

## Investigations of cytotoxic and mutagenic effects of some synthetic compounds of natural origin

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### Abstract

Investigations of cytotoxic and mutagenic effects of limonene 1, 2-epoxide (LE), hydroxycitronellal (HC), thymyl acetate (ThA), terpinyl acetate (TrA), and isopulegone (IS) were carried out. The cytotoxicity was determined with hemolytic assay and brine shrimp lethality bioassay (BSLB) while mutagenicity was determined by using *Salmonella typhimurium* TA100 and TA98 strains. Hemolytic activity of LE, HC, ThA, TrA and IS was 13, 3.07, 10.63, 22.1 and 8.73% respectively, at a concentration of 10 mg mL<sup>-1</sup> in comparison to the control. The median lethal concentration (LC<sub>50</sub>) values for these compounds as calculated with probit method were 7.95, 1.05, 6.38, 3.92 and 4.79 mg mL<sup>-1</sup>, respectively. All the tested compounds showed non-mutagenic activity to *S. typhimurium* TA100 and TA98 strains. The present investigation suggested that all the tested compounds are also non-toxic to human RBCs and brine shrimp larvae so; these chemicals might be safely used for pharmaceutical benefits.

**Keywords:** natural compounds, hemolytic activity, lethality assay, mutagenicity assay

### 1. Introduction

Toxicological screenings are routinely conducted on the chemicals intended for therapeutic purposes. Some compounds of natural origin have therapeutic value but synthetically prepared because of sparse availability. We screened a set of synthetically prepared compounds of natural origin such as limonene 1, 2-epoxide (LE), hydroxycitronellal (HC), thymyl acetate (ThA), terpinyl acetate (TrA), and isopulegone (IS) for their toxicity evaluations with *in vitro* assays.

LE (C<sub>10</sub>H<sub>16</sub>O) a colorless liquid is the principal constituent of citrus (family *Rotaceae*). It is employed in medicines to mask the taste of alkaloids and in perfumes for fragrance. It is used as an insecticide and renders lemon-orange fragrance in the hand cleansers. It is also used as medicine in gastroesophageal reflux and heartburn. LE has shown antioxidant properties because of increased activity of catalase and superoxide dismutase in mice hippocampus (De Almeida *et al.*, 2014) [7]. HC (C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>) is pale yellow in color having a sweet aroma with floral accents. It is not found in nature abundantly and is prepared by hydration of citronellal. HC is used in fragrances and in a wide variety of other products such as soaps, shampoos, detergents, cosmetics and household cleaning and maintenance products. Some of its side effects like headache, neurotoxicity, carcinogenicity etc. have also been reported (Thyssen *et al.*, 2009) [23]. TrA (CH<sub>3</sub>COOC<sub>10</sub>H<sub>17</sub>) a fragrant liquid ester is found in several essential oils but mostly prepared by reaction of alpha-terpinol with acetic anhydride. It has slightly sweet smell of chayote and lavender. Essential oils of *Laurus nobilis*, contain α-TrA that has also anticonvulsant, anticholinergic, antibacterial and antifungal properties (Patrakar *et al.*, 2012) [18]. ThA (C<sub>12</sub>H<sub>16</sub>O<sub>2</sub>) is extracted by hydro distillation from *Aeolanthus pubescens* with antioxidant properties and revealed to be useful in food

and medicines (Alitonou *et al.*, 2013) [1]. IS (C<sub>10</sub>H<sub>16</sub>O) is soluble in alcohol and difficult to dissolve in water at room temperature. It has been isolated as mixture of different volatile oils from different plants. Extracts of pulegone have significant antibacterial and antifungal activities (Duruva *et al.*, 2004) [8].

These compounds are widely used in cosmetic and topical pharmaceutical preparations. Usually, toxicological screenings are conducted on natural plant extracts rather than individual components. It has been observed that individual components have the potential to modulate the dermal absorption and penetration of topically applied drugs (Muhammad and Jim, 2015) [17]. These may cause noxious effects when come in contact with bio systems. There is limited published data on the toxicological evaluations of above mentioned compounds. Thus the present study has been planned to evaluate these compounds for their toxicities with some available *in vitro* tests.

### 2. Material and Methods

#### i) Chemicals and reagents

Compounds of natural origin (LE, HC, ThA, TrA, and IS) were prepared (≥ 98% purity) according to the literature (Lima *et al.*, 2014; Machado *et al.*, 2015) [11, 13] in the laboratory of Prof. Dr. Damião Pergentino de Sousa. Ethanol, D-glucose, potassium dichromate, sodium chloride and potassium dihydrogen phosphate were purchased from Merck, Germany. Disodium hydrogen phosphate and potassium chloride were from Bio Basic Inc, Canada. Bromocresol purple, D-biotin, L-histidine and sodium azide were purchased from Scharlau Chemie S.A, Spain. Triton X-100 was from IBI scientific, U.S.A. While, D-biotin, magnesium sulfate, trisodium citrate, sea salt and ammonium sulfate were bought from Sigma-Aldrich, Germany.

### ii) Hemolytic activity

Hemolytic activity of these compounds was studied according to the method used by Shahid *et al.* (2013)<sup>[19]</sup> and Zuber *et al.* (2014)<sup>[27]</sup>. Fresh blood (3 ml) was collected from volunteers with their consent. Blood was centrifuged for 5 min at 1000g, plasma was discarded and cells were washed three times with 5 ml of chilled (4°C) sterile isotonic phosphate-buffered saline (PBS) pH 7.4. Red blood cells (RBCs) were maintained at 10<sup>8</sup> cells mL<sup>-1</sup> for each assay. Stock solutions were prepared by adding 100 mg of each test compound in 1 ml DMSO, separately. Dilutions of 10, 20 and 40 mg mL<sup>-1</sup> were prepared out of these stock solutions. 100 µl of each test dilution was mixed with washed RBCs (10<sup>8</sup> cells mL<sup>-1</sup>) separately. Samples were incubated for 35 min at 37°C and agitated after 10 min. Immediately after incubation the samples were placed on ice for 5 min then centrifuged for 5 min at 1000g. Supernatant (100 µl) was taken from each tube and diluted 10 times with chilled (4°C) PBS. Triton X-100 (0.1% v/v) was used as positive control and PBS was used as negative control. The absorbance of test and control samples were noted at 576 nm using µQuant (Biotech, USA). The % RBCs lysis for each sample was calculated.

$$\% \text{ Hemolysis} = \frac{A_b}{A_c} \times 100$$

Where, A<sub>b</sub> is sample absorbance and A<sub>c</sub> is control absorbance

### iii) Brine shrimp lethality bioassay (BSLB)

BSLB was performed by following the procedure given by Ullah *et al.* (2013)<sup>[25]</sup>. Shrimp eggs were hatched in a plastic jar of 3 liter capacity. Egg suspensions (2 mg) were introduced into the plastic jar. Sea water (3.8% sea salt), oxygen and light were provided to the environment for hatching of eggs to produce nauplii after 24 h of incubation. A stock solution of each test compound was prepared at 1 g in 10 ml normal saline, separately. Subsequently, 0.25, 0.5, 1, 2, and 4% dilutions were prepared from each stock solution. Larvae (n=10) were added into each sterilized test tube along with 4.5 ml of sea water then 0.5 ml of each sample concentration was poured into test tubes. These test tubes were incubated for 24 h at room temperature with proper aeration. In this assay, water and methanol were taken as negative controls. Potassium dichromate was used as positive control. After 24 h, the nauplii were examined against a lightened background with a magnifying glass and an average number of survived larvae were determined. The compounds of natural origin were classified as toxic if median lethal concentration (LC<sub>50</sub>) value was less than 1 mg mL<sup>-1</sup> and non-toxic if LC<sub>50</sub> value was more than 1 mg mL<sup>-1</sup>. The LC<sub>50</sub> value was calculated with probit method.

### iv) Mutagenic activity

Mutagenic activity was based on the validated Ames bacterial reverse-mutation test but was performed entirely in liquid culture by using fluctuation test (Mohd-Fuat *et al.*, 2007)<sup>[16]</sup>. Two mutant strains of *Salmonella typhimurium* TA98 and

TA100 were used in this assay. The bacteria were maintained on nutrient agar at 3 ± 1°C. The bacteria were inoculated in nutrient broth and incubated at 37°C for 18-24 h prior to the test. Following chemicals were used in this test: Davis-mingioli salt (5.5 times concentrated), D-glucose (40%, w/v), bromocresol purple (2 mg mL<sup>-1</sup>), d-biotin (0.1 mg mL<sup>-1</sup>) and l-histidine (0.1 mg mL<sup>-1</sup>). Sodium azide (NaN<sub>3</sub>, 0.5 µg 100 µl<sup>-1</sup>) and potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, 30 µg 100 µl<sup>-1</sup>) used as positive control for *S. typhimurium* TA100 and for *S. typhimurium* TA98, respectively. All chemicals were kept at 3 ± 1°C until used.

### v) Preparation of reagent mixture

Davis-mingioli salt (21.62 ml), d-glucose (4.75 ml), bromocresol purple (2.38 ml), d-biotin (1.19 ml) and l-histidine (0.06 ml) was mixed aseptically in a sterile bottle. Reagent mixture, test compounds (10 mg in 1 ml of DMSO), sterile distilled water and standard mutagen were mixed in several bottles in specific amounts and inoculated with an overnight culture broth of *S. typhimurium* test strains. The content of each bottle was dispensed into each well of a 96-wells micro titration plate and the plates were incubated at 37°C for 4 days. Observations were made by analyzing the color changes of the dye. For the test compounds to be mutagenic, the number of positive wells has to be significantly higher than the number of positive wells in the background plate (spontaneous mutations).

### Statistical analysis

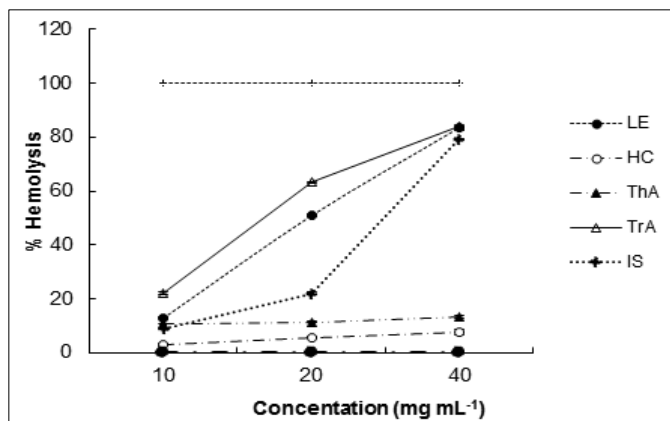
Data obtained was subjected to microsoft excel 2007 for analysis. Probit analysis was applied on BSLB data.

### 3. Results

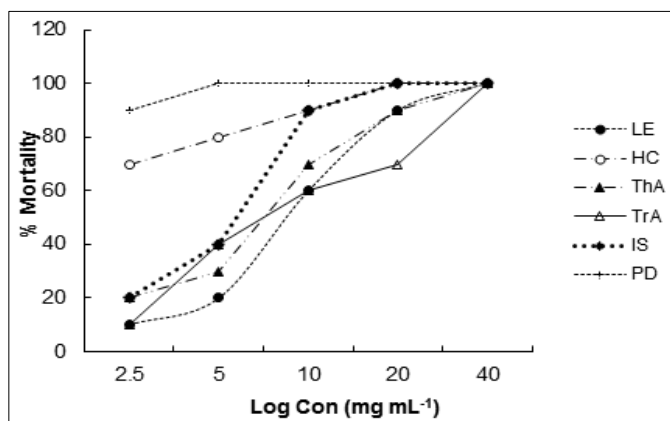
The percent hemolysis at different concentrations of tested compounds is presented in Figure 1. HC and ThA produced about 10% hemolysis at all the tested concentrations. An increase in percent hemolysis was observed with increased concentrations of IS, LE and TrA but all these compounds produced less than 20% hemolysis at a concentration of 10 mg mL<sup>-1</sup>. The synthetic compounds of natural origin caused mortality of shrimp larvae after 24 h.

The log concentration of the chemicals and percent mortality is presented in Figure 2. Potassium dichromate showed higher mortality at the lowest concentration (0.29 mg mL<sup>-1</sup>) and was used as positive control while negative control showed no mortality. The LC<sub>50</sub> values for these compounds as calculated with probit method were 7.95, 1.05, 6.38, 3.92 and 4.79 mg mL<sup>-1</sup> for LE, HC, ThA, TrA and IS, respectively. All the tested compounds were found to be nontoxic as the LC<sub>50</sub> were more than 1 mg mL<sup>-1</sup>.

In the mutagenic activity, yellow or turbid wells were scored as positive, while purple wells were scored as negative. The test compounds were considered toxic to the test strains if all wells in the test strain showed purple coloration. All test compounds were found non-mutagenic but some compounds were toxic to bacterial strains as presented in Table 1.



**Fig 1:** Percent RBC hemolysis for five synthetic compounds of natural origin, PBS and T-X100 at concentrations of 10, 20 and 40 mg mL<sup>-1</sup>.



**Fig 2:** Plot of log concentration of five synthetic natural compounds and PD versus percent shrimp mortality after 24 h of exposure.

**Table 1:** Mutagenicity test of five synthetic compounds of natural origin by using *S. typhimurium* TA98 and TA100.

Treatments	Number of positive wells/total number of wells		Results	
	TA98	TA100	TA98	TA100
Background 1	20/96	25/96		
Background 2	20/96	27/96		
Standard*	94/96	93/96	+	+
LE	20/96	0/96	-	t
HC	1/96	0/96	-	t
ThA	0/96	0/96	t	t
TrA	0/96	0/96	t	t
IS	1/96	0/96	-	t

+ Significant increase in positive wells, compared to the related background ( $p < 0.05$ ); - no significant effect observed, t toxic to TA98 and T toxic to TA100. \*Sodium azide for *S. typhimurium* TA100 and potassium dichromate for *S. typhimurium* TA98.

**4. Discussion**

Amin and Dannenfeler (2006) [2] gave a guideline to correlate a formulation’s toxicity with their hemolytic values. If the hemolytic value is less than 10%, then the formulation is considered as non-hemolytic but if the value is more than 25%, then considered as hemolytic. Furthermore, the hemolytic assay has been reported as valid assay for *in vitro* toxicity in literature (Singh and Kaur 2008). With this assay, LE was found non-toxic to human RBCs because at a concentration of 1% v/v LE produced 13% hemolysis which is less than the

toxic limit. But at higher concentrations the percent hemolysis increased above the toxic level. Present study was supported by the findings that high concentrations of citral showed 100% hemolysis probably by a non-specific steroid-terpenoid or glutathione depletion mechanism (Tamir *et al.*, 1984) [22]. HC was found non-toxic at all the tested concentration because the percent hemolysis remained lower than the prescribed toxic level. In most of pharmaceutical and cosmetic products HC is used below 1% that is non-toxic level. ThA was also found non-toxic as percent hemolysis was 10.63, 11.5, and 13.48% at concentrations of 10, 20 and 40 mg mL<sup>-1</sup>, respectively. The hemolytic assay showed that 1% TrA produced 22.1% hemolysis. With increase in the concentration of TrA, hemolytic activity was increased to 61.9 and 84.25%. (Figure 1). This is supported by the fact that reducing xenobiotics such as phenols, are capable of promoting hemolysis through oxidation of hemoglobin, forming methemoglobin (Bukowska and Kowalska 2004) [5]. Although TrA showed alarming percent hemolysis but at lowest concentration it was below the toxic level so it must be used in concentrations below 1%. Surfactants cause hemolysis through dissolution of the erythrocyte plasma membrane which ruptures due to increased fragility or due to osmotic lysis caused by increased permeability of the plasma membrane (Aparicio *et al.*, 2005) [3]. The erythrocyte lysis in the rats has been reported due to peroxy radical formation. These above mechanisms may account for some of the hemolytic activity reported in this study (Cheung *et al.*, 2003) [6].

To evaluate the possible cytotoxic potential of crude extracts and isolated natural compounds the brine shrimp lethality bioassay is of utmost importance. The crude extracts and pure substances classified as toxic if LC<sub>50</sub> value less than 1 mg mL<sup>-1</sup> and non-toxic if LC<sub>50</sub> value more than 1 mg mL<sup>-1</sup> (Meyer *et al.*, 1982). The results showed 100% mortality of all shrimp’s larvae at 40 mg mL<sup>-1</sup> concentrations of LE, HC, ThA, TrA and IS. In the present study, LC<sub>50</sub> of HC was 1.05 mg mL<sup>-1</sup>. In the present study LC<sub>50</sub> of LE is 7.95 mg mL<sup>-1</sup> and ThA is 6.38 mg mL<sup>-1</sup> which is also more than the prescribed toxic limit for this assay. The LC<sub>50</sub> for IS was 3.00 ml kg<sup>-1</sup> (Luebke 2011) [12] in comparison to the present investigation where LC<sub>50</sub> for IS and TrA is 4.79 and 3.92 mg mL<sup>-1</sup>, respectively.

Since mutagenic compounds can potentially induce cancer, so the identification of chemicals or compounds which are capable of inducing mutation is crucial in the safety assessment (Sugimura 2000) [21]. The Ames test is conducted by using *Salmonella typhimurium*. It is a widely used bacterial assay with high predictive value when comparing with rodent carcinogenicity test and for the identification of chemicals that can produce gene mutations (Leach *et al.*, 1993) [10]. The *S. Typhimurium* TA98 strain was used to observe frame shift mutation while TA100 strain identified base-pair substitution. It was found that there were no yellow wells in the blank plate which indicated no contamination during test procedure but in the background plate, there were 20 yellow wells for TA98 and 25-27 yellow wells for TA100 which indicated spontaneous mutation (Table 1). In the present study 10 mg mL<sup>-1</sup> concentration of all the tested compounds was non-mutagenic. For a compound to be mutagenic in this assay, number of positive valves in test plate should be significantly higher than the number of positive valves in the background plate. The US national toxicology program (NTP) reported that gavage exposure of male rats to limonene was linked with dose-

responsive increases in nephropathy, renal hyperplasia and renal tumors. The mechanism of tumorigenesis was the result of an accumulation of  $\alpha$ 2u-globulin in renal proximal tubules (Turner *et al.*, 2001) [24]. LE was non-mutagenic because the numbers of yellow wells were less than the number of positive wells in the background that is 20 yellow wells against TA98 which were not double than the positive well in the background. 10 mg mL<sup>-1</sup> of LE was toxic to TA100 because there was no change in the color which means it is bactericidal (Mohd-Fuat *et al.*, 2007) [16]. Alkaloids  $\beta$ -carboline was reported to be mutagenic and genotoxic as well (Boeira *et al.*, 2002) [4]. *Salmonella* mammalian microsome test (Ames test) was used to test the mutagenic properties of seventy six compounds which are used as artificial flavoring substances in food products (including HC). HC was found non mutagenic (Wild *et al.*, 1983) [26] similar to our results (Table 1). The Ames *salmonella*/microsome test was performed to evaluate the antigenotoxic and genotoxic properties of essential oils of *Origanum onites* L (contain TA). No mutagenic activity was found in screening with *Salmonella typhimurium* strains TA98 and TA100 (Ipek *et al.*, 2005) [9] similar to our findings. Our findings are further supported by the fact that 19  $\mu$ g TrA was found non mutagenic when tested against *Bacillus subtilis* H17 and M45 (Marnett *et al.*, 2014) [14].

## 5. Conclusion

The results of the present investigation suggested that all the tested compounds are non-toxic to human RBCs and brine shrimp larvae where as non-mutagenic to *S. typhimurium* TA100 and *S. typhimurium* TA98 strains. So these chemicals might be safely used for their pharmaceutical or cosmetic benefits.

## 6. References

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