Cytotoxicity and Oral Acute Toxicity Studies of Litsea glutinosa C. B (ROB) Stem Bark Ethanol Extract

Arunodaya Hosahalli Sumithregowda, Krishna Venkatarangaiah, Kumaraswamy Malleshappa Honnenahally, Vinaykumar Nagenahlall Manjunath

ABSTRACT

Background: Litsea glutinosa (Lauraceae) stem bark is widely used in folk medicine as a hepatoprotective, anti-diarrheal and anti-dysenteric drug but there is a lack of information about its toxicity. Objective: To evaluate cytotoxicity and acute toxicity of the stem bark ethanol extract (BEE). Materials and Methods: In vitro cytotoxicity of BEE was measured against breast adenocarcinoma, prostate, and colon carcinoma cell lines. In the acute toxicity tests, rats received oral doses of BEE as 1000, 2000, and 3000 mg/kg body weight. Mortality, signs of toxicity, body weight, food consumption, and gross findings were observed for 14 days. Blood samples were collected from anesthetized animals and used for hematological and biochemical parameters. Histopathological study was performed using liver and kidney samples. Results: The BEE does not show significant cytotoxic effect against the tested cell lines up to the range from 5 to 320 µg/ml. In acute toxicity study, also lethality was not observed up to 3000 mg/kg b.w. No significant differences were noticed in body and organ weights and histopathology examinations between the control and treated groups. Conclusion: This study authenticates stem BEE may contain bioactive compounds of potential therapeutic significance which are relatively safe from toxic effects, and evidences the medicinal use of this plant in folk medicine.

Key words: Acute toxicity, Litsea glutinosa, MTT assay, Breast adenocarcinoma cell line, Haematology.

INTRODUCTION

Medicinal plants from time immemorial have been the backbone of traditional medicine and are widely used to treat acute and chronic diseases. The demand for such medicines is increasing day by day for the management and treatment of various health problems. Despite this rapid growth, there is limited evidence for the effectiveness and toxicity of such medicines; much more needs to be done to validate the ethnopharmacological claims with an evidence base for phytomedicines, botanicals, and all-natural folklore-originated medicines. However, few studies have addressed the toxicity of natural plants, although many questions have been raised regarding their safety. The importance of plant species for therapeutic applications is well established but studies on certain plant-induced toxicity are scarce. However, the rationale for the utilization of medicinal plants has rested largely on long-term clinical experience with little or no scientific data on their efficacy and safety.

Chemotherapy is one of the potential treatments for prolonging the patient’s life. Almost 60% of anticancer drugs are of natural origin, such as plants (i.e., camptothecins, irinotecan, and vincristine) and microorganisms (i.e., bleomycin, dactinomycines, doxorubicin and mitomycin). However, many chemotherapeutic drugs are presently placed in a predicament of reduced therapeutic effect due to the problem of drug-resistance. Chemotherapeutic drugs also exert toxicity to normal cells, which in turn causes the unpleasant side effects to the patients. For these reasons, research and development of new classes of anticancer agents which exhibit efficient and selective toxicity in tumour cells are enticing increased attention.

Presently, herbal medicines are gaining interest because of their cost effective and eco-friendly attributes. L. glutinosa C.B. Rob is an evergreen tree species belonging to the family Lauraceae. The traditional practitioners residing near Bhadra Wild Life Sanctuary of the Western Ghats are using the stem bark extract to cure alcoholism-related liver disorders.

The leaves are aromatic and mucilaginous used in diarrhea and dysenter as well as for the treatment of wounds and bruises. The leaves and berries yield an essential oil which is used by traditional practitioners in the treatment of rheumatism. The previous investigators have reported the pres-
ence of phytoconstituents: Tannin, β-sitosterol, boldine, norboldine, laurotetanine, n-methyl laurotetanine, n-methylactinodaphnine, sebiferine, litseferine from the methanol extract of the bark. However, scanty data are available on the cytotoxicity and lethal toxicity of this species. But in traditional medicine the leaves and stem bark aqueous extract administered orally to the patients for diarrhea and dysentery. Considering ethnopharmacological applications of the plant, the investigation was undertaken to assess the toxic effects of BEE using male Albino rats and ATCC cancer cell lines.

MATERIALS AND METHODS
Plant materials
The stem bark of *L. glutinosa* was harvested from Kuvempu University campus, Karnataka, India, in December 2013 and was identified by Dr. Tariq Husain, Head and Scientist, Biodiversity and Angiosperm Taxonomy, National Botanical Research Institute, Lucknow, India, where the voucher specimen was deposited (No. 97294).

Preparation of *L. glutinosa* ethanol extract
The plant sample was ground to a coarse powder, subjected to sequential extraction using hot Soxhlet extraction technique. The extract was filtered through Whatman paper no. 1 and then concentrated using rotary flash evaporator (Buchi, Flawil, Switzerland). The yield of the ethanol extract was 23.7% based on dry weight. The dried residue of plant extract was resuspended in Mili-Q water for further biological assay.

Cytotoxicity assay
*In vitro* cytotoxic activity was performed to determine cell viability by measuring the metabolism of tetrazolium substrate MTT. The effect of BEE was assessed against breast adenocarcinoma cell line (MDA-MB-231), prostate cancer cell line (DU145) and colon carcinoma cell lines (HCT-116).

Cells were seeded into triplicate wells of 96-well microplate at a density of 4×10⁴ viable cells/ml. Cells were incubated with BEE at different concentrations ranging from 5-320 µg/ml along with a parallel control of 4×10⁻⁴ MTT (5 mg/10 ml of MTT in 1X PBS) in each well. The culture plates were fixed gently, incubated for 4 h. The blue formazan crystals being formed within cells were solubilized with 100 µl of DMSO and absorbance of blue formazan was determined at 590 nm in an automated plate reader. Percentage inhibition of the growth was calculated and expressed as mean ± SEM. IC₅₀ of the BEE on different cell lines were calculated from the concentration v/s percentage inhibition curves.

Experimental animals
Male Albino rats of Wistar strain, weighing about 160–220 g was used for the acute toxicology studies. The animals were acclimatized to laboratory conditions for 14 days prior to the experiments. The rats were maintained at a room temperature of 22–24°C, with 12 h light/dark cycle. During acclimatization, animals were housed in polycarbonate cages with a standard pellet diet and water ad libitum. The food pellets for the experimental animals were purchased from Scientist’s Choice Laboratory animal feed, Chennai, India. All procedures in this study were performed according to the guidelines of the CPCSEA (REG.NO.144/1999/CPCSEA/ddt:10/04/2000). The experimental protocol was approved by the Institutional Ethical Committee (Reg. No: NCP/IAEC/CL/242/2013-14).

Acute oral toxicity study
Acute toxicity test was performed according to the Organization of Economic Cooperation and Development (OECD) guideline 423 for testing of chemicals. In the sighting study, an overnight fasted male rat was administered orally with a single dose of 1000, 2000 and 3000 mg/kg BEE prepared in Mili-Q water, whereas, the control group received only Mili-Q water as a vehicle. After administration of *L. glutinosa* BEE, rats were observed for 24 h, with special attention given to the first 4 h and once daily further for a period of 14 days. The rats were weighed and visual observations for mortality, behavioural pattern (weakness, aggressiveness, diarrhea, salivation, discharge from eyes and ears, noisy breathing, changes in locomotor activity, clonic convulsion, fur, lethargy, and sleep), changes in physical appearance, injury, pain, and signs of illness were conducted once daily during the period. At the termination day, animals were sacrificed under mild anesthesia and blood sample was collected through the retro-orbital puncture into a sterilized centrifuge tubes and EDTA-containing AcCuvet-PLUS non-vacuum blood collection tube (Peerless Biotech Pvt. Ltd.) for biochemical and hematological analyses, respectively. b.w. and weight of the organs from the control and the test groups were measured and recorded. The relative organ weight of each animal was then calculated as follows. Relative organ weight: (absolute organ weight × 100%)/ body weight of rat on the day of sacrifice.

Haematological and biochemical analysis
The haematological parameters measured were hemoglobin (HB), total count, polymorphonuclear leukocytes, lymphocytes, eosinophils, monocytes, red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets. The biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin total, bilirubin direct, creatinine, urea, total cholesterol (TC), triglycerides (TG), glucose and total protein were estimated in the serum of experimental animals using assay kits and which were obtained from the Robonik India Pvt. Ltd, New Mumbai. The haematological parameters were determined using semi-automated haematology analyzer (Sysmex, Hamburg, Germany) and serum biochemistry tests were performed using semi-auto analyzer (Robonik India Pvt. Ltd., New Mumbai).

Histopathological study
After sacrificing the rats, all the vital organs heart, kidneys, liver, lung and spleen were autopsied and examined macroscopically for any lesions or abnormalities. The liver and kidney tissues were washed with normal saline and fixed immediately in 10% formaldehyde buffer for a period of 18 h. The tissues were dehydrated in graded (50-100%) ethanol, normal saline and fixed immediately in 10% formaldehyde buffer for a period of 18 h. The tissues were dehydrated in graded (50-100%) ethanol, followed by washing with xylene. Paraffin (56-58°C) embedding was done at 58 ± 1°C for 4 h and sections of 5 µm were taken using a rotary microtome. The sections were deparaffinised with alcohol xylene series, stained with haematoxylin–eosin dye for photo-microscopic observation, mounted in DPX with a cover slip and histological changes were observed and photographed under Nikon microscope (Model-YS2-H, Japan) at 40X magnification and images were processed in Nikon DSLR Camera (Model-D5100, Japan). The microscopic features of the organs were compared with the control group.

Statistical analysis
All values are expressed as mean ± SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests using GraphPad Prism (version 5) statistical software.

RESULTS
Cytotoxicity assay
MTT assay studies revealed that BEE does not show any cytotoxic effect on breast adenocarcinoma cell line, prostate cancer cell line and colon
carcinoma cell line. The percentage of inhibition of cell proliferation was less than 50% and the data is shown in the Figure 1.

**Acute toxicity tests**

*L. glutinosa* stem BEE extract at a tested dose of 1000, 2000 and 3000 mg/kg b.w. had no adverse effect on the behavioral responses of the tested rats up to 14 days. Physical observations also indicated no signs of behavioural pattern (weakness, aggressiveness, diarrhea, salivation, discharge from eyes and ears, noisy breathing, changes in locomotor activity, clonic convulsion, fur, lethargy, and sleep), changes in physical appearance, injury, pain, and signs of illness of the rats. There was no mortality and weight loss observed at all the tested doses Figure 2 and no significant differences observed in the relative organ weights Table 1.

However, slight weight differences (P < 0.05) were seen in the liver and heart of the animals treated with 3000 mg/kg of the extract.

**Haematological and biochemical analysis**

The haematological parameters such as HB, total count, polymorphonuclear leukocytes, lymphocytes, eosinophils, monocytes, RBC, PCV, MCV, MCH, MCHC, and platelets did not show any significant differences between the control and BEE treated groups at all test doses, Table 2. Likewise, there was no statistically significant differences observed in the biochemical parameters such as, ALT, AST, ALP, bilirubin total, bilirubin direct, creatinine, urea, TC, TG, glucose and total protein were found to be in normal range for all animals at the end of the study Table 3.

**Table 1: Effect of stem bark ethanol extract of Litsea glutinosa on the relative (%) and absolute (g) weights of organs.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>BEE-1000 mg/kg b.w.</th>
<th>BEE-2000 mg/kg b.w.</th>
<th>BEE-3000 mg/kg b.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.81±0.09</td>
<td>3.13±0.06**</td>
<td>2.90±0.02**</td>
<td>3.27±0.15*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.56±0.01</td>
<td>0.58±0.02**</td>
<td>0.60±0.03**</td>
<td>0.57±0.04**</td>
</tr>
<tr>
<td>Lung</td>
<td>0.63±0.09</td>
<td>0.75±0.03**</td>
<td>0.83±0.11**</td>
<td>0.74±0.11**</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.47±0.03</td>
<td>0.50±0.07**</td>
<td>0.36±0.15**</td>
<td>0.40±0.07**</td>
</tr>
<tr>
<td>Heart</td>
<td>0.29±0.01</td>
<td>0.34±0.003**</td>
<td>0.33±0.01**</td>
<td>0.39±0.01**</td>
</tr>
<tr>
<td>Body weight (gm)</td>
<td>213.2±9.44</td>
<td>211.0±7.75</td>
<td>211.2±3.56</td>
<td>207.3±1.33</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n = 3 for each group). *P<0.05, **P<0.01, ***P<0.001 were considered significant. Asterisks denote significant difference compared to control.

BEE: Bark ethanol extract; ns: Not significant.

**Macropathology and Histopathology**

Macroscopic observation of the vital organs of BEE treated animals also revealed no abnormalities in the colour or texture when compared with the organs of the control group. The microscopic examination of the transverse section of liver and kidney of the control and BEE treated group rats are shown in Figure 3. Histopathological examination of the control group and BEE treated rats showed normal architecture and absence of any gross pathological lesion in organs. The LD<sub>50</sub> of this plant was therefore estimated to be more than 3000 mg/kg.

**DISCUSSION**

Natural products including their derivatives and analogues still represent a major part of therapeutic alternatives. The consumers believe that traditional herbal remedies are safe, whereas, they could cause some adverse effects so their safety and efficacy evaluation is required. The
main purpose of toxicity study is to establish the adverse effect caused by the phytochemicals, investigate any possible dose-effect relationship and to identify the responsible phytochemicals causing the toxicity. The pharmacological effects of *L. glutinosa* were reported in previous studies. The present study was conducted to evaluate the possible cytotoxicity and acute oral toxicity of stem BEE of this plant.

The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC$_{50}$) of proliferation of less than 30 mg/ml after an exposure time of 24 h. IC$_{50}$ values below this stringent point have not been noted with any of the three studied cancer cell lines. The BEE does not inhibit the proliferation of the tested cancerous cells up to the level of 50%. This suggests that the constituents of the BEE do not induce cytotoxic effect on the tested cancerous cell lines. However, the previous investigators reported the cytotoxic property of *L. glutinosa* stem bark chemicals against the selected cancerous cell lines. Wang *et al.*, evaluated the cytotoxic effect of leaves and twigs of *L. glutinosa* against myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7 and colon cancer SW480 cells for which it was proved to be inactive (IC$_{50}$ > 40 mM). The present study also shows that the stem BEE of *L. glutinosa* was found to be inactive against adenocarcinoma, prostate cancer and colon carcinoma ATCC cell lines. Even at the concentration of 5–320 µg/ml the BEE was found to be inactive against the selected cancerous cell lines. On the contrary, Agrawal *et al.*, reported methanol extract of the heartwood of *L. glutinosa* showed significant cytotoxic activity. This is due to the biosynthesis and accumulation of phytoconstituents at different region of the plant parts and toxic specificity of the compounds against the selected cell lines.

The traditional practitioners of this area orally administered the stem bark extract to cure liver diseases (Jaundice), diarrhea and dysentery by the phytochemicals, investigate any possible dose-effect relationship and to identify the responsible phytochemicals causing the toxicity.

The present study was conducted to evaluate the possible cytotoxicity and oral acute toxicity of stem BEE of this plant.

<table>
<thead>
<tr>
<th>Test</th>
<th>Unit</th>
<th>Control</th>
<th>Test 1000 mg/kg</th>
<th>Test 2000 mg/kg</th>
<th>Test 3000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB</td>
<td>g/dL</td>
<td>10.65± 0.37</td>
<td>13.60± 0.28*</td>
<td>11.80± 1.32**</td>
<td>11.90± 1.09**</td>
</tr>
<tr>
<td>Total count</td>
<td>10³/µl</td>
<td>3500± 1589</td>
<td>18467± 5272**</td>
<td>14467± 3700**</td>
<td>15800± 1914**</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10³/µl</td>
<td>60.67± 3.48</td>
<td>64.33± 3.84**</td>
<td>55.67± 3.38**</td>
<td>59.60± 6.35**</td>
</tr>
<tr>
<td>Monocytes</td>
<td>10³/µl</td>
<td>1.33± 0.33</td>
<td>1.00± 0.57**</td>
<td>0.66± 0.33**</td>
<td>1.00± 0.00**</td>
</tr>
<tr>
<td>RBC</td>
<td>10⁶/µl</td>
<td>6.26± 0.31</td>
<td>7.63± 0.49**</td>
<td>7.40± 0.56**</td>
<td>8.20± 0.20*</td>
</tr>
<tr>
<td>PCV</td>
<td>%</td>
<td>38.97± 2.56</td>
<td>46.50± 1.18**</td>
<td>41.70± 2.42**</td>
<td>45.17± 0.35**</td>
</tr>
<tr>
<td>MCV</td>
<td>fl</td>
<td>62.08± 1.96</td>
<td>61.37± 2.58**</td>
<td>58.03± 1.53**</td>
<td>47.83± 3.18***</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>17.43±0.29</td>
<td>18.07±0.86**</td>
<td>16.80±0.96**</td>
<td>17.40±0.57**</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>30.60± 0.90</td>
<td>28.80± 0.51**</td>
<td>28.63± 1.63**</td>
<td>30.77± 0.23**</td>
</tr>
<tr>
<td>Platelets</td>
<td>10³/µl</td>
<td>6.10± 1.51</td>
<td>6.20±0.78**</td>
<td>5.30±1.95**</td>
<td>7.80±0.15**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n = 3 for each group). *P<0.05, **P<0.01, ***P<0.001 were considered significant. Asterisks denote significant difference compared to control.

HB: Hemoglobin; PNL: Polymorphonuclear leukocytes; RBC: Red blood cells; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration. ns: Not significant.
Effect of **Litsea glutinosa** stem bark ethanol extract on biochemical parameters in acute oral toxicity study.

**Table 3**: Effect of **Litsea glutinosa** stem bark ethanol extract on biochemical parameters in acute oral toxicity study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>Bilirubin (mg/dL)</th>
<th>Direct (mg/dL)</th>
<th>Total Protein (g/dL)</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>Glucose (mg/dL)</th>
<th>Bilirubin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>120±50</td>
<td>84±11</td>
<td>25±15</td>
<td>21±12</td>
<td>6.1±1.9</td>
<td>85±6.8</td>
<td>26±1.2</td>
<td>48.9±4.34</td>
<td>6.46±0.16</td>
</tr>
<tr>
<td>BEE (1000 mg/kg)</td>
<td>147±25</td>
<td>238±28</td>
<td>238±28</td>
<td>238±28</td>
<td>55±2±5</td>
<td>238±28</td>
<td>55±2±5</td>
<td>289±16.7</td>
<td>289±16.7</td>
</tr>
<tr>
<td>BEE (2000 mg/kg)</td>
<td>238±28</td>
<td>238±28</td>
<td>238±28</td>
<td>238±28</td>
<td>55±2±5</td>
<td>238±28</td>
<td>55±2±5</td>
<td>289±16.7</td>
<td>289±16.7</td>
</tr>
<tr>
<td>BEE (3000 mg/kg)</td>
<td>238±28</td>
<td>238±28</td>
<td>238±28</td>
<td>238±28</td>
<td>55±2±5</td>
<td>238±28</td>
<td>55±2±5</td>
<td>289±16.7</td>
<td>289±16.7</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SEM, n=3, *P<0.05, **P < 0.01, ***P<0.001. Experimental groups compared with Control.

**CONCLUSION**

The current study is valuable since it could indicate about the non-toxic parts of the plant may help to employ the plant as an antimicrobial or antioxidant agent. **Litsea glutinosa** stem bark ethanol extract was found to be nontoxic when acute oral toxicity study was performed. These results may primarily suggest **Litsea glutinosa** BEE to be consumed as a drug to treat liver diseases (jaundice), diarrhea and dysentery in known dosages, especially in poor rural communities, where conventional drugs are expensive and unaffordable.

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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ABBREVIATION USED

BEE: Bark ethanol extract; b.w: Body weight; ATCC: American type culture collection; OECD: Organization of Economic Cooperation and Development; HB: Hemoglobin; RBC: Red blood cells; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TC: Total cholesterol; TG: Triglycerides; NCI: National cancer institute; LD½: Lethal Dose 50%; ns: Not significant.

REFERENCES


GRAPHICAL ABSTRACT

SUMMARY

• Evaluation of cytotoxicity and oral acute toxicity of L. glutinosa stem BEE was performed for a period of 14 days.
• BEE did not show any significant cytotoxic effect against the tested cell lines. Whereas, acute toxicity study of BEE showed no deaths or any sign of toxicity up to a dose of 3000 mg/kg b.w. In addition, there was no change in hemato-

2. logical, biochemical and histological investigation.
3. • The present study shows that the oral administration of BEE was found to be safe.
Arunodaya et al.: Cytotoxicity and Oral Acute Toxicity Studies of Litsea glutinosa

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