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A Possible Phytol-cytoprotective Trait through Reactive Species-Induced Oxidative Stress Ebbing Pathway

ORIGINAL

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Abstract

This study aims at investigating a possible pathway of cytotoxicological status of the diterpenoid essential oil, phytol (PYL). For this brine shrimp lethality bioassay (BSLB) and hemolysis (HL) test systems were selected. In the BSLB, PYL either alone or co-treated with ethylenediaminetetraacetic acid (EDTA), potassium di-chromate ($K_2Cr_2O_7$; KD), copper sulphate ($CuSO_4.5H_2O$; CS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, TRO) as membrane lyser, strong oxidizer, oxidizer-cytogenotoxicant and antioxidative-cell-protestant, respectively. The HL was carried out in rat erythrocytes (RBCs) taking TRO as a standard. In addition, to view a time-dependent cytotoxic activity of PYL, the mortality of the shrimps was counted at 24 and 48 h. Results suggest PYL is non-cytotoxic at low (40-160 µM) but toxic at high concentration (2-8 mM) to the shrimps and RBCs. An increased cytotoxicity was observed for 24 h to 48 h in brine shrimps. In both cases groups co-treated with cytotoxicants/protestant suggest that PYL is cytoprotective in the presence of oxidizer. The cytoprotectivityof PYL may be connected to its antioxidant potential and cytotoxicity for antioxidant-mediated pro-oxidative effects. In conclusion, PYL is cytoprotective at low concentration but toxic at high, activities found, however, may be linked to the radical scavenging pathway.

Keywords
Brine Shrimp; Cytotoprotective; Oxidative Stress; Phytol; Toxicity

Introduction

Phytol (3, 7, 11, 15-tetramethylhexadec-2-en-1-ol, PYL) is the diterpenoid with evident antioxidant, antimicrobial, cytotoxic and weak mutagenic effects [1]. A substance with such type of activities may

promote overall health [2]. Frequently, essential oils (EOs) are more antioxidative and cytotoxic rather than genotoxic. The diterpenic EOs are also well-known for such type of activities. However, they often have no specific cellular targets may be due to their high hydrophobicity leading to readily permeable of the cell membranes [3].

The brine shrimp lethality bioassay (BSLB) is a popular test system for a wide variety of synthetic, semi-synthetic, and natural substances from inorganic and organic origins due to its rapidity, reliability, economy, simplicity, convenience, and applicability [4,5]. Arthropods, the live nauplii (shrimps) are the test system in this purpose. Artificially, the shrimp cysts are undergone for the production of nauplii in brine solution. Live naupli of 24-48 h aged are considered for the better susceptible to the test substances. Finally, the percentage lethality is determined by comparing the mean-surviving larvae of tested and control marked samples.

Otherwise, the hemolysis (HL) test system in rat's erythrocytes (RBCs) is also a popular method to assay the antioxidant behavior of a wide variety of substances. It is due to the rapidity, economy, simplicity, convenience and its wider range of applicability [6]. The stability RBCs is greatly affected by a number of exterior/interior factors such as – temperature, type and charges of chemical substances, and so on. In this occasion hydrogen peroxide (H_2O_2), a strong hydroxyl radical ($^{\bullet}OH$) inducer is used to produce oxidative stress to the RBCs. The $^{\bullet}OH$ eventually binds with metal ions randomly distributed in the DNA, thus implicates DNA damage through the single strand break (SSB) [7].

In this paper, we use a number of cytotoxicants, and a marked cytoprotective to determine the possible cytotoxicological behavior of PYL.

Materials

Reagents and chemicals

Tween-80 (0.05%) dissolved in saline solution (0.9% NaCl) served as the vehicle for PYL and TRO, while distilled water (DW) was used as solvent for others. Phytol (PYL), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TRO), ethylenediaminetetraacetic acid (EDTA), potassium dichromate ($K_2Cr_2O_7$; KD), copper sulfate ($CuSO_4.5H_2O$; CS) and all the necessary reagents and chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Shrimp cysts

For *Artemia salina* preparation, shrimp cysts were purchased from the local market of Teresina, Brazil.

Experimental animals

The HL inhibition test was performed in eight week-old Wister albino rats (*Rattus norvegicus*; body weight: 180–220 g) from the Central Animal House of the Federal University of Piaui, Brazil. The animals were allowed free access to food (Purina, Brazil) and water *ad libitum* and were kept under controlled lighting (12 h dark/light cycles) at 24 ± 2 °C.

Preparation of test samples

In BSLB test, PYL, cytotoxicants (EDTA, KD, CS) and antioxidant/cytoprotective (TRO) were prepared in 40, 80 and 160 µM in step 1, while in the step 2 it was 2, 4 and 8 mM. Step 3 was continued with PYL at 40-160 µM and 2-8 mM concentrations. HL test was performed with PYL and/or TRO at 40-160 µM and 2-8 mM concentrations. In all cases, all the lateral co-treatments were considered at 80 µM.

Methods

Brine shrimp lethality bioassay (BSLB)

This a slight modification of the method described by Meyer et al. [4]. Briefly, brine shrimp (*Artemia salina*) were hatched using shrimp eggs in a beaker (0.5 L), filled with artificial sea water containing a 50:50 mixture of brine solution (23.0 gm NaCl, 11.0 gm $MgCl_2.6H_2O$, 4 gm Na_2SO_4, 1.3 gm $CaCl_2.2H_2O$, 0.7 gm KCl in 1L distilled water

and adjusted to pH 8.5 using 1N Na_2CO_3) and mineral water under constant aeration for 48 h at 27 ± 3 ºC. After hatching, active nauplii free from egg shells was collected from a brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a Pasteur's pipette and placed in each test tube containing 4.5 mL of the brine solution. In each experiment, 0.5 mL of the test sample was added and maintained at the same temperature as hatching under the light and surviving larvae were counted.

Step 1. Grouped as: PYL*, EDTA*, KD*, CS*, PYL*+EDTA, PYL*+KD, PYL*+CS, TRO*+KD, TRO*+CS, KD*+PYL+ TRO, CS*+PYL+ TRO for Gr-I, Gr-II, Gr-III, Gr-IV, Gr-V, Gr-VI, Gr-VII, Gr-VIII, Gr-IX, Gr-X and Gr-XI, respectively. Staric (*) mark indicates treatment with 40, 80 and 160 µM; while un-starics for 80 µM.

Step 2. Grouped as: PYL*,PYL*+EDTA, PYL*+KD, PYL*+CS, PYL*+ TRO, PYL*+ TRO+KD and PYL*+ TRO+CS for Gr-XII, Gr-XIII, Gr-XIV, Gr-XV, Gr-VI, Gr-XVII and Gr-XVIII respectively. Staric (*) mark indicates treatment with 2, 4 and 8 mM; while un-starics for 80 µM.

Step 3. Gr-I and Gr-XII from the step 1 and 2 were preceded on to the next day.

The mortality of *A. salina* was counted at 24 h in step 1 and 2; while 24 and 48 h were considered for step 3. Experiments were conducted along with a negative control (vehicle treated, NC), different concentrations of the test substances in a set of three tubes per dose. Along with percentage lethality, the LC_{50} (concentration causes 50% mortality) values were obtained from the best-fit line plotted concentration verses percentage lethality.

Hydrogen peroxide (H_2O_2) inducedhemolysis (HL) test inraterythrocytes (RBCs)

To test the inhibition of HL induced by H_2O_2, blood was collected from the retro-orbital plexus of anesthetized (sodium pentobarbital 35 mg/kg; intra-peritoneal) Wistar rats, and the RBCs were prepared for 10% suspension in phosphate buffer solution (PBS) (pH 7.4). Next, 0.15 mL H_2O_2 (200 mM in PBS; pH7.4) and 0.2 mL of the sample solution was added to 0.5 mL of the 10% RBC suspension. The reaction mixture was incubated at 37 °C for 30 minutes and immediately centrifuged at 2,500 rpm for 3 minutes. Then, 0.2 mL of the supernatant was mixed with 2.8 mL PBS (pH 7.4) and the absorbance was measured at 475 nm [6]. For the NC, 0.2 mL of the vehicle was added. The percent rate of hemolysis was calculated using the following formula, taking into account that 100% hemolysis was induced by H_2O_2 (blank):

$$\text{Inhibition of hemolysis (\%)} = [(Abs_{blank} - Abs_{sample}) \times 100] / Abs_{blank}$$

where, Abs_{blank} is the control absorbance with 100% hemolysis induced by H_2O_2 and reactive Abs_{sample} is the absorbance of aliquots containing various concentrations of the sample being studied.

This experiment was conducted with the groups as – PYL*, TRO* and PYL*+ TRO. Staric (*) mark indicates treatment with 40-160 µM and 2-8 mM; while un-staric for 80 µM.

Statistics

Results were expressed as mean ± standard deviation (SD). In order to determine differences, data were compared by One-way Analysis of Variance (ANOVA) followed by the Newman-Keuls test ($p < 0.05$)

Results

BSLB test

Table 1 indicates the step 1 treatment, where groups (Gr-I to Gr-XI) are treated with PYL, membrane destabilizer (EDTA), oxidant (KD), cytogenotoxicant (CS) and cytoprotectant (TRO). Mortality was counted after a 24 h exposure, indicating PYL (Gr-I) at all

Table 1. Effect of cytotomodulators and phytol at low concentration in the brine shrimps.

Treatments	% mortality of nauplii			IC_{50} (CI, R^2)
	40 µM	80 µM	160 µM	
Gr-I: PYL*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
Gr-II: EDTA*	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 0.58	104.4 (-,-)
Gr-III: KD*	13.33 ± 0.58	23.33 ± 0.58	26.67 ± 0.58	40.08 ± 0.27 (28.80-55.79, 0.99)
Gr-IV: CS*	3.33 ± 0.58	13.33 ± 1.15	20.00 ± 1.00	64.62 ± 0.51 (35.22-118.6, 0.99)
Gr-V: PYL*+EDTA	0.00 ± 0.00	3.33 ± 0.58	6.67 ± 0.58	80.10 ± 1.02 (very wide, 1.00)
Gr-VI: PYL*+KD	6.67 ± 0.58	10.00 ± 1.00	16.67 ± 0.58	53.62 ± 0.99 (3.05-943.0, 0.89)
Gr-VII: PYL*+CS	3.33 ± 0.58	10.00 ± 1.00	16.67 ± 0.58	66.86 ± 0.79 (20.74-215.5, 0.98)
Gr-VIII: TRO*+KD	3.33 ± 0.58	6.67 ± 0.58	13.33 ± 0.58	70.58 ± 1.25 (6.50-765.9, 0.98)
Gr-IX: TRO*+CS	3.33 ± 0.58	6.67 ± 0.58	16.67 ± 0.58	82.83 ± 2.14 (7.86-872.8, 0.92)
Gr-X: KD*+PYL+ TRO	3.33 ± 0.58	3.33 ± 0.58	10.00 ± 1.00	78.08 ± 2.06 (0.51-11986, 0.72)
Gr-XI: CS*+PYL+ TRO	3.33 ± 0.58	6.67 ± 0.58	16.67 ± 0.58	82.83 ± 2.14 (7.86-872.8, 0.92)
NC (0.5 mL of vehicle)		0.00 ± 0.00		-

*indicates test concentration 40-160 µM; non-star indicates treatment with 80 µM (except NC); PYL: phytol; EDTA: ethylene-di-amine tetraacetate; KD: potassium dichromate ($K_2Cr_2O_7$); CS: copper sulphate ($Cu_2SO_4.5H_2O$); TRO: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); NC: negative control (0.05% tween-80 dissolved in 0.9% NaCl solution) values are mean ± standard deviation (SD); LC_{50}: concentration produced 50% lethality; CI: confidence interval (95% confidence levels); R_2: coefficient of determination.

the tested doses remain non-cytotoxic. EDTA (Gr-II), the membrane destabilizer produces 3.33% mortality with the 160 µM dose. Highest cytotoxicity was observed in the Gr-III, treated with KD. The KD at 40, 80 and 160 µM exhibited percentage mortality by 13.33 ± 0.58, 23.33 ± 0.58 and 26.67 ± 0.58, respectively. The group treated with CS (Gr-IV) was also produced cytotoxicity to the brine shrimps. However, the activity was lower than the Gr-III. CS produced highest percentage mortality with the dose 160 µM by 20.00 ± 1.00. The PYL groups treated with 80 µM of EDTA (Gr-V), KD (Gr-VI) and CS (Gr-VII), percentage mortality increased in the order of Gr-VI>Gr-VII>Gr-V, where Gr-V was almost similar to the group treated alone with EDTA (Gr-II). TRO at 40-160 µM when co-treated with 80 µM of KD (Gr-VIII) and CS (Gr-IX), Gr-VIII was more active in cytoprotection than the Gr-IX. The KD (Gr-X) and CS (Gr-XI) were co-treated with PYL (80 µM)+ TRO (80 µM) in which Gr-XI other than Gr-X remained unchanged in mortality rate of the naupli when compared to the Gr-VII and Gr-IX.

In the step 2, Gr-XII treated with PYL in 2, 4 and 8 mM exhibited percentage mortality by 10.00 ± 1.00, 23.33 ± 0.58 and 43.33 ± 1.15, respectively, at 24 h. with an exception with the dose 8 mM, PYL in the Gr-XIII co-treated with 80 µM of EDTA produced similar mortality rates to the Gr-XII. However, PYL when co-treated with KD (80 µM) (Gr-XIV), it decreased the mortality rates, where percentage mortality was calculated as 6.67 ± 0.58, 20.00 ± 1.00, and 33.33 ± 0.58 with the 2, 4 and 8 mM of PYL, respectively. Group XV suggests that PYL (2-8 mM) co-treated with CS (80 µM) produces the mortality rates similar (with an exception with dose 4 mM) to that of the Gr-XIII, treated with EDTA. PYL co-treated with TRO (80 µM) (Gr-XVI) increased the mortality rates of shrimps at all doses, however; where the highest percentage of mortality was observed at 8 mM by 46.67 ± 0.58. Gr-XVII tells the PYL co-treatment with TRO (80 µM) + KD (80 µM) in which the mortality was found to be between the Gr-XIV and Gr-XII. However, PYL when co-treated with TRO (80

Table 2. Effect of cytotomodulators on high concentration of phytol in the brine shrimps.

Treatments	% mortality of nauplii			IC_{50} (CI, R^2)
	2 mM	4 mM	8 mM	
Gr-XII: PYL*	10.00 ± 1.00	23.33 ± 0.58	43.33 ± 1.15	3.46 ± 1.07 (0.53-22.56, 0.95)
Gr-XIII: PYL*+EDTA	10.00 ± 0.00	23.33 ± 0.58	40.00 ± 1.00	3.25 ± 0.89 (0.61-17.19, 0.96)
Gr-XIV: PYL*+KD	6.67 ± 0.58	20.00 ± 1.00	33.33 ± 0.58	3.34 ± 0.79 (1.04-10.78, 0.98)
Gr-XV: PYL*+CS	10.00 ± 1.00	26.67 ± 0.58	40.00 ± 1.00	3.01 ± 0.60 (1.11-8.20, 0.99)
Gr-XVI: PYL*+ TRO	16.67 ± 0.58	33.33 ± 0.58	46.67 ± 0.58	3.13 ± 1.28 (0.08-118.6, 0.84)
Gr-XVII: PYL*+ TRO+KD	13.33 ± 0.58	30.00 ± 1.00	36.67 ± 0.58	2.41 ± 0.29 (1.63-3.56, 0.99)
Gr-XVIII: PYL*+ TRO+CS	13.33 ± 0.58	30.00 ± 1.00	43.33 ± 0.58	2.79 ± 0.58 (0.93-8.31, 0.98)
NC (0.5 mL of vehicle)	0.00 ± 0.00			-

*indicates test concentration 2-8 mM; non-star indicates treatment with 80 µM (except NC); PYL: phytol; KD: potassium dichromate ($K_2Cr_2O_7$); CS: copper sulphate ($Cu_2SO_4.5H_2O$);); TRO: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); NC: negative control (0.05% tween-80 dissolved in 0.9% NaCl solution) values are mean ± standard deviation (SD); LC_{50}: concentration produced 50% lethality; CI: confidence interval (95% confidence levels); R_2: coefficient of determination.

Table 3. Time and concentration dependent cytotoxicity of phytol in brine shrimps..

Treatment at hour	% mortality of nauplii			IC_{50} (CI, R^2)
	40 µM	80 µM	160 µM	
PYL at 24 h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
PYL at 48 h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
	2 mM	4 mM	8 mM	
PYL at 24 h	10.00 ± 1.00	23.33 ± 0.58	43.33 ± 1.15	3.46 ± 1.07 (0.53-22.56, 0.95)
PYL at 48 h	13.33 ± 0.58	30.00 ± 1.00	46.67 ± 0.58	2.97 ± 0.72 (0.74-12.01, 0.97)
NC (0.5 mL of vehicle) at 24 h	0.00 ± 0.00			-
NC (0.5 mL of vehicle) at 48 h	0.00 ± 0.00			-

PYL: phytol; NC: negative control (0.05% tween-80 dissolved in 0.9% NaCl solution) values are mean ± standard deviation (SD); LC_{50}: concentration produced 50% lethality; CI: confidence interval (95% confidence levels); R_2: coefficient of determination.

µM) + CS (80 µM) (Gr-XVIII) causes an increase in mortality by 2 and 4 mM concentrations, keeping 8 mM unchanged in comparison to the Gr-XII. An increased mortality rate of Gr-XVIII also observed when compared to the Gr-XV (PYL treated with CS alone) **(Table 2)**.

The data shown in the **Table 3** are for the step 3, in which Gr-I and Gr-XII were continued with a 24 more to count the mortality of the nauplii. Data suggest, PYL at low concentrations (40-160 µM, Gr-I) produces zero (0) mortality to the live nauplii. Otherwise, at 2, 4 and 8 mM of PYL increased the mortality rates by 33.3, 66.7 and 33.4%, respectively.

No mortality of the shrimps was observed in the NC group treated with the vehicle at 24 and 48 h.

HL test

Data presented in **Table 4** indicates that all the treatment groups inhibit lysis of RBCs. In PYL* group the highest inhibition of HL was observed with 160 µM by 58.85 ± 0.01, while the lowest with 8 mM by 10.61 ± 0.01. The lysis inhibition by the PYL observed ordered by the concentrations as 160 µM>2

Table 4. Oxidative stress-induced erythrocytes lysis assay.

Treatment at hour	% mortality of nauplii			% mortality of nauplii		
	40 µM	80 µM	160 µM	2 mM	4 mM	8 mM
PYL*	22.91 ± 0.01[a]	30.54 ± 0.01[a]	58.85 ± 0.01[a]	40.41 ± 0.01[a]	25.51 ± 0.01[a]	10.61 ± 0.01[a]
	IC50: 58.74 ± 1.24 (CI: 1.27-2709, R2: 0.83)			IC50: 5.21 ± 0.73 (CI: 1.35-20.07, R2: 0.97)		
TRO*	32.22 ± 0.01[a,b,d]	40.60 ± 0.01[a,b]	64.80 ± 0.01[a,b]	60.89 ± 0.01[a,b,d]	48.04 ± 0.01[a,b,d]	21.79 ± 0.01[a,b,d]
	IC50: 44.37 ± 1.03 (CI: 0.79-2498, R2: 0.83)			IC50: 6.49 ± 0.36 (CI: 3.73-11.28, R2: 0.99)		
PYL*+ TRO	26.26 ± 0.01[a,b]	46.93 ± 0.01[a,b,c]	69.46 ± 0.01[a,b,c]	49.91 ± 0.01[a,b]	28.31 ± 0.01[a]	15.27 ± 0.01[a,b]
	IC50: 51.98 ± 0.72 (CI: 9.58-282.1, R2: 0.96)			IC50: 5.14 ± 1.00 (CI: 0.52-50.83, R2: 0.93)		

[a]: p<0.05 compared to NC (0.05% tween 80 in 0.9% NaCl); [b]: p<0.05, compared to Gr-I; cp<0.05, compared to Gr-II; d: p<0.05, compared to Gr-III (ANOVA and *t*-Student-Newman-Kewls as *post hoc* test); PYL: phytol; TRO: trolox; IC_{50}: half minimal inhibitory concentration; CI: confidence interval (95% confidence levels);); R_2: coefficient of determination; values are the mean ± standard deviation (SD) (n=5).

mM> 80 µM> 4 mM> 40 µM> 8 mM. Otherwise, TRO* showed lysis inhibition in the order of 160 µM> 2 mM> 4 mM> 80 µM> 40 µM> 8 mM. PYL, when co-treated with TRO (80 µM) it, exhibited highest percentage inhibition of HL with 160 µM by 69.46 ± 0.01 then followed by 49.91 ± 0.01, 46.93 ± 0.01, 28.31 ± 0.01, 26.26 ± 0.01 and 15.27 ± 0.01 with 2 mM, 80 µM, 4 mM, 40 µM and 8 mM, respectively.

Discussion

The BSLB is extensively used test system in the primary concerning in the toxicity assessment of crude as well as isolated compounds in brine shrimps. Furthermore, the cytotoxic compounds having significant sensitivity towards the shrimps, this assay can be recommended as a guide for the detection of antitumor and pesticidal compounds [4]. In addition, this bioassay has a great correlation to the human solid tumor cell lines. Specifically, BSLB acts through the inhibition in the embryonic development stages as the agents tested in this system are considered to have ovicidal and larvicidal properties [8]. Mainly, the mortality of the larvae in BSLB relates to- a) detergent/destabilization activity of the test substances which may cause lysis/ protrusion of the cell membrane, b) overproduction of reactive oxygen/nitrogen species (ROS/RNS) leading to the oxidative damage and/or prooxidative effects; causing destruction of the genetic materials such as proteins, lipids and DNA, and RNA other related phenomena. It is to be mentioned that those are the main causes of cyto-/genotoxic damage of the eukaryotic cells.

In this study, we firsty performed a cytotoxicological study of PYL in BSLB. In addition, for further clarification, we carried out HL analysis, which is a well-known •OH-induced hemolysis test system for substances with antiradical capabilities. Although, PYL is evident for its antimicrobial and cytotoxic activities [1] but the possible action mechanisms are yet to be found out. After going through this broad viewpoint, we carried out a three-step study, which was further divided into a number of treatment groups at different concentrations.

In the step 1 (data shown in **Table 1**), PYL (Gr-I) at low concentrations (40-160 µM) produced no mortality to the brine shrimps. In Gr-II, EDTA exhibited poor mortality only at the highest treated dose (160 µM), while in Gr-V with 80 and 160 µM. The EDTA mainly acts through membrane destabilization by fluidization and expansion effects. The intercalation of EDTA into the lipid membrane induces membrane curvature. Further loss of material from the lipid membrane facilitates membrane instability,

leading to membrane permeabilization and cell lysis [9]. Although the EDTA action on live nauplii was insignificant, but PYL at 80 µM, co-treated with EDTA did not inhibit the event. PYL may have no antagonistic interaction with EDTA. Furthermore, insignificant increased in mortality rates may be due to non-toxic effects of PYL at 80 µM as the membrane potential (MP) is an important parameter for illustrating the mechanism of action of cytotoxic molecules. Treatments that alter the MP can also change cellular functions. There are agents that may cause the MP to approach or become zero, such as carbonyl cyanidem-chlorophenylhydrazone [10], with the ultimate result being the loss of cellular materials, irregular cellular metabolism, and finally cell death. EDTA, other than PYL may act through this pathway.

Chromium (VI) compounds have been reported to be toxic and carcinogenic as it can readily pass through the cell membranes. Once inside the cell, Cr (VI) is reduced to its lower oxidation states [Cr (V)] and [Cr (IV)] and then Cr (III) by low molecular weight molecules, enzymatic and non-enzymatic reductants. These reactive chromium intermediates are capable of generating a whole spectrum of ROS; in excess of those can cause injury to cellular proteins, lipids and DNA lead to a state known as oxidative stress [11]. In a recent study, Rasool et al. [12] suggests that KD produces high malondialdehyde (MDA) and low levels of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] in male *Swiss* mice, leading to DNA damage in testicular cells, thus the adverse reproductive abnormalities in animals. Otherwise, ROS coming from the substances may implicate DNA damage; one of them is the $^{\bullet}OH$ which eventually binds with metal ions randomly distributes in the DNA; the ultimate result is the single strand break (SSB) [7], whichever also evident for oxidative DNA modification [13]. Pejin et al. [14] suggested that PYL is a potent radical scavenger, which potentially scavenged $^{\bullet}OH$, superoxide anion ($^{\bullet}O_2^-$), methoxy ($^{\bullet}CH_2OH$), carbon-dioxide anion ($^{\bullet}CO_2^-$), nitric-oxide ($NO^{\bullet}$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. In our study, Gr-III treated with KD exhibits higher mortality rates at all tested concentrations. However, KD in Gr-VI, Gr-VIII and Gr-X when co-treated with PYL (80 µM), TRO (80 µM) and PYL (80 µM) + TRO (80 µM) reduced the mortality rates to the shrimps. The observed activity was more prominent with the order of PYL + TRO>TRO>PYL. TRO is a well-known antioxidant for its reactive oxidisable species capturing capacity. Thus, PYL and TRO may protect the nauplii through the pathway above mentioned.

It is noteworthy that the formation of ROS in biological systems can be accomplished by copper-(II) (Cu^{2+}) catalysis, with the consequent cytotoxic response [15]. The genotoxicity of CS in *Allium cepa* root cells in a mild alkaline comet assay (pH 12.3), is evident for the single strand breaks DNA [16]. Cu (II) is also an inflammatory mediator to the cells [17].A study conducted with copper oxide nanoparticles (CuONPs) and CS in the arthropod, *Drosophila* indicates that both CuONPs and CS are genotoxic. There is a suggestion for oxidative stress- mediated damage to the cell macromolecules [18]. In our study, by considering Gr-V, VII, IX and XI, it should be said that CS has toxic effects inthe brine shrimps. Although, there is a reduced mortality rate observed in the Gr-VII, Gr-IX and Gr-XI co-treated with PYL (80 µM), TRO (80 µM) and PYL (80 µM) + TRO (80 µM) but the activity was almost similar except 40 and 80 µM doses treated with 80 µM PYL in the Gr-VII. Thus, the activities of both PYL and TRO may be more concerned with the neutralization of strong oxidizer, KD-mediated oxidizing effects.

In the step 2 (data shown in **Table 2**), high concentrations of PYL alone as well as co-treatments exhibited a dose-dependent cytotoxicity. In comparison to Gr-XII (PYL), Gr-XIII (PYL+EDTA), Gr-XIV (PYL+KD), Gr-XV (PYL+CS) and Gr-XVII (PYL+TRO+KD) decreased the mortality rates of the brine shrimps. The decreased order of the mortality rate was Gr-XIV> Gr-XVII>Gr-XIII>Gr-XV. Otherwise, PYL co-treated with 80 µM of TRO (Gr-XVI) exhi-

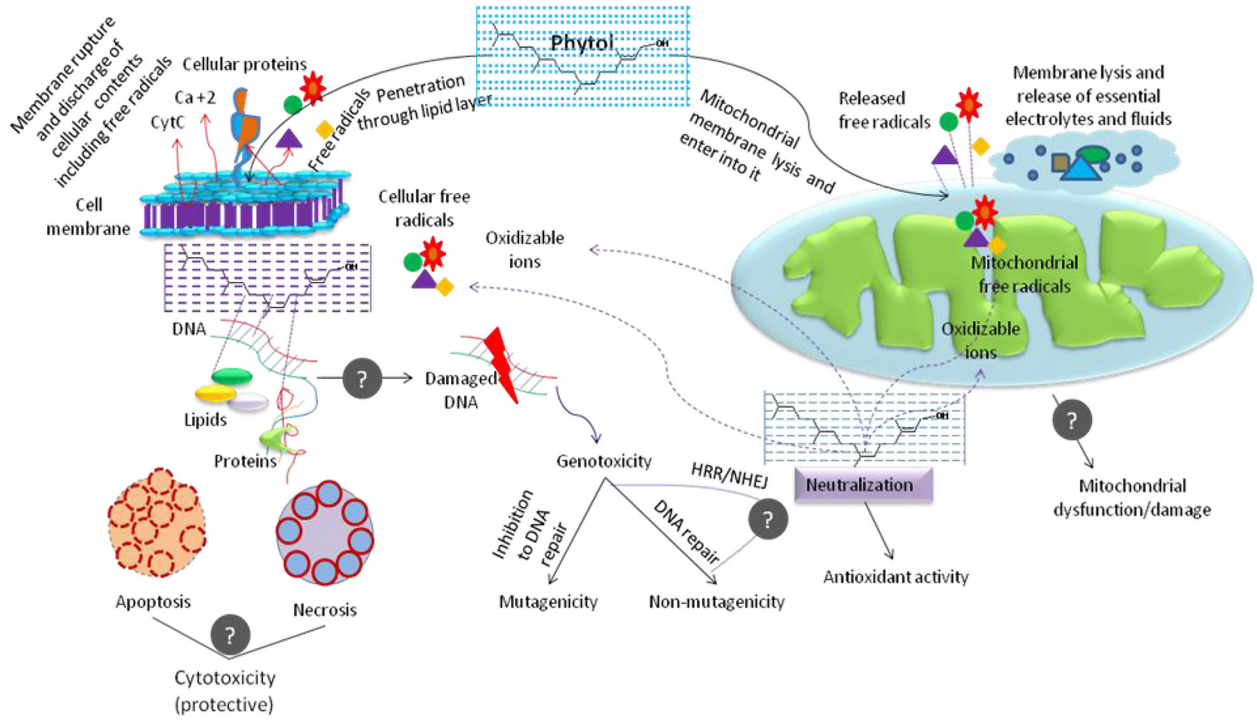
bited an increased percentage of mortality of the shrimps. The similar event was observed in case of Gr-XVIII; where PYL at 2 mM and 4 mM exhibited increased mortality rates remaining unchanged with 8 mM in comparison to the Gr-XII. The terpenoid EOs are the good examples of antioxidants. At low doses, this kind of EOs can act ascellular protectants as they scavenge reactive species harmful to the cell membrane. But EOs are hydrophpobic in nature, which at high concentrations capable to cross the cell as well as mitochondrial membranes and may destroy the cellular essential molecules and genetic materials [3]. It may be due to the fact that at low concentration there is a balance between the oxidative species and antioxidant molecules; but at high concentration oxidative radicals may contribute an obstacle to the molecules to be available to interact and cross the cell membranes. In addition, it may cause an increased in membrane permeability to external toxicants and internal cellular essentials. Bakkali et al. [3] suggested that in eukaryotic cells, EOs can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential. This affects ionic Ca^{++} cycling and other ion channels, eventually reducing the pH gradient, affecting the proton pump and the ATP pool, and changing the fluidity of membranes. The latter effect causes the membranes to become abnormally permeable, resulting in leakage of radicals, cytochrome C, Ca^{++}, and proteins, thus resulting in oxidative stress and bioenergetic failure. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis. Antioxidant-mediated this type of activity is called the prooxidative (protective) activity. As Gr-XIII and Gr-XV were treated with the oxidant, KD; the reduced mortality of the shrimps may be due to both antioxidant and prooxidant capabilities of PYL. Furthermore, results from the Gr-XIV are the confirmation about the PYL-mediated prooxidative effect at high concentrations.

However, by considering the Gr-IV and Gr-XI we can say that CS produces toxic effects on the brine shrimps, which may relate to its oxidative stress-mediated cytogenotoxicity. According to Lo´pez-Camarillo et al. [19], eukaryotic double strand breaks (DSBs) can be repaired by homologous recombination repair (HRR) and nonhomologous end-joining (NHEJ). Thus the groups (Gr-VII, Gr-IX, Gr-XI and Gr-XV) co-treated with PYL and/or TRO may protect the shrimp cells from oxidative stress-mediated this type of detrimental effect.

However, the cytotoxic activities of EOs or their major components are sometimes activated by light. There, they may act through adduct formation. The phototoxic EOs are capable of crossing the cell membrane without causing damage to it or to the cellular macromolecules, therefore reactions forming radicals inside the cell upon exposure to light may be occurring. However, phototoxicity of EOs is dependent on the type of compounds present in EOs, concentration and their localization in the cell, as well as whether or not they produce different types of radical upon exposure to light [3]. According to Ghaneian et al. [20] PYL produced time dependent toxicity in albino mice. It is well-knownthat BSLB is a light-dependent test system as for continuous light and temperature maintenance is essentialalong with electrolyte balance and pH for the livelihood of the shrimps. In this study, the Gr-I and Gr-XII in the step 3 **(Table 3)** confirming that PYL at higher rather than low concentrations is cytotoxic; and the toxicity depends on the time of exposure of this diterpenic EO. Thus, our findings may link to the cytotoxic activity observed in Schistosoma mansoni by Moraes et al. [21] and Ghaneian et al. [20]. However, exposure to EOs could induce mitochondrial and DNA damage due to the formation of respiratory deficient cytoplasmic petite mutants, in which exponentially growing cells are more sensitive to [3]. PYL, in this occasion may also produce death to the shrimps by this way.

Otherwise, PYL at 80 µM as a co-treatment with the Gr-VI, Gr-VII, Gr-X and Gr-XI other than alone reduced the percentage of mortality of the shrimps; whichever also seen in the Gr-XIV and Gr-XVII co-

Figure 1: Postulated toxicological events of phytol.



PYL at low concentration: acting as a radical scavenger and protecting cell from oxidative stress-induced detrimental effects, thus the antioxidant activity.
PYL at high concentration: due to high hydrophobicity, readily crossing the cellular as well as mitochondrial membranes, causing loss of cellular essentials with the permission to the exterior toxicants, changing membrane potential, neutralizing reactive species and eventually causing self-destruction to the cell macromolecules such as – proteins, lipids and DNA; thus leading cyto-/genotoxicity and mitochondrial dysfunction. Genotoxic damage, if remains un-repaired it may turn to mutagenic event.

treated with KD (80 µM) and TRO (80 µM)+KD (80 µM), respectively.

PYL has a hydroxyl (-OH) group at its C1 position. According to the Gyawali and Ibrahim [22] substances having active -OH group with antioxidative potential may cause cytotoxicity. Terpenoid EOs are the best examples in this occasion [3]. Hydrogen peroxide (H_2O_2) is a well-known strong oxidizer as it potentially produces $^{\bullet}OH$, which is causing stress to the RBCs in HL assay [6]. Our finding suggests PYL to have significant ($p < 0.05$) $^{\bullet}OH$ scavenger up to 4 mM. Although, the observed activity was lower than the TRO treated with similar concentrations, but PYL when co-treated with TRO (80 µM); it significantly increased the percentage inhibition of HL with all the test concentrations in comparison to the NC and PYL* groups. Interestingly, PYL in addition to TRO (80 µM) at 2-8 mM concentrations produces inhibition rate within the PYL* and TRO* groups. By considering the HL inhibition, the treatments with 40 µM we can say that PYL at very low concentration is incapable to combat potential of the $^{\bullet}OH$ coming from the H_2O_2; thus the diminishing the protection of RBCs from oxidative stress-mediated lysis. However, PYL at high concentrations (4 mM and 8 mM) also reduces the protectivity, this may relate to the high concentration-mediated membrane lysis and cytotoxicity observed in brine shrimps.

Finally, by considering Gr-VI to Gr-IX, Gr-X, Gr-XI, Gr-XVI to Gr-XVIII in BSLB test and all the test groups shown in HL assay we can tell that PYL produced TRO-*like* activity. According to the above findings a PYL-mediated cyto-events has been shown in **Figure 1.**

Conclusion

PYL induced cytotoxicity on brine shrimps especially at higher concentrations andinhibited HL in low as well as high concentrations. The activity found in

low concentration may be due to its cytotoxic effect of it seems capability, which may relate to its radical scavenging power and the cytoxicity due to antioxidant-mediated prooxidative effects. Cytoprotectivity of PYL is concentration-dependent while the cytoxicityseems to be concentration and time-dependent.

Abbreviations

BSLB: brine shrimp lethality bioassay; CAT: catalase; $^{\bullet}CH_2OH$: methoxy radical; $^{\bullet}CO_2$-: carbon-dioxide anion radical; CS: copper sulphate ($CuSO_4.5H_2O$); CuONPs: copper oxide nanoparticles; $^{\bullet}$DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; DW: distilled water; EDTA: ethylenediaminetetraacetic acid; EO: essential oil; GPx: glutathione peroxidase; HL: hemolysis; KD: potassium di-chromate ($K_2Cr_2O_7$); MP: membrane potential; $NO^{\bullet}$: nitric-oxide radical; RNS: reactive nitrogen species; $^{\bullet}O_2^-$: superoxide anion radical; PYL: phytol (3, 7, 11, 15-tetramethylhexadec-2-en-1-ol); RBC: red blood corpuscles; ROS: reactive oxygen species; SOD: superoxide dismutase; TRO: trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid).

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Conflict of interest

We have no competing interest from any single point of view.

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