Research Article

Evaluation of Antioxidant Activity and Acute Toxicity of Clausena excavata Leaves Extract

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Clausena excavata (Lour.), locally known as “Kemantu hitam,” is a common plant in Malaysian folklore medicine. This study evaluated the antioxidant properties of the solvent extracts of C. excavata leaves and determined the acute toxicity of methanolic extract C. excavata (MECE) leaves in Sprague-Dawley rats. Harvested leaves were dried and subjected to solvent extraction using petroleum ether, chloroform, ethyl acetate and methanol in succession. The antioxidant activity of each extract was determined using the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The total phenolic content (TPC) and total flavonoids content (TFC) were estimated by Folin-Ciocalteu and ethanolic aluminium chloride method, respectively. The chloroform extract was found to be highest in flavonoid content, while the methanolic extract showed the highest TPC and antioxidant activity. There was no mortality in rats treated with MECE leaves even at a high dose of 5000 mg/kg body weight. However, the MECE leaves produced mild to moderate pathological changes in the liver and kidneys, shown by mild degenerative changes and leucocyte infiltration. The extract did not affect the haematological parameters or relative weights of the liver or kidneys. Overall, the MECE leaves have potent antioxidant activity and are presumed safe to be used orally as a health-promoting product at low to moderate doses.

1. Introduction

Free radicals are harmful by-product of cellular oxidative phosphorylation and energy production. These radicals damage various intracellular macromolecules to include DNA, protein, and lipids [1]. Antioxidants have the ability to prevent oxidative damage and inhibit inflammatory conditions by nullifying the activities of free radicals [2]. Currently, the search for plant sources of antioxidants is gaining momentum with Clausena excavata (Rutaceae family) among the plants targeted. C. excavata is a medicinal plant widely distributed in Southeast Asia and is known by unique local names, such as Chamat in Thailand and Jia huang pi in China. In Malaysia the plant is locally known as “Cherek hitam” and “Kemantu hitam.” The leaves of the plant are used in folklore medicine for the treatment of several illnesses such as malaria, headache, abdominal pain, dysentery, pulmonary tuberculosis, diarrhoea, cold, wound, snake-bite, and poisoning. Recent studies showed that the plant also possessed immune-modulatory [3], analgesic [4], anti-inflammatory, antivirus, anticancer [5], antioxidant [6], antimycobacterial [7], and antifungal [8] activities. C. excavata has been reported to exhibit one of the highest beneficial biological activities among Clausena genus [9]. These activities are attributed to its high phenolic compounds such as furanocoumarins and flavonoids. The plant also contains other pharmacologically active compounds like coumarin, carbazole alkaloid, and flavonoid glycosides [7, 10–12].

It is imperative that compounds from plants for human use are screened for potential toxicity, particularly for
repeated and prolonged applications. Toxicity following overdose of active principles from plant candidates poses limitations to its use, since it may result in deaths in some cases [13]. Although a recent study reported that repeated oral doses of the methanol extract of C. excavata stem [14] did not cause significant pathological changes in rats, there is currently a paucity of information on the safe use of the plant leaves. This present study aims to determine the antioxidant properties and the safety of oral application of methanol extract of C. excavata leaves in rats.

2. Materials and Methods

2.1. Collection, Identification, and Processing of Plant Material. C. excavata leaves were collected from Pendang, Kedah, Malaysia. The plant was identified and authenticated by Dr. Shamsul Khamis of the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia. The leaves were sorted, washed, and dried at room temperature for two weeks before being ground into powder and stored in air tight plastic bags.

2.2. Extraction of Plant Material. Extraction was done at room temperature using petroleum ether, chloroform, ethyl acetate, and methanol in succession. The extraction at a 1:5 dried plant weight to volume ratio began with petroleum ether for 3 days. The filtrate was collected and the residues were subject to further extraction with chloroform, ethyl acetate, and methanol, and the filtrate was collected after each solvent extraction. All filtrates were evaporated to dry under reduced pressure using a rotary evaporator at 45 to 50°C to obtain crude extracts.

2.3. In Vitro Assessment of Antioxidant Activity

2.3.1. 2,2-Diphenyl-1-picryl Hydrazyl Free Radical Scavenging Method. DPPH scavenging activity of C. excavata petroleum ether, chloroform, ethyl acetate, and methanol extracts were estimated using the method of Lim and Murti jaya [15] and Alnajar et al. [16]. Briefly, 195 μL DPPH reagent was added to 5μL of samples or quercetin standard. The mixtures were vigorously shaken and incubated in the dark for 2 hours at room temperature. Absorbance reading was done using a spectrophotometer at 515 nm against a blank.

2.3.2. Ferric Reducing Antioxidant Power Assay Method. Ferric-reducing antioxidant power (FRAP) of each fraction was estimated according to the method of Alnajar et al. [16] and Mayakrishnan et al. [17]. Briefly, the freshly prepared reaction mixture containing 25 mL of acetate buffer, 2.5 mL of 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution and 2.5 mL of FeCl₃·6H₂O was incubated for 5 minutes at 37°C water-bath and absorbance was measured using a spectrophotometer at 593 nm. Thirty microliter of extract and ninety microliter of distilled water were added to nine hundred microliters of FRAP working reagent and mixed before absorbance was measured at 593 nm.

2.3.3. Total Phenol Content. The total phenol content (TPC) of each fraction was determined as described earlier using Folin-Ciocalteu reagent and Gallic acid as a standard [16, 18]. Absorbance was measured spectrophotometrically at 750 nm and results were expressed as mg Gallic acid equivalent (GAE) per g of extract (mg GAE/g).

2.3.4. Total Flavonoid Content. Total flavonoid was estimated using the method developed by Adedapo et al. [19]. Briefly, 0.5 mL of the sample was added to 0.5 mL of 2% AlCl₃ dissolved in ethanol. After incubation for one hour at room temperature, the absorbance was measured at 420 nm. A calibration curve was prepared using (+) quercetin equivalent per gram (QE/g) extract.

2.3.5. Phytochemical Characterization of the Methanolic Extract of C. excavata. The LC-MS/MS analysis was carried out as described earlier by Mayakrishnan et al. [18], using an AB Sciex 3200 Q trap with Perkin Elmer UHPLC FX15.

2.4. Experimental Animals. Thirty-six adult male and female Sprague-Dawley rats, 8 to 10 weeks old, weighing between 165 and 200 g, were procured from the Animal House of the Faculty of Medicine, University of Malaya, Malaysia. The rats were maintained in clean stainless steel cages at an ambient temperature of 25 ± 2°C and fed standard rat/mouse pellet (Specialty Feeds, Glen Forrest, Western Australia) and water ad libitum for one week before experimentation. All studies performed were approved by the Institutional Animal Care and Use Committee, University of Malaya (ISB/22/007/2013/I111/SFA).

2.4.1. Animal Grouping and Acute Oral Toxicity Study. The Animals were subdivided into 2 groups, males (n = 16) and females (n = 16). The groups were designated as control male (CM), control female (CF), given 10% Tween-20 orally, and treatment groups, administered extract orally as follows: male 1 (M1, 2000 mg/kg body weight extract), male 2 (M2, 5000 mg/kg body weight extract), female 1 (F1, 2000 mg/kg body weight extract), and female 2 (F2, 5000 mg/kg body weight extract). The acute oral toxicity of the methanolic extract C. excavata (MECE) leaves was evaluated following Organization for Economic Cooperation and Development (OECD-423) guidelines [20] with slight modification. The M1 and F1 rats were each administered one dose of 2000 mg/kg orally and M2 and F2 rats were administered 5000 mg/kg body weight of the extract suspended in 10% Tween-20 (5 mL/kg). The rats were monitored for mortality for 48 hours and humanely euthanized after 14 days.

2.4.2. Blood Parameters. The rats were fasted overnight with free access to water. Blood samples were collected in plain and EDTA coated tubes. Serum was separated by centrifugation of whole clotted blood at 3000 r/min (Hettich, EBA 20) for 10 minutes and used to determine concentrations of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, creatinine (Cr), urea (BUN), sodium (Na⁺), potassium (K⁺),
Table 1: Total phenolic and flavonoid contents of *Clausena excavata* extracts.

<table>
<thead>
<tr>
<th>Extract content</th>
<th>Solvent</th>
<th>Methanol</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Petroleum ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC (mg GAE/g extract)</td>
<td>Methanol</td>
<td>522.0 ± 11.6</td>
<td>497.0 ± 7.1</td>
<td>373.0 ± 6.9</td>
<td>157.0 ± 7.5</td>
</tr>
<tr>
<td>TFC (mg QE/g extract)</td>
<td>Ethyl acetate</td>
<td>96.7 ± 2.0</td>
<td>142.5 ± 1.0</td>
<td>188.6 ± 3.0</td>
<td>25.8 ± 0.8</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

\(^{a,b,c,d}\)Mean ± SD with different superscripts are significantly different at \(P < 0.05\).

TPC = total phenolic content; TFC = total flavonoid content; GAE = Gallic acid equivalent; QE = quercetin equivalent.

and chloride (Cl\(^-\)). Non-coagulated blood samples collected in EDTA tubes were used for hematological evaluation; red blood cell count (RBC), hemoglobin (Hb) concentration, and white blood cell count (WBC).

2.5. Histopathology. All the rats were humanely sacrificed and the gross pathology of internal organs was evaluated. Tissue samples of the liver and kidney were collected from all the experimental groups, weighed, and then fixed in 10% buffered formalin. The fixed tissues were embedded, sectioned, and stained with hematoxylin and eosin stain and observed under light microscopy.

2.6. Statistical Analyses. All data obtained from this study were analysed using SPSS computer statistical software package, version 19, and expressed as mean ± standard deviation (SD). Significance within groups was determined using one-way ANOVA and Tukey's posttest analysis at \(P < 0.05\) for significance.

3. Results

Extraction of air-dried ground *C. excavata* leaves with petroleum ether, chloroform, ethyl acetate, and methanol produces extract yield (wt/wt) of 1.56, 2.57, 0.38, and 0.94%, respectively.

3.1. Ferric Reducing Antioxidant Power. The increase in FRAP absorption suggested that the extract has free radical scavenging properties. The study showed that methanolic and ethyl acetate extracts at 3 mg/mL possessed the highest antioxidant activity (Figure 1).

3.2. DPPH Free Radical Scavenging of *Clausena excavata* Extracts. The DPPH radical scavenging activities of different extracts of *C. excavata* leaves are presented in Figure 2. The study showed that the methanolic extract possessed the highest radical scavenging activity.

3.3. Total Phenolic and Flavonoid Contents of *Clausena excavata* Extracts. Table 1 shows the phenolic and flavonoid content of the different crude leaf extracts. The MECE leaves had the highest amount of phenolic compounds, while the chloroform extract had the highest total flavonoid content.

3.4. Phytochemical Characterization of the Methanolic Extract of *C. excavata*. Based on LCMS/MS analysis, the MECE contains pipecolic acid, myricetin glucoside conjugate, quercetin-rhamnose-hexose-rhamnose, kaempferol conjugate, phenolic acid, flavonoids, furocoumarin, and 8-geranyloxypсорalen (Table 2).

3.5. Acute Oral Toxicity Study. There were no mortality or behavioural abnormality in rats treated orally with 2000 and 5000 mg/kg body weight of the methanolic fraction of the plant extract showing that the oral LD50 of MECE leaves...
Table 2: Compound identified from MECE via LCMS/MS analysis.

<table>
<thead>
<tr>
<th>Retention time</th>
<th>Molecular weight</th>
<th>MS/MS fragments ions</th>
<th>Tentative Identification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.69</td>
<td>756.2</td>
<td>(–) 755, 300, 271.0, 255.1, 179.0, 151.0</td>
<td>Quercetin-rhamnose-hexose-rhamnose</td>
<td>Chua et al., 2011 [21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kachlicki et al., 2008 [22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Del Rio et al., 2004 [23]</td>
</tr>
<tr>
<td>3.9</td>
<td>772.2</td>
<td>(–) 771, 316, 271.1, 179.0, 151.0</td>
<td>Myricetin glucoside Conjugate Myricetin 3-O-rhamnosyl-glucoside 7-O-rhamnoside</td>
<td>Chua et al., 2011 [21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kachlicki et al., 2008 [22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Del Rio et al., 2004 [23]</td>
</tr>
<tr>
<td>4.3</td>
<td>740.2</td>
<td>(–) 739.2, 284.1, 255.0, 179.0, 151.0</td>
<td>Kaempferol conjugate</td>
<td>Chua et al., 2011 [21]</td>
</tr>
<tr>
<td>7</td>
<td>179.3</td>
<td>(+) 161.2, 135.1, 133.1</td>
<td>Caffeic acid</td>
<td>Chua et al., 2011 [21]</td>
</tr>
<tr>
<td>6.72</td>
<td></td>
<td>(+) 77.0, 91.0, 95.0, 105.0, 119.1, 134.1, 148.3, 149.2, 156.3, 161.3, 174.4, 177.3, 179.3, 190.4, 202.4, 204.4, 206.4, 218.4, 221.4, 230.4, 232.4, 243.4, 245.4, 246.4, 256.4, 270.4, 284.4, 288.4, 298.4, 316.4, 326.4, 344.4, 362</td>
<td>8-Geranyloxy psoralen</td>
<td></td>
</tr>
<tr>
<td>5.95</td>
<td></td>
<td>(+) 77.91, 91.0, 95.0, 111.0, 119.0, 133.1, 147.2, 149.2, 161.2, 177.2, 179.3, 191.3, 203.3, 243.3, 245.4, 247.3, 255.3, 271.4, 273.4, 289.4, 291.4, 313.4, 323.4, 331.4, 341.4, 359.4, 377.4</td>
<td>Unidentified furoucoumarin</td>
<td></td>
</tr>
<tr>
<td>5.27</td>
<td></td>
<td>(+) 117.1, 134.2, 146.2, 147.2, 150.2, 160.3, 163.3, 180.3, 202.3, 246.4, 473.4, 491.5, 509.5, 527.6</td>
<td>Unidentified flavonoids</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td></td>
<td>(+) 119.0, 134.1, 146.2, 147.2, 162.3, 164.3, 180.3, 201.3, 229.3, 245.3, 240.4, 246.4, 294.4, 341.3, 404.4, 491.4, 523.3, 531.5, 541.6, 559.6</td>
<td>Unidentified flavonoids</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td></td>
<td>(+) 146.2, 179.3, 194.4, 203.3, 247.3, 292.4, 324.4, 324.4, 370.4, 388.4, 470.4, 557.6, 735.7</td>
<td>Unidentified flavonoids</td>
<td></td>
</tr>
<tr>
<td>4.72</td>
<td></td>
<td>(–) 107.0, 123.0, 137.1, 139.1, 163.1, 179.1, 195.2, 197.2, 241.1</td>
<td>Unidentified phenolic acid</td>
<td></td>
</tr>
</tbody>
</table>

was greater than 5000 mg/kg body weight. Metabolic studies showed that body weight was lower in the group treated with high doses of extract. The kidneys and liver of rats treated with the extract did not show gain in relative organ weights (Table 3).

3.6. Serum Biochemistry and Hematology

3.6.1. Liver Function Test. The liver function tests showed slightly increased serum enzyme concentrations in both male and female rats fed MECE leaves. M2 had elevated serum ALP, AST, and ALT concentrations, while in F2, only the ALT and ALP concentration increased slightly ($P < 0.05$). Total serum bilirubin concentration increased in both F1 and F2 rats (Table 4).

3.6.2. Renal Function Test. Serum creatinine concentrations of M1 and M2 rats were significantly ($P < 0.05$) lower than that of NC male rats. However, the BUN concentration of the M2 rats were not significantly ($P > 0.05$) different from that of the control. The mean BUN concentration of F2 rats

Table 3: Relative weights of selected organs after administration of a single dose methanolic extract of Clausena excavata.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sex</th>
<th>MECE treatment (mg/kg body weight)</th>
<th>Untreated</th>
<th>2000</th>
<th>5000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative organ weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Male</td>
<td>2.33 ± 0.10</td>
<td>2.55 ± 0.19</td>
<td>2.58 ± 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>2.60 ± 0.07</td>
<td>2.78 ± 0.14</td>
<td>2.82 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Male</td>
<td>0.68 ± 0.01</td>
<td>0.74 ± 0.02</td>
<td>0.70 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.55 ± 0.01</td>
<td>0.61 ± 0.02</td>
<td>0.63 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD. MECE = methanolic extract Clausena excavata; untreated = rats given normal diet.
Table 4: Effect of methanolic extract of Clausena excavata extract on liver biochemical parameters.

<table>
<thead>
<tr>
<th>Treatment group (MECE leaves)</th>
<th>ALT (U/I)</th>
<th>AST (U/I)</th>
<th>ALP (U/I)</th>
<th>Albumin (g/dL)</th>
<th>Bilirubin (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>57.0±2.2</td>
<td>196.0±3.6</td>
<td>1777±75</td>
<td>12.2±0.3</td>
<td>2.19±0.01</td>
</tr>
<tr>
<td>M1 (2000 mg/kg b.wt)</td>
<td>59.1±2.3</td>
<td>183.4±11.7</td>
<td>228.2±27.0</td>
<td>13.0±0.3</td>
<td>2.73±0.17</td>
</tr>
<tr>
<td>M2 (5000 mg/kg b.wt)</td>
<td>64.7±4.3</td>
<td>256.6±20.4</td>
<td>248.6±23.4</td>
<td>13.8±0.3</td>
<td>2.42±0.22</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>46.8±2.5</td>
<td>165.1±6.2</td>
<td>143.3±10.7</td>
<td>14.7±0.7</td>
<td>1.92±0.03</td>
</tr>
<tr>
<td>F1 (2000 mg/kg b.wt)</td>
<td>47.8±2.1</td>
<td>181.6±15.9</td>
<td>109.0±5.2</td>
<td>15.2±0.2</td>
<td>2.76±0.19</td>
</tr>
<tr>
<td>F2 (5000 mg/kg b.wt)</td>
<td>53.7±5.2</td>
<td>195.1±26.4</td>
<td>193.2±19.3</td>
<td>14.8±0.3</td>
<td>2.83±0.16</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

For each sex, means within column with different superscript are significant at \( P < 0.05 \).

ALT = alanine transaminase; AST = aspartate transaminase; ALP = alkaline phosphatase.

MECE = methanolic extract Clausena excavata; CM = untreated control male; CF = untreated control female; M1, M2, F1, and F2 are treatment groups; b.wt = body weight.

Table 5: Effects of methanolic extract of Clausena excavata on kidney biochemical parameters.

<table>
<thead>
<tr>
<th>Treatment Group (MECE leaves)</th>
<th>Sodium (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>144.3±0.7</td>
<td>4.90±0.10</td>
<td>104.8±0.1</td>
<td>6.42±0.34</td>
<td>635.6±21.4</td>
</tr>
<tr>
<td>M1 (2000 mg/kg b.wt)</td>
<td>145.8±0.2</td>
<td>4.66±0.06</td>
<td>103.6±1.0</td>
<td>4.61±0.21</td>
<td>379.6±48.7</td>
</tr>
<tr>
<td>M2 (5000 mg/kg b.wt)</td>
<td>146.2±0.4</td>
<td>5.00±0.25</td>
<td>105.0±0.4</td>
<td>5.90±0.21</td>
<td>415.5±25.7</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>144.4±0.7</td>
<td>4.70±0.06</td>
<td>104.3±1.1</td>
<td>5.79±0.17</td>
<td>576.8±31.1</td>
</tr>
<tr>
<td>F1 (2000 mg/kg b.wt)</td>
<td>144.2±0.7</td>
<td>4.64±0.29</td>
<td>104.4±0.4</td>
<td>5.81±0.24</td>
<td>424.6±19.3</td>
</tr>
<tr>
<td>F2 (5000 mg/kg b.wt)</td>
<td>146.1±0.9</td>
<td>4.90±0.15</td>
<td>106.8±0.6</td>
<td>7.70±0.29</td>
<td>561.6±29.8</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

For each sex, means within column with different superscript are significant at \( P < 0.05 \).

MECE = methanolic extract Clausena excavata; CM = untreated control male; CF = untreated control female; M1, M2, F1, and F2 are treatment groups; b.wt = body weight.

Table 6: Effects of methanolic extract of Clausena excavata extract on hematological parameters in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Untreated</th>
<th>MECE leaves treatment (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>5000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relative organ weight (g)</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte (10¹²/L)</td>
<td>Male</td>
<td>8.44±0.11</td>
<td>8.04±29.00</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>7.80±0.03</td>
<td>7.79±0.03</td>
</tr>
<tr>
<td>Leukocyte (10⁹/L)</td>
<td>Male</td>
<td>8.46±0.21</td>
<td>9.94±0.37</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6.55±0.96</td>
<td>6.20±0.71</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>Male</td>
<td>13.80±0.20</td>
<td>14.02±0.31</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13.10±0.12</td>
<td>12.63±0.48</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

MECE = methanolic extract Clausena excavata; untreated = rats given normal diet without MECE leaves treatment.

was significantly \( P < 0.05 \) higher than that of control by 24%. Similarly, serum Na⁺, K⁺, and Cl⁻ concentrations were variably elevated among the male and female treatment groups (Table 5).

3.6.3. Hematological Parameters. The erythrocyte and leukocyte counts and Hb concentration did not significantly \( P > 0.05 \) vary between the control and experimental rats following administration of C. excavata extract (Table 6).

3.7. Histopathology. Microscopic evaluation of tissue sections of M1 and M2 rats did not show abnormal histopathological changes. However, kidney sections of F1 and F2 rats showed histopathological changes that varied according to dose level.
These changes include mild tubular epithelial degeneration, increased Bowman’s capsular space as a result of constriction of the glomerulus, mild congestion, cast-like materials within the renal tubular spaces, and mild leukocytic infiltration typified by lymphocyte and neutrophil infiltration in the renal interstitium (Figure 3).

Microscopic examination of the liver sections of M1, F1 and M2, F2 rats showed central vein congestion, cloudy hepatocytes swelling, and diffuse infiltration by Kupffer cells. Multifocal areas of mild perilobular necrosis coupled with neutrophil infiltration were observed in some sections of the liver tissue of the male rats given a high dose of extract, indicating that the effect of the high dose MECE leaves was more severe in the male than female rats (Figure 4).

4. Discussion

Over the past years, a handful of pharmacologically active constituents of plants belonging to the family Rutaceae have been identified, these include methoxyflavones, furoquinoline alkaloids, furanone-coumarins, and flavonoids. These
constituents were shown to have vital effects on inflammation, viral infections, and digestive system disorders such as gastritis and enteritis [25–27]. Phytochemical screening showed that these plants contain furanone-coumarins, flavonoids, and glycosides [12, 28]. *C. excavata*, in particular, possesses a wide range of pharmacological properties which range from antioxidant properties [6], hyperglycemic, antirhinitis, anti-inflammatory [29], anti-nociceptive [4], antiviral, anti-cancer and anti-fungal activities [8, 30]. To determine the antioxidant activities of *C. excavata*, most of these studies used the bark and stem of the plant, not the leaves. In the present study, the MECE leaves were observed to strongly reduce FeCl₃ and showed the scavenging activity for DPPH, at potency close to that of quercetin. This observation suggests that the *C. excavata* extract, by its phenolic and flavonoid contents, may be able to donate electrons to the free radicals [31, 32]. It is well-documented that phenolic compounds are among principal and effective antioxidant constituents in plant foods [33]. Thus quantification of phenolic contents of an extract would be essential to determine
its antioxidant activity. Previous studies have reported that MECE leaves with high phenolic content inhibit lipid peroxidation [6, 29]. Flavonoids of the MECE leaves with their wide spectrum of medicinal actions are the major polyphenols in the scavenging of oxidizing species like hydroxyl radical, superoxide anion, or peroxyl radicals [34] (Alnajara et al., 2012). The chloroform extract of C. excavatawas shown in the current study to have the highest concentration of flavonoids, which is comparable with those reported by others [35, 36]. Thus it is not surprising that C. excavata, with its potent phenolic antioxidant, is effective in the treatment of various oxidative stress-related chronic diseases.

The MECE leaves showed good antioxidant activity and were subsequently selected for phytochemical screening and animal studies. Initial investigation using LCMS/MS confirmed that the MECE leaves are rich in phenolic/flavonoids-based compounds [11]. Notably, these compounds in the MECE leaves possess several biological activities; for example, coumarin has been used as an anti-inflammatory, anti-cholinesterase, antioxidant, and anticancer compound [25, 27, 37]; quercetin glycoside has been used as a hepatoprotective compound in CCl4-induced hepatic damage, while kaempferol and myricetin glucosides have been shown to have high anti-inflammatory and antioxidant activities [38, 39].

The toxicity of MECE leaves in rats was evaluated by an acute 14-day study. No mortality or symptom of toxicity was observed in the rats in the study. The LD50 of MECE leaves in both sexes of rats were greater than 5000 mg/kg body weight. This is in accordance with an earlier study [14], which reported that oral LD50 of the methanolic extract from the stem of C. excavata is more than 5000 mg/kg body weight in both sexes in rats. Therefore, the high LD50 of MECE leaves indicates that the extract is relatively safe, especially for oral administration. According to Ecobichon [40] and OECD, any test compound that does not produce adverse effects at a dose exceeding 5 g/kg body weight is considered nontoxic.

Change in body weight and relative organ weight of animals is an indicator of adverse effect of administered drugs or chemicals [41, 42]. The organs of these animals tend to swell or become damaged, which will subsequently alter their organ-to-body weight ratios upon exposure to toxic substances. In this study, the body weights of rats (male and female) treated with MECE leaves increased gradually but insignificantly (P > 0.05) compared with the control rats. Similarly, the relative organ weights showed no significant (P > 0.05) difference between test and control groups.

Increased serum creatinine is a good indicator of abnormal kidney function while simultaneous increases in serum AST and ALT concentrations are associated with hepatopathy [43]. Our study showed significant (P < 0.05) but variable increases in serum enzymes in male and female rats treated with MECE leaves. Serum bilirubin concentration was significantly (P < 0.05) elevated in MECE leaves-treated female rats only. In this case, increase in ALP level is associated with increased hepatic synthesis, in the presence of increasing biliary pressure and obstruction in the normal bile flow as a result of damage or inflammation [36]. In male rats treated with low dose MECE leaves, we observed that the serum bilirubin and ALP concentrations were not significantly (P > 0.05) different from that of the controls, an indication that the liver has not been compromised by the treatment.

Significant increase in BUN without abnormality in serum creatinine concentration in female rats treated with a high dose MECE leaves is indicative of hemococoncentration and/or high dietary protein rather than kidney impairment [44, 45]. This assertion is based on the fact that serum creatinine is a more accurate marker of glomerular filtration and kidney function than urea. Serum Na+ was slightly elevated in both treated male and female rats, while K+ was elevated in the male rats only and CT in female rats treated with high dose MECE leaves only. Since the concentrations of these electrolytes in blood are generally governed by diet and water intake, these changes do not reflect adverse effect of MECE leaves.

Although MECE leaves-treatment of rats produced mild to moderate pathological changes in the kidneys and liver, neither mortality nor symptoms of toxicity at the doses used in this study were observed; thus the extract is presumed safe for oral application.

5. Conclusion

The MECE leaves contain pharmacologically active compounds including flavonoids and phenolics, which are good antioxidants. The MECE leaves did not exhibit obvious toxicity at concentrations even at a high dose of 5000 mg/kg body weight. Thus the MECE leaves have potent antioxidant activity and are presumed safe to be used orally as health-promoting product at low to moderate doses.

Abbreviations

MECE: Methanolic extraction Clausena excavata
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
Cr: Creatinine
BUN: Blood urea nitrogen
OECD: Organization for Economic Cooperation and Development
CDL: Clinical Diagnostic Laboratory
UMMC: University of Malaya Medical Centre
H&E: Haematoxylin and eosin
ANOVA: Analysis of variance
SD: Standard deviation.

Conflict of Interests

The authors declare that they do not have any conflict of interests regarding the publication of this paper.

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References


