Acute skin irritation, acute and sub-acute oral toxicity studies of *Rosmarinus officinalis* essential oils in mice and rabbit

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In Ankober, Northern Ethiopia, *Rosmarinus officinalis* has been commonly used for flavoring foods as a condiment; moreover, the plant has also been widely used for different medicinal purposes. The current study was undertaken to provide data on acute and subacute toxicity in mice as well as skin irritation of *R. officinalis* essential oil in rabbit. Acute dermal and oral toxicity tests were conducted using limited dose of 2000 mg/kg. In sub-acute study, 1000 mg/kg were given by gavage to mice for 28 consecutive days. The mice were weighed and various observations like mortality, behavior, injury, or any signs of illness were conducted once daily during the study period. At the end of each study, biochemical parameters were evaluated and kidney and liver were taken after sacrifice for gross findings and histological analyses. For dermal toxicity, 10% ointment formulation of oils was applied on the rabbit skin to evaluate whether the animals sustained significant skin damage. The LD₅₀ of *R. officinalis* essential oil for both dermal and oral administration is greater than 2000 mg/kg. There was no significant difference (p > 0.05) observed in the body weights, biochemical parameters, and gross abnormalities, as compared to the control in subacute study. No mortality was recorded. Pathological studies showed that there were no any macroscopic changes in kidneys and liver and all of them have normal appearance. The data of acute skin irritation test demonstrated that 10% *R. officinalis* oils ointment formulation did not induce acute toxicity in the skin of the animals. Overall, the findings of this study indicate that *R. officinalis* essential oil is non-toxic.

**Key words:** *Rosmarinus officinalis*, skin irritation, acute dermal toxicity, acute oral toxicity, subacute toxicity, biochemical analysis, histopathology.
INTRODUCTION

Rosmarinus officinalis (Rosemary) is an evergreen shrub that belongs to the family of Lamiaceae. It is the most common household plants distributed in several parts of the world (Asressu and Tesema, 2014). Rosemary leaves are often employed for various medicinal uses in addition to its role as condiment for flavoring foods. Several ethno-medicinal study reports of Rosemary showed that the plant is widely used for management of headaches, inflammatory diseases, and physical and mental fatigue (Lulekal et al., 2013). Moreover, it has been reported for its use as a stimulant, hepatoprotective and mild analgesic agent (Raškovic et al., 2014). Ethnobotanical investigations on traditional use of *R. officinalis* in Ankober District, North-Central Ethiopia showed prominent use reports on utilizing leaves of *R. officinalis* to treat different human and livestock ailments. Moreover, people of the Ankober District frequently use crushed Rosemary leaves in salad, fish and meat as flavor and preventing food poisoning (Lulekal et al., 2013).

Various pharmacological studies on Rosemary reported its antitumour (Singletary and Nelshoppen, 1991), antispasmodic (Lis-Balchin, 1996), estrogenic (Zhu et al., 1998), antiulcer (Dias et al., 2000), antinephrotoxic (Makino et al., 2002), antitrypanosomal (Abe et al., 2002), and osteoclastic (Muhlauer et al., 2003) properties. Moreover Hur et al. (2004) reported the use of Rosemary as an immune stimulant. Recent pharmacological investigations on Rosemary also indicated its diuretic (Haloui et al., 2007), antiproliferative (Hussain et al., 2010), anti-inflammatory (Minaiyan et al., 2011), analgesic (Martínez et al., 2012), psychostimulant (Alnamer et al., 2012), hepatoprotective (Raškovic et al., 2014), antioxidant (Takayama et al., 2016), and antimicrobial (Asressu and Tesema, 2014; Mekonnen et al., 2016) properties.

Toxicity is the expression of being poisoned that occurs due to the interaction between cells and toxicants (Jothy et al., 2011). For a very long time, several herbal based preparations are supposed to be safe. Yet, many herbal products have been shown to be highly toxic when given either acutely or repeatedly for management of ailments (Prasanth et al., 2015). For instance, ethnobotanical studies in Ethiopia reported traditional herbal preparations side effects such as diarrhea and skin necrosis (Limenih et al., 2015). Hence, it is a timely effort to assess toxicological effects of traditionally used medicinal plant extracts such as that of *R. officinalis*, preclinically or clinically. This help to identify any potential toxic effect so as to minimize or avoid health-risks. So far, in Ethiopia, studies carried out to evaluate toxicity of essential oils from *R. officinalis* are lacking. Hence, the present investigation is aimed at investigating acute, sub-acute and dermatoxicity effects of *R. officinalis* leaves essential oil in order to increase the confidence in their safety to humans to treat various ailments.

MATERIALS AND METHODS

Collection and identification of plant material

*R. officinalis* fresh leaves were collected from Ankober Herbal Project Nursery Site which is located in North Shewa Zone, Amhara Regional State, Ethiopia and the identity of the plant specimens was confirmed by a taxonomist at the National Herbarium, College of Natural Science, Addis Ababa University, Ethiopia where a voucher specimen was deposited.

Preparation of essential oil

Thirty grams of shade dried *R. officinalis* fresh leaves were taken into a 1000-mL round bottomed flask and 300 mL of distilled water was added. Clevenger-type apparatus distilled the mixture for about 3 h to get colorless oil.

Preparation of drug formulation

Ointment (5 and 10% w/w) was formulated for acute dermal toxicity study and skin irritation test, where 5 and 10 g of the extract was incorporated in 100 g (for each) petroleum jelly base. Then physical parameters like physical appearance and homogeneity, viscosity, spreadability and extrudability were evaluated using procedures described elsewhere in Bora et al. (2014) and Nair et al. (2010).

Experimental animals

Female New Zealand rabbits (1.4 to 2.3 kg) and healthy adult Swiss Albino mice of both sexes (10 to 12 weeks of age) were obtained from the Ethiopian Public Health Institute and the School of Pharmacy, AAU animal house, respectively. All the animals were kept at room temperature (25 ± 2°C) in an air conditioned room at 12 h light/dark cycle and acclimatized for 5 days before the study. The animals were provided with water and food pellets *ad libitum* before and throughout the experimentation period. All the animals were cared for and treated humanely according to the Principles of Laboratory Animal Care (ILAR, 1996). Ethical approval for this study was obtained from Deber Birhan University IRB.

Rabbit skin irritation test

Acute dermal irritation tests were performed using OECD guideline 404 with little modification using two rabbits (OECD, 2002). The fur of the animal was removed by closely trimming the dorsal area of

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animal dermal toxicity study

Acute dermal toxicity tests were performed using OECD guideline 434. About 10% of the body surface area fur was shaved 24 h before the study from the dorsal area of the trunk of five rats showing normal skin texture. A limit test dose of 2000 mg/kg of formulation was applied uniformly over the shaved area for 24 h. Animals were housed individually and general behavioral changes; symptoms of toxicity and mortality were observed after treatment critically for the first 4 h, then over a period of 24 h. At the end of the exposure period, the remaining test substance was removed and the observation was continued daily thereafter for a total of 14 days (OECD, 2004).

Acute oral toxicity study

An acute toxicity study of *R.* officinalis essential oil was carried out in five female mice using the method of OECD guideline 425 (OECD, 2008). A limit test single dose of 2000 mg/kg of the test sample was given to mice using oral gavage after being deprived of food for 3 h. All the mice were observed for general behavioral changes; symptoms of toxicity and mortality after administration of essential oil for the first 4 h (critically), then over a period of 24 h, and thereafter daily for 14 days.

Sub-acute toxicity study

Sub-acute toxicity study was performed as per the OECD guidelines 407 (OECD, 2008). The treatment (5 male and 5 female) and control (5 male and 5 female) groups orally received *R.* officinalis essential oil at the dose of 1000 mg/kg and saline once daily for 28 days, respectively. All mice were observed once in day for any clinical signs of toxicity all the way during the experimental period and the body weight of each mouse was recorded once in a week. At 29th day, mice from both groups were properly anesthetized and blood was collected from a common carotid artery. The serum level of alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT) were analyzed. After blood collection, mice from both groups were immediately sacrificed and vital organs of each mouse were examined for the evidence of gross lesions. Liver and kidney were preserved in 10% neutral buffered formalin solution to undertake histopathological examination. Tissues embedded in paraffin wax were sectioned 5 μm thick, stained with haematoxylin and eosin, mounted on glass slides and examined under a standard light microscope (Olaniyan et al., 2016).

Statistical analysis

The data were analyzed by using SPSS version 20 and *P* < 0.05 was considered as statistically significant. The results were expressed as mean ± standard deviation (SD).

RESULTS

*R.* officinalis oil ointment formulation

Table 1 shows physical evaluation of *R.* officinalis essential oil ointment formulation. The ointment formulation was found to be white color, smooth and free from grittiness. The physicochemical properties studied showed satisfactory results for spreadability, extrudability, washability and viscosity.

Rabbit skin irritation test

The effect of the essential oils on dermal irritation is
shown in Table 2. From the results of acute dermal irritation study, essential oils appeared to be safe after applying 10% ointment formulation. There was no evidence of any noticeable skin irritation (no erythema and edema) and inflammation in the study period when compared with the control (Figure 1). At 1 h after removal of test substance and thereafter on the skin sites where the test substance was applied, it was found that in all rabbits, erythema and edema score was “0”.

**Acute dermal toxicity study**

No toxic effect was observed on the behavioral response of rats treated with a dose of 2000 mg/kg *R. officinalis* essential oil. All rats were dosed once and observed for 14 days. Moreover, there were no signs of changes in the behaviour patterns, skin, eyes, salivation, and diarrhea of the rats. Neither mortality nor significant weight loss was also observed.

**Acute oral toxicity study**

Oral administration of *R. officinalis* essential oil at the dose of 2000 mg/kg did not show any signs of toxicity and mortality in any of the treated female mice for 14 days during observation period. Hence, the oral lethal dose 50 (LD$_{50}$) value of *R. officinalis* essential oil was found to be greater than 2000 mg/kg.

**Sub-acute toxicity**

**General observation and effects on body weight**

Oral administration of *R. officinalis* essential oil for 28 day caused no noticeable change in the general behavior of the mice and there were no significant changes in body weight or food intake of the mice as compared to the control group. Both the control and treatment groups appeared healthy during the whole study period. There was an increase in body weight of mice in both treatment and control groups. The percentage body weight gain, however, was higher in the control group (17.07%) as compared to treatment groups (7.85%) (Table 3).

**The effect of *R. officinalis* oil on serum biochemical parameters**

Sub-acute administration of *R. officinalis* essential oil effects on biochemical parameters are shown in Table 4. Administration of *R. officinalis* essential oil for 28 days did not show any significant changes in biochemical parameters such as SGOT, SGPT and ALP when compared with the control groups. The serum level of SGOT, SGPT and ALP of treatment groups was lower as compared to the control group. Yet, the difference was not statistically significant.

**Histopathology**

Light microscopic examination of liver and kidney sections of control and treatment groups showed a normal histology (Figures 2 and 3) and absence of any gross pathological lesions after 28 days treatment with 1000 mg/kg dose of *R. officinalis* essential oil. The result showed normal hepatocellular morphology, normal peribronchial area with absence of necrosis and inflammation. All kidney sections showed normal glomerular architecture, no vascular necrosis or hyaline changes and unremarkable tubule interstitial parenchyma.

### Table 1. Physical evaluation of 10% *R. officinalis* essential oil ointment formulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Color</th>
<th>Viscosity (cP) at 100 rpm (Mean±SD)</th>
<th>Spreadability (g.Cm/min) (Mean±SD)</th>
<th>Extrudability (g) (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. officinalis</em> oil ointment</td>
<td>White</td>
<td>8158.3±30.1</td>
<td>2420.7±77.9</td>
<td>0.137±0.015</td>
</tr>
</tbody>
</table>

### Table 2. Score of erythema and edema after application of test materials on rabbit skin.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>1 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>7th day</th>
<th>15th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Edema</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Score for skin reaction of 10% *R. officinalis* essential oil ointment (three treatment site) at different time interval after removing test ointment. Primary Irritation Index (PII) = 0/3, PII=0; Category of irritation based on PII is Negligible. Con: Control; Treat: treatment.
Table 3. Effects of *R. officinalis* essential oil on body weight of mice treated orally for 28 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RO 1000 mg/kg</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0 (g)</td>
<td>28±2.7</td>
<td>24.6±2.88</td>
</tr>
<tr>
<td>Week 1 (g)</td>
<td>28±2.7</td>
<td>24.6±2.88</td>
</tr>
<tr>
<td>Week 2 (g)</td>
<td>29±0.55</td>
<td>29±1.73</td>
</tr>
<tr>
<td>Week 3 (g)</td>
<td>30±2.61</td>
<td>28.8±1.09</td>
</tr>
<tr>
<td>Week 4 (g)</td>
<td>30.2±2.28</td>
<td>28.8±1.09</td>
</tr>
<tr>
<td>Body weight gain (%)</td>
<td>7.85</td>
<td>17.07</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM, n = 10 animals/group, p > 0.05. RO: *R. officinalis*.

Table 4. Biochemical parameters of mice treated orally with *R. officinalis* oil for 21 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RO 1000 mg/kg</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT (U/L)</td>
<td>316±128</td>
<td>348±123</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>79.6±64.5</td>
<td>80.8±26.08</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>265±124</td>
<td>267±33</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM, n = 10 animals/group, p > 0.05. RO: *R. officinalis*.

**DISCUSSION**

Currently, concern regarding safety profile of traditional herbal preparations is increasing since there is no standard dosage and limited scientific studies on safety profile of traditional herbal products (Eran et al., 2016; Saad et al., 2006). Hence, adequate scientific knowledge of traditional herbal preparations oral toxicity is mandatory. This will help to reveal the possible clinical signs of adverse effect and identify doses that could be used by preparations under investigation. Irrespective of the pharmacological benefits of the *R. officinalis* essential oil, there are no detail knowledge about toxicity profile of this medicinal plant in Ethiopia. In the present acute toxicity study, administration of *R. officinalis* essential oil at 2000 mg/kg dose to the mice both orally and dermally did not reveal any signs of toxicity or mortality in any animal during the whole study period. Therefore, the LD<sub>50</sub> of *R. officinalis* essential oil for both route of administration is considered to be greater than 2000 mg/kg. *R. officinalis* essential oil is safe since agents having LD<sub>50</sub> value greater than 2000 mg/kg are considered as relatively safe (Nath and Yadav, 2015). This finding correlates with that of Fahim et al. (1999) and Nakavuma et al. (2016), who reported LD<sub>50</sub> value of 5,000 g/kg and 4,723 mg/kg respectively. On the contrary, Alnamera et al. (2012) reported *R. officinalis* essential oil LD<sub>50</sub> of 897.85 mg/kg; which is much lower than the LD<sub>50</sub> value of the present study and previously reported value. The possible reason for this discrepancy could be variation in secondary metabolites due to agro-ecological difference. The main secondary metabolite of *R. officinalis* oil used in this study were α-pinene (50.83%), camphene (5.21%), β-pinene (2.06%), β-myrcene (0.68%), 1, 8-cineole (24.425%), camphor (3.845%), broneol (1.518%), and broneol acetate (1.628%). Verbenone (0.521%), linalool (1.262%), and limonene (1.729%) (Mekonnen et al., 2016). While the major constituents of *R. officinalis* oil used in the study of Nakavuma et al. (2016) were α-pinene (26.46%); 1, 8-cineole (24.20%), verbenone (9.41%), geraniol (3.38%), linalool (3.12%), and limonene (0.02%) (Unpublished data) and Alnamera et al. (2012) study had composition of α-pinene (15.82%), camphene (6.80%), β-pinene (4.75%), myrcene (1.70%), p-cymene (2.16%), 1, 8-cineole (50.49%), camphor (11.61%), broneol (2.58%), and broneol acetate (2.08%). Higher 1, 8-cineole (50.49%) content in the study of Alnamera et al. (2012) as compared to the present study and Nakavuma et al. (2016) study might play role for the observed difference.

To make sure people using pharmaceutical formulation are safe, assessment of skin irritancy potential to human skin of any chemicals or formulations is compulsory (Kamkaen et al., 2007). The absence of any toxic sign like erythema and edema reactions in present skin irritation test asserts the non-irritant of *R. officinalis* essential oil ointment. Based on the results, the essential oil primary irritation index was found to be 0 and it was concluded that the essential oil was non-irritating to the skin (PII<0.5). Low camphor content (3.845%) of *R. officinalis* essential oil might be responsible for absence of skin irritation since camphor is an irritant substance. This finding is in good agreement with that of Hamza et al. (2017), who reported *R. officinalis* essential oil was non-irritant to the skin though the study was conducted in rat.

Information on target organ toxicity, dosage regimens and observable sign of toxicity that possibly influence life span of experimental animals can be generated by sub-acute toxicity study (Hilaly et al., 2004). In toxicity study, changes in the body weight serve as a sensitive indicator of general health of experimental animals. There were no significant body weight changes of experimental animals after completing 28 days essential oil administration as compared to the control groups (Table 3). Both treatment and control group animals showed a steady increment in body weight throughout the study period.

Besides body weight, quantification of food and water consumption is necessary during toxicity study of an agent with medicinal value. In this study, *R. officinalis* essential oil did not affect food and water consumption showing that it did not interfere with the normal digestion and metabolism of animals. For better physiological status of the animal and response to the test substance
Figure 2. Liver histology of control and *R. officinalis* essential oil treated animals. (A) Liver section of control animals revealed normal architecture and hepatic cells; (B) Liver section of *R. officinalis* essential oil (1000 mg/kg) treated animals exhibited normal architecture of hepatocytes and hepatic cells after 28 days of treatment.

Figure 3. Kidney histology of control and *R. officinalis* essential oil treated animals. (A) Kidney section of control animal showed normal size of glomeruli with normal tubules; (B) Kidney section of *R. officinalis* essential oil (1000 mg/kg) treated animals exhibit normal size of glomeruli with normal tubules after 28 days of treatment.

under investigation, proper intake of supplements is needed (Kumar et al., 2014).

Serum biochemistry such as alkaline phosphatase, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase and creatinine can be used as biomarkers to identify potential toxic effect of drugs and xenobiotics to the vital organ (Brandt et al., 2009; Kumar et al., 2014). Higher blood level of both SGOT and SGPT enzymes are a good indicator of toxicity to the liver parenchymal cells (Kumar et al., 2014). In the present study, there was no statistically meaningful difference in all biochemical parameters measured between the treatment and control groups (Table 4). *R. officinalis* essential oil did not significantly change the levels of SGOT, SGPT and ALP in experimental animal highlights that it did not affect normal liver function and metabolism of the animals. Histological assessment of the organs (Figures 2 and 3) further confirmed serum biochemical observations. Normal structural features of kidneys histopathological investigation of both treatment and control group propose the normal renal function. Normal architecture of glomeruli and renal tubules portrays the absence of renal toxicity. Liver histopathology of both treatment and control group animals was almost the same with normal morphology of hepatocytes, central vein and portal triads. This finding is in good agreement with that of Nakavuma et al. (2016), who showed lack of gross and microscopic changes in the mice tissues and organs treated with *R. officinalis* essential oil.
Conclusions
The current finding asserts that *R. officinalis* essential oil is not toxic and do not produce any adverse effect in the acute and sub-acute oral toxicity investigations. The histology examination revealed no remarkable changes in kidney and liver of the mice in both control and treatment groups. The level of the marker enzymes in the vital organs was also found to be normal. Besides, the skin tolerance test on rabbit showed ointment of *R. officinalis* essential oil is not irritant and do not show any dermal toxicity. Overall, it can be concluded that the *R. officinalis* essential oil is well tolerated in daily dose at 1000 mg/kg for a period of 28 days and safe for traditional use and as food additive.

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CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

REFERENCES


