

GAS CHROMATOGRAPH-MASS SPECTROMETER ANALYSIS AND ACUTE ORAL TOXICITY OF CINNAMOMUM BURMANNII, NESS EX BL. ESSENTIAL OILHERDWIANI W^{1*}, SOEMARDJI AA², EL-FAHMI³, TAN MI⁴

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ABSTRACT

Objective: *Cinnamomum burmannii* Nees Ex Bl. essential oil has cytotoxic effect on a lot of cancer cell lines. An investigation was carried out to analyze the possible chemical components from *C. burmannii* essential oil and evaluate its acute toxicity, before an effective formulation of *C. burmannii* essential oil as anticancer drugs.

Methods: This study was analyzed chemical components from *C. burmannii* essential oil by gas chromatograph-mass spectrometer (GC-MS) and evaluated the acute oral toxicities of *C. burmannii* essential oil in strain Balb/C mice.

Results: This analysis revealed that *C. burmannii* essential oil contains the active compound cinnamaldehyde (71.814%), trans-cinnamyl acetate (11.09%), coumarin (3.41%), and cineol (1.77%). Acute oral toxicity of *C. burmannii* essential oil with lethal dose 50 3679.11 mg/kg BW.

Conclusion: *C. burmannii* essential oil contains the active compound cinnamaldehyde, trans-cinnamyl acetate, coumarin and cineol. Acute oral toxicity conclusively indicates *C. burmannii* essential oil includes category 5 practically non-toxic.

Keywords: Gas chromatograph-mass spectrometer, *Cinnamomum burmannii*, Essential oil, Acute toxicity.

INTRODUCTION

The study of adverse effects, poisonous, and harmful of drugs and other chemicals compound in plants, which may increase weakness in the general health or the chances of mortality, mentally as well as physically is the definition of toxicology. Toxicological studies may be three types depending on the duration of drug exposure to animals such acute, subacute, and chronic toxicological studies [1].

Adverse effects of a single dose of a substance occurring following oral or dermal administration, or multiple doses given within 24 hrs, or an inhalation exposure of 4 hrs were referred by acute toxicological studies [2]. Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hrs, with special attention given during the first 4 hrs, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed [3]. All observations are systematically recorded with individual records being maintained for each animal. Additional observations will be necessary if the animals continue to display signs of toxicity. Observations should include changes in behavior pattern, autonomic and central nervous systems, somatomotor activity, eyes and mucous membranes, skin and fur, circulatory, and respiratory [3]. Salivation, diarrhea, tremors, convulsions, lethargy, sleep, and coma should be directed to observations. The principles and criteria summarized in the Humane Endpoints Guidance Document should be taken into consideration [3]. Animals should be humanely killed if found in a moribund condition and animals showing severe pain or enduring signs of severe distress. The time of animals death because of humane reasons or found dead, should be recorded as precisely as possible [4].

The bark and leaves *Cinnamomum burmannii* (Family: Lauraceae) are often added to food preparations to improve aroma and taste. It has been found to possess potent antidiabetic, antimicrobial, antioxidant, and antipyretic properties and has been used in traditional Chinese and Indian medicine. Cinnamon bark extract effectively inhibits α -glucosidase leading to suppression of postprandial hyperglycemia in streptozotocin-induced diabetic rats loaded with sucrose, maltose [5].

Review of literature indicates that *Cinnamomum* showed various cytotoxic activities in cancer cell line, namely, basal cell carcinoma (BCC-1), breast cancer (MCF7) cell line, epidermoid carcinoma (A431), human cancer promyelocytic leukemia (HL-60), human cervical carcinoma (SiHa), human colorectal carcinoma (HCT 116, HT 29, and SW 480), human epithelioid cervix carcinoma (HeLa), human glioblastoma multiforme tumor (T98G), human leukemia (K562) and leukemia rat embryonic fibroblast (5RP7), human liver cancer (Hep-1), human lymphoblast lung (U937), human melanoma (A375) cell lines, human nasopharyngeal carcinoma (NPC/HK1 and C666-1), human oral cancer (KB) lymphocytic leukemia (L1210) cells, human oral squamous cell carcinoma (Ca9-22 and SCC12) human prostate cancer cell (DU145 and PC-3), tumor cell line lymphoma melanoma, and cervix hepatoma Hep G2 cells line (Hep G2 and Hep3B) cell lines [6,7]. The effect of cinnamaldehyde on Hep G2 cell apoptosis was concluded on the CD95 (APO-1/CD95) signal transduction and p53 pathways. Several studies have shown that the B-cell lymphoma 2 (Bcl-2) family of proteins is central of apoptotic regulation [8,9].

Before an effective formulation of *C. burmannii* essential oil as anticancer drugs, evaluation of its toxic effects is required. The present work evaluated the acute oral toxicities of *C. burmannii* essential oil using OECD 423 in female *Mus musculus* mice at the dosage of 300, 500, 1000, 1500, 2000, and 3000 mg/kg BW of an animal for a period of 14 days. The main purpose of the acute toxicity study is to determine

the median lethal dose (50% death) (LD_{50}) [10,11]. The results obtained from this study will provide the safety information of this essential oil before its commercialization as a natural anticancer product.

METHODS

Plant materials and distillation

C. burmannii stem bark was obtained from Jambu Village, Salatiga, Central of Java, Indonesia. Then, stem bark was dried at the temperature (40°C) and ground to produce fine particle. The extraction of essential oil was done by a water steam distillation method. The extraction was done for at least 8 hrs, and the temperature was adjusted to maintain the boiling conditions. Sodium sulfate dehydrates (Na_2SO_4) was added to remove the remaining water in the essential oil to obtain 100% purity with the density of 1.015 mg/ml.

Gas chromatograph-mass spectrometer (GC-MS) analysis

GC-MS analysis was carried out on Agilent GC 6890N 5975B MSD system. The following conditions: Capillary column with nominal length 30.0 m, nominal diameter 250.00 μ m, nominal film thickness 0.25 μ m, and stationary phase HP-5MS 5% phenyl methyl siloxane. Helium was used as carrier gas at a constant flow of 1.0 mL/minute and an injection volume of 1 μ l was employed (split ratio of 50:1), nominal initial pressure 8.75 psi (On), initial temp 300°C (On), split flow: 49.7 mL/minutes and total flow 53.5 mL/minutes. Total GC running time was 40 minutes.

Identification of components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Animals

Female *Mus musculus* Balb/C strain mice weighing 20-30 g were obtained from the Integrated Research Development Institute Gadjah Mada University. The animals were kept in plastic cages in environmental conditions room temperature 22-24°C, with 12 hrs/12 hrs dark/light cycle, fed a mouse pellet diet (Comfeed Indonesia) and allowed to drink water ad libitum without distraction. All the animal handling protocols were approved by the Animal Ethics Committee of Faculty of Pharmacy, Bandung Institute of Technology.

Acute toxicity

Healthy mice have fasted overnight but allowed access to water *ad libitum* and were randomly divided into eight groups (n=5). The first group (control group) received distilled water only; the second group received *Oleum Olivae*. The other six groups were orally treated with a single dose of *C. burmannii* essential oil at 300, 500, 1000, 1500, 2000, or 3000 mg/kg BW, respectively. The doses in this acute toxicity study were based on the results of a range-finding study, where the observations on mortality and toxicity signs were made. All the treatments were administered by force feeding. Animals were observed for signs of toxicity, body weight, and mortality for a period of 14 days after treatment [12]. The toxicity signs and symptoms were observed in individual cages during the first 24 hrs after the essential oil administration and subsequently monitored daily throughout the duration of the study [12]. All observations are systematically recorded with individual records being maintained for each animal. Additional observations will be necessary if the animals continue to display signs of toxicity. Observations include changes in behavior pattern, autonomic and central nervous systems, somatomotor activity, eyes and mucous membranes, skin and fur, circulatory, and respiratory [3]. Salivation, diarrhea, tremors, convulsions, lethargy, sleep, and coma should be directed to observations. The principles and criteria summarized in the Humane Endpoints Guidance Document [2]. According to the mortality of rats observed within 14 days, the LD_{50} value was calculated. At day 15, all surviving animals were sacrificed, internal organs were excised, and organ weights were measured.

Statistical analysis

The LD_{50} was calculated using probit analysis (SPSS 11.5). All values were expressed as mean \pm standard error of the mean and were analyzed by one-way analysis of variance followed by *post-hoc* test, and statistically significant findings were considered those in which $sig < 0.05$.

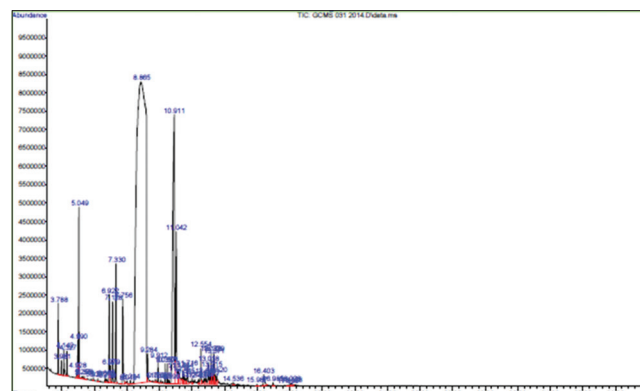


Fig. 1: Chromatogram of *Cinnamomum burmannii* essential oil

Table 1: Active compound identified in *Cinnamomum burmannii* essential oil

No	Retention time (min)	Name of the compound	Molecular weight	Peak area %
1	3.788	Alpha.-Pinene	136	0.562
2	3.981	Camphene	136	0.137
3	4.142	Benzaldehyde	106	0.371
4	4.327	Bicyclo[3.1.1]heptane	136	0.200
5	4.990	dl-Limonene	136	0.402
6	5.049	1,8-Cineole	154	1.769
7	5.258	Benzaldehyde 2-hydroxy	122	0.052
8	5.378	1,4-Cyclohexadiene	136	0.044
9	6.212	D-Fenchyl alcohol	154	0.026
10	6.669	Camphor	152	0.061
11	6.739	Propene	134	0.082
12	6.922	Benzenepropanal	134	1.329
13	6.979	1-Borneol	154	0.234
14	7.330	3-Cyclohexene-1-methanol	154	2.533
15	8.016	Benzoic acid (CAS)	122	0.059
16	8.214	2-Decenal	154	0.062
17	8.865	Cinnamaldehyde	132	71.814
18	9.284	2-Propen-1-ol	134	0.359
19	9.912	alpha.-Copaene	204	0.206
20	10.357	Bicyclo[3.1.1]hept-2-ene	204	0.225
21	10.501	Caryophyllene	204	0.203
22	10.911	Trans-cinnamyl acetate	176	11.094
23	11.042	2H-1-Benzopyran-2-one	146	3.712
24	11.170	Naphthalene	204	0.843
25	11.545	4-Chloro-3,5-Dimethylisoxazole	131	0.190
26	11.716	.delta.-Cadinene	204	0.301
27	12.011	.alpha.-Calacorene	200	0.101
28	12.554	(-)-Caryophyllene oxide	220	0.507
29	12.747	1H-Cycloprop[e] azulene	204	0.305
30	13.077	.alpha.-Gurjunene	204	0.120
31	13.178	tau.-Muurolol	222	0.472
32	13.215	1-Naphthalenol	222	0.165
33	13.329	.alpha.-Cadinol	222	0.424
34	13.371	.alpha.-Amorphene	204	0.301
35	13.520	Caryophyllenol-II	220	0.203
36	14.536	Ethyl 3-nitropropionate	147	0.049
37	15.987	Hexadecanoic acid	270	0.032
38	16.403	Hexadecanoic acid (CAS)	256	0.337
39	18.028	4,8,12-Trimethyltridec-3-enoic acid	268	0.043
40	18.128	Nerol	154	0.071

RESULTS

Chromatogram of *C. burmannii* essential oil (Fig. 1)

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Active compound of *C. burmannii* essential oil (Table 1)

The chromatogram of essential oil of cinnamon can be seen in Fig. 1. Active compound identified in *Cinnamomum burmannii* essential oil was shown on Table 1

Body weight

Individual weights of animals were determined shortly before the test substance is administered and weekly thereafter. Weight changes were calculated and recorded. Weight changes were presented in Table 2.

Sign of toxicity

Observation of sign of toxicity was presented in Table 3.

Platform in all treatment was significant difference (sig=0.04). The control treatment (aquadest) significant difference with dose 3000 mg/kg BW (sig=0.024) and dose 2000 mg/kg BW (sig=0.09) (Graph 1).

Grooming in all treatment was a significant difference (sig=0.01). There was significant difference the control group (aquadest) with dose 300 mg/kg BW (sig=0.006) (Graph 2).

Urination significant difference in all treatment (sig=0.000). However, there was no significant difference with control treatment (Graph 3).

Mortality

Mortality of mice in all treatment for 24 jam, the LD₅₀ value was analyzed by Reed-Muench method.

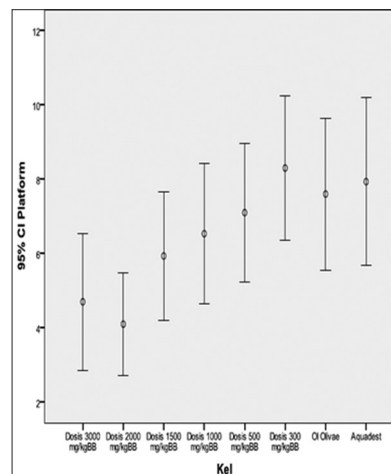
Table 2: Body weight changes of mice in acute toxicity study in control and group treated with different doses of *Cinnamomum burmannii* essential oil

Dose (mg/kg BW)	Body weight changes	
	Delta 1	Delta 2
5000	Not observed	Not observed
3000	1.4±1.52	2.3±2.6
2000	2.2±0.45	2.8±1.3
1500	2±1.73	3.2±1.64
1000	1.6±1.14	2±2.45
500	1.8±1.48	2.2±1.64
300	1.2±1.3	2.8±0.45
OI Olive	2.6±0.55	3.6±0.89
Aquadest	2±1.22	2±1.22
Sig	0.515	0.421

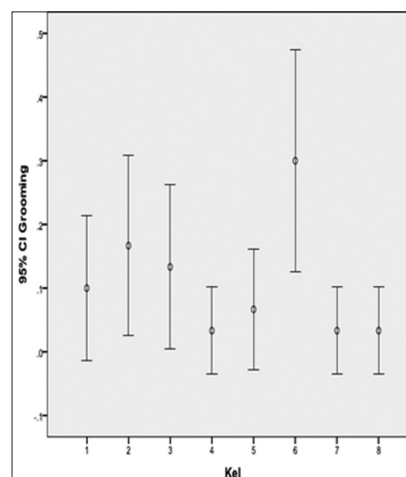
Delta 1=Body weight day-7–body weight day-0. Delta 2=Body weight day-14–Body weight day-0. There were no significant body weight changes in day 7 and day 14 with significant=0.515 and 0.421, respectively

Table 3: Observation of sign of toxicity in all group treatment except group 5000 mg/kg BW

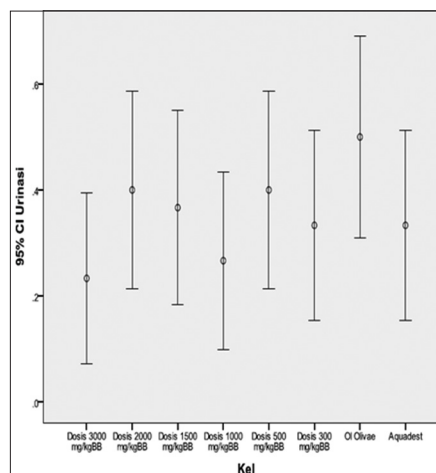
Observation	Result (%)	Conclusion
Straub	0	No significant difference
Piloerection	0	No significant difference
ptosis	0	No significant difference
R Pineal	100	No significant difference
R cornea	100	No significant difference
Lacrimation	0	No significant difference
Catalepton	0	No significant difference
Posture	100	No significant difference
Hanging	100	No significant difference
Retablissement	100	No significant difference
Flexi	100	No significant difference
Hafner	100	No significant difference
Mortality	0	No significant difference
Respiratory	100	No significant difference
Defecation	Sig=0.065	No significant difference



Graph 1: Platform in all treatment



Graph 2: Grooming in all treatment



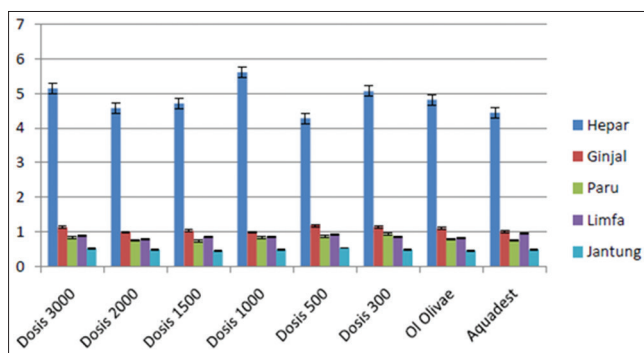
Graph 3: Urination in all treatment

Table 4: Mortality in all treatment

Dose (mg/kg BW)	Sum		Cumulative			%
	Dead	Life	Dead	Life	Sum	
5.000	5	0	5	0	5	100
3.000	0	5	0	5	5	0
2.000	0	5	0	5	5	0
1.500	0	5	0	5	5	0
1.000	0	5	0	5	5	0
500	0	5	0	5	5	0
300	0	5	0	5	5	0

Table 5: Index of organ

Dose (mg/kg BW)	Index of organ (%)				
	liver	Renal	Spleen	Limfe	Heart
3000	5.15±0.7	1.13±0.13	0.83±0.33	0.88±0.18	0.52±0.06
2000	4.58±0.71	0.99±0.07	0.75±0.11	0.78±0.13	0.49±0.16
1500	4.71±0.42	1.03±0.14	0.73±0.11	0.86±0.14	0.45±0.1
1000	5.61±0.75	0.99±0.13	0.83±0.09	0.86±0.24	0.49±0.11
500	4.28±1.78	1.18±0.07	0.87±0.23	0.93±0.13	0.54±0.13
Dosis 300	5.06±1.03	1.14±0.24	0.93±0.25	0.85±0.13	0.49±0.06
Ol Olivae	4.82±0.85	1.1±0.16	0.78±0.25	0.83±0.09	0.46±0.12
Aquadest	4.44±0.56	1±0.2	0.76±0.12	0.94±0.11	0.49±0.1
Sig	0.96	0.65	0.596	0.589	0.633



Graph 4: Index of organ

Mortality was observed in the groups receiving 5000 mg/kg BW with five deaths (Table 4). From the acute toxicity data, using probit analysis, LD₅₀ of *C. burmannii* Nees Ex Bl essential oil was estimated to be 3679.11 mg/kg BW.

Index of organ

After observation for 14-15 days, then the test animals were turned off and weighted organs and organ index is calculated obtained the following results shown in Table 5.

There is no significant difference on index of the organ in all treatment. Graph of index organ was presented in Graph 4.

DISCUSSION

A total of 40 different compounds were identified in *C burmannii* essential oil by GC-MS analysis. The active principles with their retention time, molecular weight, and concentration (%) are presented in Table 1 and Fig. 1. The prevailing compounds were cinnamaldehyde (71.814%), trans-cinnamyl acetat (11.09%), coumarin (3.41%), and cineol (1.77%).

In this acute toxicity study, using Reed-Muench method, LD₅₀ of *C. burmannii* essential oil was estimated to be 3679.11 mg/kg BW. Reed-Muench method based on cumulative of life and died animals.

There was assumed that the died animals on that dose will have died on higher dose, and the life animals in the smaller dose [13].

LD₅₀ of Cinnamon oil was 3679.11 mg/kg BW thus the essential oil can be classified by Environmental Protection Agency (EPA) in practically non-toxic [10]. Categories of toxicity by EPA toxicity are very highly toxic with LD₅₀ value is <10 mg/kg BW, highly toxic with LD₅₀ value is 0-50 mg/kg BW, moderately toxic with LD₅₀ value is 51-500, slightly toxic with LD₅₀ value is 501-2000, and practically non-toxic with LD₅₀ value is >2000 [10]. Based on Gassel, 1995 category of toxicity are super toxic with LD₅₀ value is <5 mg/kg BW, extremely toxic with LD₅₀ value is 5-50 mg/kg BW, very toxic with LD₅₀ value is 50-500 mg/kg BW, moderate toxic with LD₅₀ value is 500-5000 mg/kg BW, mild toxic with LD₅₀ value is 5000-15000 mg/kg BW, and practically non-toxic with LD₅₀ value is >LD 15000. So, based on this classification of *C. burmannii* essential oil can be classified mild toxicity [14].

Based on International Labour Organization, chemicals can be allocated to one of five toxicity categories based on acute toxicity by the oral, dermal, or inhalation route according to the numeric criteria expressed as (approximate) LD₅₀ (oral, dermal) or LC₅₀ (inhalation) values. Acute toxicity hazard categories oral (mg/kg) exposure route LD₅₀ values defining categories are the category 1 until category 5 with LD₅₀ are 5, 50, 300, 2000, and 5000, respectively. *C. burmannii* essential oil includes category 5 with LD₅₀ the range of 2000-5000 mg/kg. Criteria for category 5 are intended to enable the identification of substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. The specific criteria for category 5 are: First, reliable evidence is already available that indicates the LD₅₀ or LC₅₀ to be in the range of category 5 values or other animal studies or toxic effects in humans indicate a concern for human health or an acute nature. Second, through extrapolation, estimation, or measurement of data, if assignment to a more hazardous category is not warranted, and reliable information is available indicating significant toxic effects in humans, or any mortality is observed when tested up to category 4 values by the oral, inhalation, or dermal routes, or - where expert judgment confirms significant clinical signs of toxicity, when tested up to category 4 values, except for diarrhea, piloerection, or an ungroomed appearance, or - where expert judgment confirms reliable information indicating the potential for significant acute effects from other animal studies. Recognizing the need to protect animal welfare, testing in animals in category 5 ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health [15].

The acute oral toxicity study of *C. burmannii* essential oil was not caused a significant decrease in body weight at all treatment. An insignificant increase in body weight of test animals indicates that the administration of the essential oil does not affect the growth of the animals. No significant changes were observed in wellness parameters used for evaluation of toxicity. Behavioral pattern, skin, fur, eyes, mucous membrane, salivation, the sleep of the treated as well as the control animals were found to be normal. Tremors, lethargy, diarrhea, and coma did not occur in any of the animals. The body weights of all the mice were increased after the oral administration of *C. burmannii* essential oil, but the changes in the body weights were found to be statistically insignificant.

Administration of a single oral dose of the *C. burmannii* essential oil had produced some toxicity symptoms that there was the significant difference in all group treatment such as platform, grooming, and urination with significant difference 0.04, 0.01, and 0.000, respectively. Platform there was a significant difference between dose 3000 and 2000 mg/kg BW with control treatment (aquadest) and significant difference 0.024 and 0.09, respectively. It was shown curiosity mice. Changes in motor activity are a manifestation sedative activity, central nervous depressants, muscle relaxants, paralysis, or anesthesia [16].

Grooming in all treatment was a significant difference. There was the significant difference the control group (aquadest) with dose 300 mg/kg BW (sig=0.006). Urination significant difference in all treatment (sig=0.000). However, there was no significant difference with control treatment. There was indicated that cinnamon oil did not affect to urination.

Some toxicity symptoms which probably were the result of active compound in *C. burmannii* include cinnamaldehyde. At higher concentrations, cinnamaldehyde induces an oral irritant sensation that decreased in magnitude (self-desensitization) on repeated application at short (1-min) interstimulus intervals [16-19]. When applied topically to the skin, whereas cinnamaldehyde elicited burning pain [20]. Intraoral application of cinnamaldehyde excites cold-sensitive trigeminal subnucleus caudalis (Vc) neurons in rats in a manner exhibiting self-desensitization [21,22] and reciprocal cross-desensitization [23].

Cinnamaldehyde elicits oral irritation that desensitizes across repeated applications and shows for the first time that these chemicals induce reciprocal cross-desensitization of oral irritation. Cinnamaldehyde has been previously reported to elicit oral irritation in a temporally desensitizing pattern [17-19,24]. The self-desensitizing effects of cinnamaldehyde may be mediated peripherally because TG cells exhibited reduced responses to repeated application of the higher concentration of cinnamaldehyde at 5 minutes interstimulus interval [25].

Coumarin is one of active compounds in *C. burmannii* caused mutations in bacterial assays [26-29]. However, in a UDS test, rats were reported negative results, and no adduct formation was found in rats after coumarin treatment. There was not observed induction of micronuclei in three micronucleus tests *in vivo* in mice [29-32]. It suggests that coumarin is not genotoxic in rodents [33].

According to Liang, 1996, one of the toxic symptoms shown to be a result of the muscarinic effect of cholinergic poisoning is lacrimation. Hypoactivity was suggested to be due to a decrease in locomotor activity controlled by the Central Neuro System [34]. In the literature, research said that coumarin is one of the active compounds in *C. burmannii* essential oil is toxic to the liver and kidney [35]. This chemical compound has been banned as a food additive in the United States and Western Europe. There is research since the mid-1800s on the toxic effects of coumarin on animals. In 1875 and 1877, it was found coumarin caused liver deterioration and blood vessel dilation in a variety of warm and cold-blooded animals [27].

There is two procedures in OECD guidelines for testing of chemical acute toxicity procedure; they are up-down procedure (UDP) and fixed dose procedure (FDP). UDP only between 6 or 10 animals of one sex (fewer than either the LD₅₀/the FDP). In this research, we used one sex, i.e., female mice. Available literature the sexes are usually similar in their acute toxicity response and that of females are often more sensitive than males when acute toxicity differences do exist, thus obviating the need for both sexes to be tested in most cases. Unlike the FDP the UDP also estimates an LD₅₀, thus providing data directly applicable to all current hazard classification systems based on acute toxicity [36].

CONCLUSION

C. burmannii essential oil contains mainly compound cinnamaldehyde (71.814%), trans-cinnamyl acetate (11.09%), coumarin (3.41%), and cineol (1.77%). Acute oral toxicity conclusively indicates *C. burmannii* essential oil include category 5 practically non-toxic with LD₅₀ 3.679.11 mg/kg BW.

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