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TOXICITY TEST ON THE COMBINATION OF Caesalpinia sappan AND Zingiber officinale IN Rattus norvegicus INDUCED BY COMPLETE FREUND’S ADJUVANT

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ABSTRACT

Caesalpinia sappan and Zingiber officinale are plant species that have been studied for their efficacy in treating inflammation related to rheumatoid arthritis. This study aimed to examine the effects of combining sappanwood and red ginger in order to determine the potential toxicity of the herbal extracts in medicine. The toxicity testing was carried out in vivo using 32 Wistar strain male white rats (Rattus norvegicus) grouped into eight groups of four. The rats were injected with complete Freund's adjuvant to induce a chronic inflammatory effect. The eight groups consisted of the negative control group, the positive control group, the normal group, and five treatment groups. This study was conducted by observing the animals for toxic symptoms and death to determine the safety of the extracts and drugs. The observation results were analyzed using a one-way analysis of variance (p<0.05). The analysis results showed that weight gain and relative organ weight among the groups had no significant differences (p>0.05). Microscopic examination of the organ preparations observed under a light microscope revealed no significant changes or adverse effects in rats treated with the extracts or drugs. In conclusion, a combination of sappanwood and red ginger ethanol extracts administered orally has no toxic effect in rats injected with complete Freund's adjuvant.

Keywords: Rheumatoid arthritis; red ginger; sappanwood; toxicity test; good health and well-being


INTRODUCTION

Rheumatoid arthritis (RA) is the most common autoimmune rheumatic disease. It is a chronic inflammatory disease that progresses and causes permanent joint damage. Systemic inflammation in rheumatoid arthritis is strongly associated with several extra-articular comorbidities, including cardiovascular disease, metabolic syndrome, osteoporosis, interstitial lung disease, infection, malignancy, fatigue, depression, and cognitive impairment, thereby increasing morbidity and mortality in rheumatoid arthritis patients (Al-Saadany et al. 2016, Firestein & McInnes 2017, Panagopoulos & Lambrou 2018).

Early diagnosis, management, and timely treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying antirheumatic drugs (DMARDs) are essential to achieve rapid disease control and prevent further joint damage and disability. It is often challenging to distinguish joint manifestations from the inflammatory process causing arthritis, particularly in patients with early onset. The essential principle of rheumatoid arthritis therapy is to achieve a
therapeutic goal, which is to relieve or at least reduce disease activity (Hidayat et al. 2021). Rheumatoid arthritis drugs can relieve pain and slow the disease progression, but they do not repair tissue damage caused by rheumatoid arthritis. Currently, no known treatment can completely cure rheumatoid arthritis. The use of drugs is also limited because they can cause dangerous side effects such as bleeding in the gastrointestinal tract, cardiovascular complications, impaired kidney function, nausea, dyspepsia, pulmonary toxicity, myelosuppression, liver fibrosis, stomatitis, cirrhosis, immune reactions, and local injection site reactions (Ravikumar 2014, Choudhary et al. 2021). Curative treatment for rheumatoid arthritis is relatively expensive and long-term. It also causes side effects with a high risk of infection. Therefore, 90% of patients are estimated to choose herbal medicines for treating rheumatoid arthritis (Rambod et al. 2018).

Plant species that have been studied for their efficacy in treating inflammation and reducing pain associated with rheumatoid arthritis are sappanwood (Caesalpinia sappan) and red ginger (Zingiber officinale). Sappanwood contains brazillian and sappanone A, while red ginger contains 6-poradol, 6-shogaol, and 1-dehydro-6-gingerol, which act as anti-arthritis compounds (Jung et al. 2015, Ezza et al. 2018). There is an increasingly high interest in herbal medicines, which encourages further research on sappanwood and red ginger as rheumatoid arthritis treatments. This study examined the toxic effects of combining sappanwood and red ginger ethanol extracts as a treatment for rheumatoid arthritis. This is essential to provide information and a reference for considering the use of sappanwood and red ginger as effective ingredients in anti-arthritis drugs. Sappanwood and red ginger are anticipated to be upgraded as standardized herbal medicines and phytopharmaceuticals.

MATERIALS AND METHODS

The main materials used in this study were sappanwood and red ginger rhizomes. The sappanwood samples were obtained from Rengganis Jamu Shop, Gresik, Indonesia (7°09'06.7"S 112°39'25.0"E), while the red ginger samples were purchased from Wonokromo Market, Surabaya, Indonesia (7°18'07.8"S 112°44'17.1"E). Other materials used were 96% ethanol, distilled water, complete Freund’s adjuvant (CFA) made in the USA by G-Biosciences (38°41’18.251”S 90°22’24.34”E) with batch number 02J#200F#201601, diclofenac sodium made in Indonesia by PT Novell Pharmaceutical Laboratories (3°19’4.44”S 115°25’2.8’E) with batch number ALU/02/250, carboxymethylcellulose (CMC) made in Indonesia by PT Gunacipta Multirasa (6°10’41.016”S 106°37’47.999”E) with batch number 3000-01, haematoxylin-eosin stain kit, and 10% neutral buffer formalin (NBF). The test animals used were male Wistar rats (Rattus norvegicus) (Barkia et al. 2020). The tools used in this study were syringes produced by Terumo (USA), oral syringes, animal scales, analytical balances, rotary evaporators, volume pipettes, glassware produced by Iwaki (Indonesia), and camera microscopes.

The first method performed in this study was sample extraction. Sappanwood samples were obtained in a dry state. Red ginger rhizomes were washed under running water, sliced thinly, and then dried. The dried samples were ground into a fine powder (Nurhidayah et al. 2022). The samples were extracted by maceration for three, with the fresh solvent being changed every 24 hours. In the maceration process, 1,000 mL of 96% ethanol was used for every 500 g of the samples. Afterwards, the solvent was evaporated using a vacuum rotary evaporator at a temperature of 40°C until it had completely evaporated. The concentrated extract was cooled and stored in a closed container (Aristyanti et al. 2017).

The toxicity testing was carried out in vivo using 32 Wistar strain male white rats (Rattus norvegicus) obtained from the Pharmacology Laboratory of Universitas Airlangga, Surabaya, Indonesia. The rats weighed ±150–200 g and were 3–4 months old. The animal models were acclimatized for 14 days and provided with standard rodent commercial feed and water ad libitum. The categorization of the animal models was performed on the last day of the acclimatization period by weighing and grouping them into eight groups of four in separate cages (Idang et al. 2019).

The in vivo study was conducted through the experiment of administering predetermined doses to the animal models. Signs of toxicity were observed in the percentage of weight gain, relative organ weight, and histopathology of organs. Further monitoring was carried out for 16 days to observe any occurrence of toxic symptoms or death (Pereira et al. 2019). Figure 1 shows the process of toxicity testing. On the first day of the toxicity testing, the rats in seven groups received a subplantar injection of 0.1 mL of complete Freund’s adjuvant reagent on their left hind legs to induce rheumatoid arthritis or subchronic inflammatory conditions (Noh et al. 2021).

The toxicity test was observed for 16 days, followed by measuring body weight on day 17. On days 16 to 29, the rats received oral treatment at predetermined doses. Two experimental groups were administered exclusively with sappanwood extract and red ginger extract, respectively. Three groups of rats received a
Combination of sappanwood and red ginger extracts in 0.5% carboxymethylcellulose orally with varying doses, i.e., F1 (30 mg/200 g BW of sappanwood extract and 30 mg/200 g BW of red ginger extract), F2 (60 mg/200 g BW of sappanwood extract and 30 mg/200 g BW of red ginger extract), and F3 (30 mg/200 g BW of sappanwood extract and 60 mg/200 g BW of red ginger extract). As a comparison, the three other groups served as the positive control, negative control, and normal groups (Martina et al., 2019). The positive control group received diclofenac sodium in 0.5% carboxymethylcellulose at a dose of 2.7 mg/200 g BW and a combination of 30 mg/200 g BW of each extract (Martina et al., 2019). The negative control group only received 0.5% carboxymethylcellulose. Lastly, the normal group did not receive any treatment (Martina et al., 2019, Amalia et al., 2021).

**RESULTS**

On day 15, the plantar condition of the rats’ left legs showed that arthritis had developed with symptoms of swelling of the feet, redness of the toes, and changes in the shape of the sole. All of the animals involved in the experiment survived until the day of euthanasia. The body weight of the rats in each group was measured on seven occasions: prior to induction with complete Freund’s adjuvant on day 1 and between days 15 and 30, as detailed in Table 1.

Table 2 shows that significant weight gain was observed among rats in the normal group, with a maximum increase of 19.0893%, followed by the sappanwood extract group at 17.3174%. In the groups treated with the combination of extracts (F1, F2, and F3), the percentage of body weight gain on the last day of measurement was 7.7660%, 7.6299%, and 14.4067%, respectively. The results were compared between the treatment and normal groups. There was no statistically significant difference (p>0.05) observed in the average body weight changes.

**Figure 1. Toxicity testing of extracts using the adjuvant-induced arthritis method.**

Body weight gain in the rats was the first sign of toxicity. The rats’ body weight was measured on days 1, 15, 18, 21, 24, 27, and 30. Changes in body weight compared to the initial body weight (day 1) were expressed as a percentage using an analytical balance with the following formula (Sunil et al., 2013):

\[ \text{Weight gain percentage} = \frac{W_b - W_a}{W_b} \times 100\% \]

Note:
- \( W_a \): body weight before FCA injection (g)
- \( W_b \): body weight during the treatment period (g)

The second sign of toxicity was identified according to the assessment of organ weight. Quantitative data on the rats’ vital organs (liver, kidney, and spleen) were assessed by weighing the organs of euthanized animals using an analytical balance. The following formula was used for determining the relative organ weight, including the liver, kidney, and spleen (Attanayake et al., 2013):

\[ \text{Relative organ weight} = \frac{W_o}{W_b} \times 100\% \]

Note:
- \( W_o \): Absolute organ weight (g)

The third sign of toxicity was evaluated using histopathological assessment via hematoxylin-eosin (HE) staining. Observation of the liver, kidney, and spleen tissue was performed using a light microscope with the entire field of view and a magnification of 40X and 400X. The observations showed cell degeneration and necrosis in the kidneys, hydropic and fatty degeneration and necrosis in the hepatocytes of the liver, and bleeding and necrosis in the spleen (Fitmawati et al., 2018, Hidayati et al., 2018, Jannah & Budijastuti, 2022).

The data obtained from this study were statistically analyzed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, N.Y., USA). Statistical significance among the experimental groups was assessed through one-way analysis of variance (ANOVA) at 5% confidence intervals. The results were expressed as the mean ± standard deviation. A significance value below 0.05 (p<0.05) was considered significantly different (Amalia et al., 2021).
Table 1. Measurement of the rats' body weight in each group.

<table>
<thead>
<tr>
<th>Samples</th>
<th>D1</th>
<th>D15</th>
<th>D21</th>
<th>D27</th>
<th>D30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>187.0</td>
<td>189.7</td>
<td>193.5</td>
<td>193.0</td>
<td>193.7</td>
</tr>
<tr>
<td>Positive</td>
<td>187.2</td>
<td>189.1</td>
<td>193.2</td>
<td>193.0</td>
<td>191.7</td>
</tr>
<tr>
<td>Sappanwood</td>
<td>187.5</td>
<td>190.7</td>
<td>192.2</td>
<td>193.0</td>
<td>190.0</td>
</tr>
<tr>
<td>Red ginger</td>
<td>224.2</td>
<td>224.5</td>
<td>230.2</td>
<td>231.2</td>
<td>230.7</td>
</tr>
<tr>
<td>F1</td>
<td>230.4</td>
<td>230.7</td>
<td>231.3</td>
<td>234.2</td>
<td>231.6</td>
</tr>
<tr>
<td>F2</td>
<td>192.7</td>
<td>193.0</td>
<td>193.2</td>
<td>192.2</td>
<td>193.0</td>
</tr>
<tr>
<td>F3</td>
<td>198.2</td>
<td>199.2</td>
<td>194.0</td>
<td>198.2</td>
<td>197.5</td>
</tr>
<tr>
<td>Normal</td>
<td>161.7</td>
<td>168.2</td>
<td>191.2</td>
<td>191.0</td>
<td>195.7</td>
</tr>
</tbody>
</table>

Table 2. Body weight increase of the rats in each group (%).

<table>
<thead>
<tr>
<th>Samples</th>
<th>D1</th>
<th>D15</th>
<th>D21</th>
<th>D27</th>
<th>D30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0.16</td>
<td>0.62</td>
<td>1.29</td>
<td>2.84</td>
<td>7.84</td>
</tr>
<tr>
<td>Positive</td>
<td>1.21</td>
<td>7.90</td>
<td>1.10</td>
<td>2.68</td>
<td>6.74</td>
</tr>
<tr>
<td>Sappanwood</td>
<td>1.47</td>
<td>7.82</td>
<td>2.09</td>
<td>4.84</td>
<td>11.40</td>
</tr>
<tr>
<td>Red ginger</td>
<td>8.54</td>
<td>10.94</td>
<td>11.99</td>
<td>12.73</td>
<td>16.73</td>
</tr>
<tr>
<td>F1</td>
<td>0.80</td>
<td>3.28</td>
<td>2.91</td>
<td>5.67</td>
<td>7.90</td>
</tr>
<tr>
<td>F2</td>
<td>0.45</td>
<td>0.03</td>
<td>5.70</td>
<td>4.90</td>
<td>9.99</td>
</tr>
<tr>
<td>F3</td>
<td>5.66</td>
<td>7.91</td>
<td>9.41</td>
<td>11.82</td>
<td>12.890</td>
</tr>
<tr>
<td>Normal</td>
<td>15.99</td>
<td>15.66</td>
<td>15.99</td>
<td>17.30</td>
<td>18.40</td>
</tr>
</tbody>
</table>

The liver, kidney, and spleen weights were measured to observe any potential effects of the extracts, as the organs are the parameters for assessing toxic effects. The statistical test using one-way ANOVA showed that the relative organ weights of the rats' liver, kidney, and spleen were significant (p>0.05). As seen in Table 3, measurement of the organ weight between the treatment and control groups showed that there was no significant difference.

The histopathology of vital organs was also observed to determine any potential effects of the extracts. At the end of the 30-day toxicity testing, a histopathological examination was conducted on the liver, kidney, and spleen of the rats in all groups. The histopathological test assessed the presence of cell degeneration and necrosis in the kidney, hepatocytes and necrosis in the liver, as well as bleeding and necrosis in the spleen. There was no difference in the histopathological characteristics of the liver, kidney, and spleen among the rats in each experimental group, as presented in Table 4.

Table 3. Organ weight index of the rats' liver, kidney, and spleen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>4.27</td>
<td>0.90</td>
<td>0.16</td>
</tr>
<tr>
<td>Positive</td>
<td>4.32</td>
<td>0.83</td>
<td>0.17</td>
</tr>
<tr>
<td>Sappanwood</td>
<td>4.11</td>
<td>0.69</td>
<td>0.10</td>
</tr>
<tr>
<td>Red ginger</td>
<td>5.00</td>
<td>2.29</td>
<td>0.26</td>
</tr>
<tr>
<td>F1</td>
<td>4.53</td>
<td>0.80</td>
<td>0.15</td>
</tr>
<tr>
<td>F2</td>
<td>4.12</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>F3</td>
<td>4.43</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>Normal</td>
<td>4.15</td>
<td>0.13</td>
<td>0.08</td>
</tr>
</tbody>
</table>

As shown in Figure 2, the most severe damage to the kidney organs was observed in the negative control group. Rats in the negative control group that did not receive any drugs exhibited 40% organ damage. In the treatment groups, the F3 group experienced the lowest rate of organ damage, at 15%. It was followed by the F1 and F2 groups, with an organ damage rate of 20%. The groups that received sappanwood and red ginger extracts exclusively, however, suffered 25% organ damage.

Table 4. Histopathological examination of the rats’ liver, kidney, and spleen.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Moderate hydropic degeneration (55%)</td>
<td>Moderate cell degeneration (40%)</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>Mild hydropic degeneration (30%)</td>
<td>Mild cell degeneration (30%)</td>
<td>0</td>
</tr>
<tr>
<td>Red ginger</td>
<td>Mild hydropic degeneration (30%)</td>
<td>Mild cell degeneration (25%)</td>
<td>0</td>
</tr>
<tr>
<td>Sappanwood</td>
<td>Mild hydropic degeneration (25%)</td>
<td>Mild cell degeneration (20%)</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>Mild hydropic degeneration (25%)</td>
<td>Mild cell degeneration (20%)</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td>Mild hydropic degeneration (25%)</td>
<td>Mild cell degeneration (15%)</td>
<td>0</td>
</tr>
<tr>
<td>F3</td>
<td>Mild hydropic degeneration (20%)</td>
<td>Mild cell degeneration (15%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3 exhibits the reading of the liver organs. The negative control group that did not receive any drugs suffered the most significant damage, with a rate of 55%. The F3 group experienced the lowest rate of organ damage (20%) among the treatment groups, followed by the F2 and F3 groups (25%) as well as the sappanwood, red ginger, and positive control groups (30%). Meanwhile, in the reading of the spleen organs, there was no organ damage.

DISCUSSION

Several studies have reported that sappanwood contains phytochemical constituents. Some of the phytochemical constituents are xanthone, coumarin, chalcone, flavone, homoisoflavonoid, brazilin, brazilein, campesterol, stigmasterol, and β-sitosterol (Nirmal et al. 2015; Bukke et al. 2015). Brazilin is the main phytochemical constituent in sappanwood. It has been reported to have pharmacological activities useful in treating rheumatoid arthritis, including anti-inflammatory, analgesic, antioxidant, and anticonvulsant activities (Jung et al. 2015, Kim et al. 2015).
Figure 2. The results of microscopic observations on the kidney organs.
Red ginger contains several bioactive compounds, i.e., 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, 10-shogaol, zingerone, 6-paradol, 6-dehydrogingerdione, 10-dehydrogingerdione, 6-gingerdione, 10-gingerdione, and 1-dehydro-6-gingerol. The pharmacological effects of red ginger include analgesic, anti-inflammatory, hepatoprotective, nephron protector, and antioxidant properties, which are useful in treating rheumatoid arthritis (Mbueng & Kuete 2017, Ezzat et al. 2018).

*Rattus norvegicus* rats injected with complete Freund's adjuvant were used to evaluate the toxicity of the combination of sappanwood and red ginger extracts. Significant weight changes in the body and internal organs are considered a sensitive indicator of exposure to toxic substances. Due to their sensitivity in predicting toxicity and correlation with organ histopathological changes, the organ weight of the liver, kidney, and spleen can determine toxic effects. Increased liver, kidney, and spleen weight indicates organ hypertrophy, directly indicating chemical or biological toxicity (Amna et al. 2013, Ping et al. 2013). In this study, there was no organ hypertrophy in all test groups.

The toxic effects of xenobiotics can be seen in the liver and kidneys. The liver plays a role in drug
metabolism, transportation, and the clearance of foreign substances (Corsini & Bortolini 2013). The kidney is the main organ for drug secretion. While the spleen is essential in regulating the body's immunity, damage to the organ can cause immune disorders and splenomegaly (Bronte & Pittet 2013, Wang et al. 2019, Lees et al. 2020). In this study, the microscopic examination revealed no significant changes according to the observed parameters. Organ preparations observed under a light microscope in all fields of view did not indicate any adverse effects in the rats treated with extracts.

Damage to the kidney can be caused by toxic substances that enter the body. The primary function of the kidney is to excrete the remnants of the digestive system, which can be identified through histological structural changes, including cell degeneration and necrosis. Cell degeneration is an abnormality in cells that occurs due to light injury, which affects the structure of the cell. It causes disruptions to metabolic processes in the body. The destruction of proximal tubular epithelial cells is a symptom of cell necrosis, a form of cell death (Miller & Zachary 2017).

Viral infections can lead to the growth of benign or, more often, cancerous viruses that cause histological changes in the kidney. Due to the large number of compounds entering the tubules, the malignant effect may be heightened. This can cause the cells in the kidney to undergo necrosis. Various factors that can induce this condition include high levels of toxins such as phosphorus, poisonous mushrooms, and arsenic (Suhi et al. 2013).

Hepatic damage is indicated by two histological changes: hepatocytes and necrosis. The two types of damage in hepatocytes are hydropic degeneration and fatty degeneration. Hydropic degeneration is a condition in which cell damage is characterized by swelling of the cytoplasm, which is an excessive accumulation of fluid due to the inability of cells to maintain homeostasis (Berata et al. 2015). Fatty degeneration is the final stage of hydropic degeneration, which has suffered permanent (irreversible) damage. Fatty degeneration occurs when fat accumulates in the cytoplasm of liver cells (Sangi 2016). If the cell condition worsens over time, it can cause permanent damage. Furthermore, the cells may experience death or necrosis, which can cause symptoms such as nuclear chromatin. Nuclear changes that may happen include agglomeration (pyknosis), rupture (karyorrhexis), and dissolution (karyolysis) (Kumar et al. 2013).

Strength and limitations

This study evaluated the toxic effects caused by the combination of sappanwood and red ginger ethanol extracts for the treatment of rheumatoid arthritis in rat models. However, due to the limited dose of extracts in the combination formula, this study was unable to provide more representative findings for determining the optimal combination dose. Further research is required to determine the optimal dose for the safe treatment of rheumatoid arthritis in humans.

CONCLUSION

There was no fatality recorded during toxicity testing by administering the combination of sappanwood and red ginger ethanol extracts to rats injected with complete Freund's adjuvant. The measurement of body weight and organ weight (i.e., the liver, kidney, and spleen) did not reveal any toxic effects. Additionally, the observed organs did not show any histopathological changes in rats treated with the extract.

Acknowledgment

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Conflict of interest

None.

Ethical consideration

This study obtained ethical clearance from the Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, with registration No. 147/EC/KEPK/FKUA/2022 on 8/8/2022.

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Author contribution

NAS collected and analyzed the data and wrote the manuscript. T collected the data and wrote the manuscript. S collected and analyzed the data. FIS also collected and analyzed the data.

REFERENCES


