.0001 (****), P<0.001 (***), P<0.01 (**), and P<0.05 (*) as compared to the negative control. Data were analyzed using ANOVA followed by Dunnett's test.

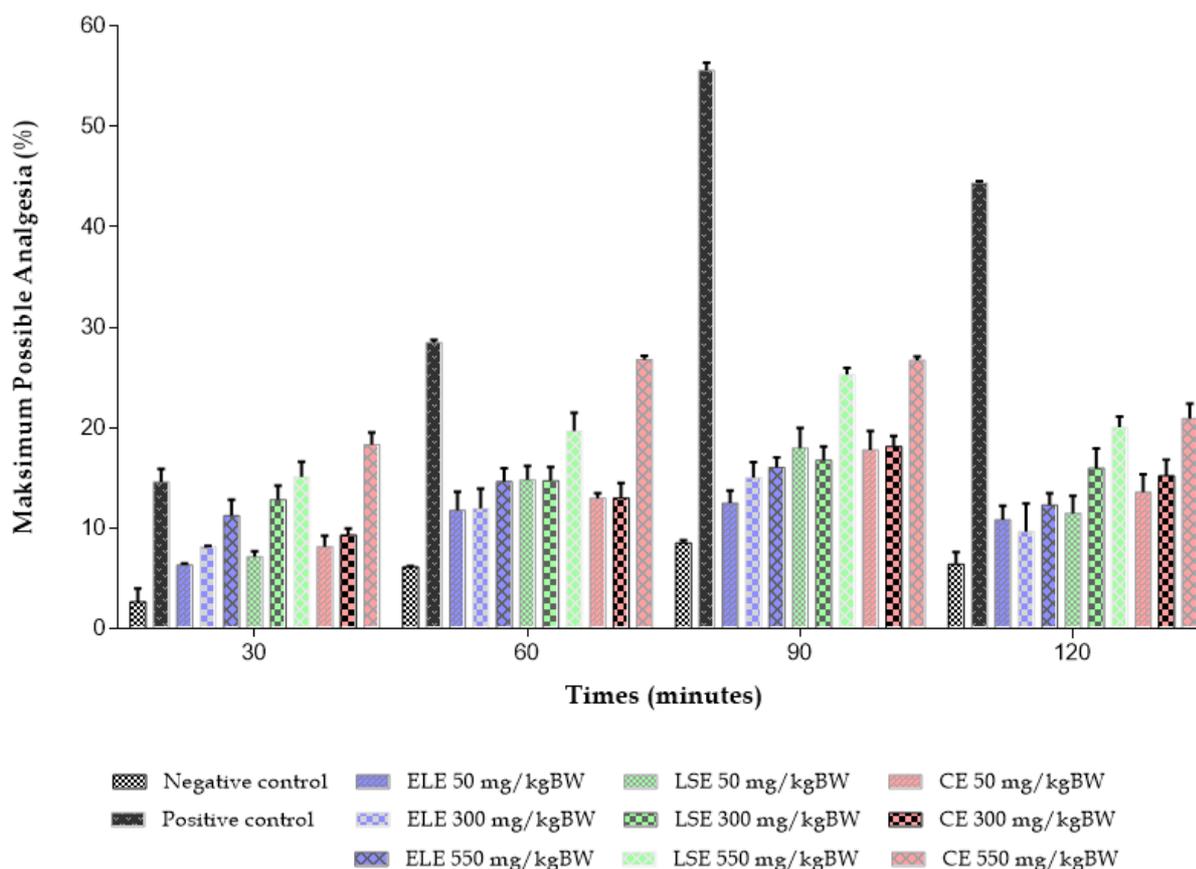


Figure 4. The Percent of maximum possible analgesia (MPA) of control and all extracts with different doses. Data are presented as mean±SEM. The number of mice (n)=5.

3. DISCUSSION

Free radicals are produced in response to the normal physiological process in the living organism. However, the overproduction of ROS can bring about oxidative harm to biological molecules such as lipids, proteins, and DNA, leading to severe disease[25]. This harmful effect can be neutralized by either cellular mechanisms or by the intake of antioxidants from external sources, including plants. Compounds can act as antioxidants, such as hydrogen donors, free radical scavengers, reducing agents, and singlet oxygen quenchers[26].

This present study was designed to evaluate the antioxidant and analgesic effect of the *Luvunga sarmentosa* extract (LSE), *Eurycoma longifolia* extract (ELE), and a combination of extract (CE) to verify the claims of its traditional uses. The antioxidant activity of the extracts was carried out by measuring DPPH free radical scavenging. The result of the DPPH test showed that CE possessed moderate antioxidant activity with an IC₅₀ value of 162.5±0.21 µg/mL. The LSE and ELE possessed weak antioxidants with an IC₅₀ value of 293.45±0.32 and 200.20±0.23 µg/mL, respectively. The result proved that treatment with combination increases the pharmacological effect.

Analgesic models can be divided into categories according to the mechanism of action, and there is peripheral and central analgesia[27]. Writhing test-induced acetic acid was conducted to examine the peripheral analgesic effect, and a hot plate test was conducted to assess the involvement of the central analgesic effect[28]. Acetic acid-induced pain indirectly releases endogenous mediators, such as serotonin, bradykinin, histamine, and prostaglandins E₂ (PGE₂), to stimulate nociceptive neurons[29]. Two in vivo pain models were used in this study. Results of the present study suggest that LSE, ELE, and CE displayed a significant reduction in writhing count after oral administration in a dose-dependent manner compared to the negative control (P<0.00001) through interfering peripheral mechanisms of pain inhibition. After forty-five minutes of the test period, the three extracts at 550 mg/kg BW demonstrated the highest analgesic activity by reducing the number of writhes by 67.60±2.76, 56.45±2.34, and 68.64±1.65%, respectively. CE has higher analgesic activity than LSE and ELE. There is no significant difference in analgesic activity at 550 mg/kg BW dose between the positive control and CE.

In the hot plate test, the extracts increased the latency time from 30 to 120 min observation compared to baseline values (0 min). The extracts and negative control observed a significant difference in the latency time. The highest increase in the reaction time of extracts (LSE, ELE, and CE) and positive control were served on a 550 mg/kg BW dose (90 min) with an MPA of 25.32%, 16.04%, 26.72%, and 55.58%, respectively. Based on these data, we conclude that the analgesic effect of the CE is due to the LSE. The extracts had lower analgesic activity in the measurement using the hot plate than in the acetic acid tests. Therefore, the extracts may be more effective for peripheral analgesia, and their mechanism may be related to inhibiting inflammatory mediators' release.

Phytochemical studies showed that LSE, ELE, and CE revealed the presence of triterpenoids, flavonoids, tannins, and coumarin. The measurement of the total phenolic and flavonoid of the extracts showed that the CE has a higher amount of phenolic and flavonoid than LSE and ELE. These data correlate with the antioxidant and analgesic activity of CE. The CE has a higher ability to scavenge free radicals and analgesic activity compared to the LSE and ELE. Several phenolic compounds have been reported to show antioxidant activities due to redox's ability to absorb and neutralize free radicals, decompose peroxide, and quench singlet or triplet oxygen. Phenolics and flavonoids have potential biological activities, including hepatoprotective, anti-inflammatory, analgesic, antipyretic, anti-cancer, anti-arthritic, and antimicrobial [9,10]. Both compounds are essential agents for antioxidant activity in the medicinal plant because of their hydrogen donating the property to neutralize free radicals[30].

4. CONCLUSION

The combination extract of *L. sarmentosa* and *E. longifolia* showed higher antioxidant and analgesic activity than the single extract of *L. sarmentosa* and *E. longifolia*. This proved that treatment with the combination could increase the pharmacological effect. The combination extract possessed moderate antioxidant activity and was more effective for peripheral analgesia.

5. MATERIALS AND METHODS

5.1. Plant collection and authentication

The stems of *L. sarmentosa* (Bl.) Kurz and *E. longifolia* Jack were collected from traditional healers in the Pager, Rakumpit district, Palangka Raya City, Central Kalimantan, Indonesia, on September 2019. The identification and authentication were carried out at Purwodadi Botanical Garden, East Java, Indonesia. A voucher specimen number is 1048/IPH.06/HM/IX/2019 and 1049/ IPH.06/HM/IX/2019. Raw material has been stored at the herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

5.2. Plant extraction

The stem of *L. sarmentosa* (Bl.) Kurz and *E. longifolia* Jack were shade dried after cutting into small pieces and were ground into a fine powder using a grinder. Four hundred grams of powder was extracted by maceration using 70% ethanol three times (24 h each). For combination extract, each 200 g dried powdered *L. sarmentosa* and *E. longifolia* (1:1) was extracted with the same methods. The crude extract was filtered and dried in a vacuum.

5.3. Phytochemical screening

The extracts were submitted to phytochemical analysis using standard methods[31,32]. Briefly, the presence of alkaloids was determined with Dragendorff's test; flavonoids were detected with NaOH; a sulphuric acid test measured triterpenoids, steroids, and carotenoids; tannins were estimated with ferric chloride; coumarins were measured with sodium hydroxide.

5.4. Determination of Total Phenolic Content (TPC)

The total phenolic content of the ethanol extract was measured according to the method by Herald et al. (2012) and Zhang et al. (2006) with slight modifications[33,34]. Briefly, 25 μ L extract solutions (1000 μ g/mL) or gallic acid standard (12.5-500 μ g/mL) were added to a 96-well microplate, followed by water (75 μ L) and Folin & Ciocalteu's phenol reagent (25 μ L). The well plate was covered with aluminum foil and incubated for 6 minutes. After incubation, 100 μ L Na_2CO_3 solution (75 g/L) was added to each well, followed by incubation for 90 mins in the dark at room temperature. The mixtures were shaken for the 30s before a microplate reader read the absorbance at 765 nm. The absorbance of the standard solution of different concentrations was used to form the standard calibration curve from where total phenol content was determined and expressed as mg gallic acid equivalent (GAE) per gram of dry extract.

5.5. Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extracts was estimated by following the method of Herald et al. (2012) and Zhang et al. (2006) with slight modifications[33,34]. Briefly, 25 μ L extract solutions (1000 μ g/mL) or quercetin standard (12.5-500 μ g/mL) were mixed with 110 μ L sodium nitrate solution (0.066 M). The well plate was covered with aluminum foil and incubated for 5 minutes. After incubation, 15 μ L of 0.75 M aluminum chloride was added to the mixture and allowed to react for 6 min. Then 100 μ L sodium hydroxide solution (0.5 M) was added to the mixture. The mixtures were shaken for the 30s before a microplate reader read the absorbance at 420 nm. TFC was calculated as mg of quercetin equivalents per gram of the extract (mg QE/g of extract).

5.6. Antioxidant activity

The antioxidant activity was carried out in triplicates based on modified protocols[33,35]. The extracts (12.5-1000 μ g/mL) and Ascorbic acid (0.78-100 μ g/mL) were separately dissolved in methanol. A 100 μ L sample or standard at different concentrations was mixed with 100 μ L of 0.25 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 96 well plates. DPPH reagent (100 μ L) was mixed with methanol (100 μ L) to serve as a control, while methanol (200 μ L) was used as a blank. The reaction mixtures were incubated for 30 min in the dark at room temperature (26°C). The absorbance of each reaction mixture was then measured using the microplate reader at 517 nm wavelength. The following formula calculated the DPPH scavenging effect.

$$\% \text{ DPPH radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard

5.7. Experimental animal

Male mice (Deutschland, Denken, and Yoken strains), weighing 25-30 g and aged 4-8 weeks, were used in the experimental studies, purchased from the Farma Veterinary Center, Surabaya, Indonesia. All animals were maintained on a standard diet and water ad libitum at the Animal Laboratory of the Institute of Tropical Disease, Universitas Airlangga, Surabaya. The animals were kept at standard temperature (25 \pm 1°C) and a 12/12 h light/dark cycle. All the animals were acclimatized to the laboratory conditions before experimentation.

5.8. Ethical consideration

Animal experiments were conducted following the experimental procedures and were approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (2.KE.117.03.202).

5.9. Acetic acid-induced writhing test

The acetic-acid writhing test was performed using the method followed by Han et al (2016) and Ilmi et al(2021)[36,37]. Mice were randomly divided into 11 groups, taking five mice in each group, where group 1 received carboxymethyl cellulose (CMC-Na 0.5%), and group 2 received Diclofenac-sodium as a positive control at a 40 mg/kg body weight dose. Groups 3, 4, and 5 were treated with *Luvunga sarmentosa* extract (LSE) at a dose of 10, 40, and 80 g of *Simplicia*, equal to extract doses of 50, 300, and 550 mg/kg body weight, respectively. Groups 6, 7, and 8 were treated with *Eurycoma longifolia* extract (doses 50, 300, and 550 mg/kg body weight), and the other three groups (groups 9,10, and 11) were treated with combination extract (CE) at a dose of 50, 300, and 550 mg/kg body weight. All treatments were administered orally. The extracts and the positive control were treated 30 minutes before injection of 1% acetic acid at a dose of 10 ml/kg body weight intraperitoneally. Afterward, 5 min later writhing number was counted for 45 min. The mean value for each group was calculated and compared with the control. The percentage of analgesic activity was calculated as follows:

$$\% \text{ Inhibition} = \frac{W_c - W_t}{W_c} \times 100$$

Where W is the number of writhing, c is the negative control, and t is the test.

5.10. Hot plate test

The analgesic activity was also evaluated using the hot plate method[36,38,39]. Mice were given oral therapy according to groups. After 30 minutes of treatment, the mice were placed on a hot plate at $55 \pm 0.5^\circ\text{C}$. The reaction time (in seconds) or latency period was determined as the time for the rats to react to the thermal pain by licking their paws or jumping. The reaction time was recorded before (0 min) and 30, 60, 90, and 120 min after administering the treatments. The maximum reaction time was fixed at 20 sec to prevent any injury to the tissues of the paws. The maximum possible analgesia (MPA) is calculated as follows:

$$\% \text{MPA} = \frac{\text{test group mean} - \text{control group mean}}{\text{cut off time 20 seconds} - \text{control group mean}} \times 100$$

5.11. Data analysis

All experimental values were expressed by the mean and standard error of the mean. GraphPad Prism 7.0 software was used to perform one-way ANOVA followed by post-hoc Dunnett's test statistical analysis in the analgesic test and probit analysis in the antioxidant test. Results were considered statistically significant at the $P < 0.05$ level. Microsoft Excel was used to perform statistical analysis of TPC and TFC.

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