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Original Research Article

Impact of *Shodhana* an Ayurvedic purification process on cytotoxicity and mutagenicity of *Croton tiglium* Linn.

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ABSTRACT

Background: *Croton tiglium* Linn. (CT) which is commonly called *Jaypal* is used in Ayurvedic preparations like *Ichhabhedi Ras, Asvakancuki Rasa.* Due to its toxic contents, seeds of *Croton tiglium* are purified before use, by the process mentioned in classical Ayurvedic texts called *Shodhana* meaning purification.
Objectives: The objective of the present study is to study the impact of Ayurvedic Purification process on cytotoxicity and genotoxicity of *Croton tiglium* Linn.
Materials and methods: *Croton tiglium* Linn. Seeds were processed for *Shodhana* by soaking in water, heating with milk (*Snehan)* and later grinding in Lemon Juice (*Bhavana*). Aqueous and Hydroalcoholic extracts were prepared before and after purification i.e. *Shodhana*. Cytotoxicity of the *Croton tiglium* was studied against Chinese Hamster Ovary cell line by MTT assay. Ames test was performed to study the mutagenicity of the extracts in *Salmonella typhi* TA 98, 100 and 102 strains. Phytoconstituents were studied by using LCMS analysis.
Results: The results indicated decrease in cytotoxic concentration (IC_{50}) of *Croton tiglium* seeds after purificatio*n* from 3.03 mg/mL to 0.99 mg/mL in aqueous extract and 18.56 mg/mL to 5.45 mg/mL. Genotoxicity study by Ames test indicated *Croton tiglium* Linn. *Croton tiglium* Linn. Seeds are non-genotoxic in strains like *S. typhi*, TA 98, 100 and 102. There was change in Phytochemical profile before and after shodhana.
Conclusion: Although both the concentrations are practically non-toxic, the decrease in cytotoxic concentration indicates Purification process as described in classical ayurvedic texts i.e. *Shodhana* has definitely increased the potency of the seeds of *Croton tiglium* Linn.

1. Introduction

Croton tiglium L. which is commonly called as *Jaypal* or *Kumbhini* in Ayurveda is traditionally used in various formulations in India and China [1]. It is toxic in nature owing to its metabolites like Phorbol Ester Myristate and Crotonic acid [2]. *Croton tiglium* Linn. is reported to have severe purgative action. It primarily impairs Intestinal tract and Kidneys [3,4]. *Croton tiglium* Linn. contains irritating oil and proteins such as crotin which is sometimes indicated as Crotanoglobulin and crotanalbumin [5]. Crotocaudin and croton oleic acid are also reported toxic metabolites of *Croton tiglium* [6,7]. Owing to its toxic properties, *Croton tiglium* Linn. is classified as a drug included in Schedule E1 of the Drugs and Cosmetics act, 1940 owing to its toxic properties [8]. For such drug materials having intrinsic toxic property, Ayurveda suggests a purification process called *Shodhana* [9].

Croton tiglium Linn. is used in various ayurvedic preparations after purification. Such purified *Croton tiglium* Linn. seeds are the

Abbreviations: CT, *Croton tiglium* Linn.; AQ, Aqueous Extract; HA, Hydroalcoholic Extract; BS, Before Shodhana; AS, After Shodhana; SMF, Spontaneous Mutation Frequency; ND, Not Detected.

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main ingredient in *ayurvedic* formulations. About 52 ayurvedic formulations contain *Croton tiglium* having 8 dosage forms used for 29 varieties of diseases [10,11]. Recently published safety studies have indicated that the *Croton tiglium* seeds may have mutagenic properties [12] which can further be attributed to its Phorbol Esters content [13]. The Ayurvedic purification process may have some effect on its cytotoxic and genotoxic properties. Hence, present study was undertaken with a purpose to study impact of ayurvedic purification process on cytotoxic and genotoxic potential of the *Croton tiglium* Linn. seeds.

2. Materials and methods

2.1. Collection of plant material and purification (Shodhana) process

Seeds of *Croton tiglium* Linn. were supplied by NRIP, Cheruthurithy along with the passport data vide Collection number NRIP/RDR/215 after identification by the botanist. The seeds were identified and were subjected to standardization as per Ayurvedic Pharmacopoeia of India. Seeds were then processed for *Shodhana* as described in Ayurvedic Pharmacopeia of India [14].

For purification, initially seeds (100 gm) were soaked in water overnight. Next day, the seeds were washed under running tap water. All the water was drained out and the outer covering and cotyledons of the seeds were removed (Fig. 1). The seeds were then tied in a muslin cloth to form a bundle which is also called Pottali/ Dola yantra. The pottali was tied on to a wooden ruler. This pottali was hung on a steel container containing cow milk (Godugdha) in such a way that 1/4th the bottom part of the pottali dipped into the milk. The milk was heated at 60 °C uninterruptedly for 3 h on an induction stove. The seeds were washed under running tap water and air-dried for some time. The cotyledons of the seeds were removed using forceps. The seeds were crushed into a fine paste using a mortar pestle by adding around 150 mL of fresh lemon juice to it. The grinding process was done for around 1 h. The paste was spread on a glass plate and kept for air drying. This process is called *Bhawana*. The process of *Bhawana* included grinding with lemon juice in a mortar pestle, for an hour. This procedure was repeated for 3 days. The paste was air-dried until all moisture was evaporated and then subjected to aqueous and hydro-alcoholic extraction.

Seeds powder was soaked in distilled water (1:5 W/V) for 24 h for aqueous extract preparation [15]**. For Hydro-alcoholic extract preparation, alcohol and water were mixed in 60: 40 (V/V) ratios, and seeds were soaked in this solvent in 1:5 (W/V) ratio.** After 24 Hours the preparation was filtered through a muslin cloth. The samples were kept in a rotary shaker incubator at 40 °C at 80 rpm for 6 h and then allowed to stand for 18 h at 37 °C. The filtrate obtained was lyophilized and the powder was stored in an airtight container at room temperature. The aqueous and hydro-alcoholic lyophilized extracts were dissolved at different concentrations in phosphate-buffered saline containing 1% Dimethyl Sulfoxide. Drug stock was prepared as aqueous extract 2 mg/mL in 1% DMSO before

Fig. 1. Process of Purification, extraction and Solubility studies of *Croton tiglium* seeds Aqueous and Hydroalcoholic Extract Preparation.

Shodhana and 5 mg/mL in 1% DMSO after *Shodhana.* Solubility of hydro-alcoholic extract was 5 mg/mL in 1% DMSO before and after *Shodhana.*

2.2. Standardization of Croton tiglium seeds

Standardization of seeds before extract preparation was carried out by proximate analysis of Ash Values i.e., total ash, acid Insoluble ash, Extractive value i.e. Alcohol soluble extractive and water soluble extractive, Loss on drying and pH value. The results obtained were compared with the standards given in Ayurvedic Pharmacopoeia of India.

Table 1
Standardization of *Croton tiglium linn.*

Sr. no.	Test	Results	API Standards
1. Ash value			
1a	Total ash value:	2.35%	Not more than 3
1b	Acid insoluble ash:	0.08%	not more than 0.5
2 Extractive value			
2a	Alcohol soluble extractive:	29.2%	Not less than 15%
2b	Water soluble extractive:	8%	Not less than 7%
3	Loss on drying	5.8	–
4	pH	6.17	–

Aqueous and hydro-alcoholic extracts were subjected to LCMS analysis before and after purification process. Chromatographic analysis was performed using the Agilent 6500 system with a C18 column (2.1 × 150 mm, 1.8 μm). The mobile phase was composed of acetonitrile (A) and water (B) in a gradient manner. The flow rate was 0.40 mL·min−1 and the fluid temperature was kept at 40 °C; the sample injection volume was 20 μL. LCMS depicted abundance of the metabolite and its molecular mass. Based on this, metabolites were identified by using Metlin software [16].

2.3. Cytotoxicity studies

Cytotoxicity studies were performed on aqueous and hydro-alcoholic extracts before and after the purification process on the Chinese Hamster Ovary (CHO) cell line as described by Mossman 1983 [17]. The cell line was obtained from the Cell Repository of the National Center for Cell Sciences, Pune after a mutual Material Transfer Agreement to use the cell line solely for research purposes.

Table 2
Metabolites detected in *Croton tiglium* Linn by LC-MS analysis before and after purification (Shodhana) in aqueous and hydro-alcoholic extracts.

Sr No.	Name of the phytoconstituent	m/z	Abundacne AQ before shodhana	Abundacne AQ After shodhana	Abundacne HA Before shodhana	Abundacne HA After shodhana	Comment/
1	Myristic acid	246.24	19,248	210,593	21,663	741,151	Shodhana increases leaching of Phytochemical, more quantity in Hydro-alcoholic extract
2	(2S)-2-hydroxyphytanic acid	346.33	5075	482,622	8438	83,419	Shodhana increases leaching of Phytochemical in more quantity in aqueous extract
3	Sinapic acid	247.05	ND	96,024	ND	32,861	Shodhana increases leaching of Phytochemical
4	Phosphatidyl glycerol	269.03	ND	83,173	ND	10,664	Shodhana increases leaching of Phytochemical, more quantity in aqueous extract
5	Clocortolone pivalate	495.23	24,628	9944	ND	3490	Shodhana causes decrease in quantity of phytochemical
6	Cyclic-3,20-bis(1,2-ethanediyl acetal)-11alpha-(acetyloxy)-5alpha,6alpha-epoxypregnane-3,20-dione	499.26	58,856	14,242	ND	2713	Shodhana causes decrease in quantity of phytochemical, less solubility in Hydro-alcoholic extract.
7	1-Aminocyclohexanecarboxylic acid		216,578	7839	ND	ND	Shodhana causes decrease in quantity of phytochemical, no solubility in hydroalcoholic extract.
8	(3S,5R,6R,6′S)-6,7-Didehydro-5,6-dihydro-3,5,6′-trihydroxy-13,14,20-trinor-3′-oxo-beta,epsilon-caroten-19′,11′-olide 3-acetate	337.15	22,601	19,283	ND	11,754	Shodhana causes decrease in quantity of phytochemical, less solubility in Hydro-alcoholic extract.
9	Sphinghanine	274.27	151,290	2,259,748	131,732	1,620,730	Shodhana increases leaching of Phytochemical
10	(Z)-5-[(5-Methyl-2-thienyl) methylene]-2(5H)-furanone	215.01	ND	ND	ND	11,193	Shodhana increases leaching of Phytochemical in hydroalcoholic extract.
11	Tridecanoic acid, 4,8,12-trimethyl-; 4,8,12-Trimethyltridecanoic acid	279.22	ND	ND	ND	10,332	Shodhana increases leaching of Phytochemical in hydroalcoholic extract.
12	Palmitic Acid	279.22	ND	ND	ND	8702	Shodhana increases leaching of Phytochemical in hydroalcoholic extract.
13	Myricanol	278.14	ND	5431	ND	3725	Shodhana increases leaching of Phytochemical.
14	crotin	341.38	ND	2156	13,037	1105	Hydroalcoholic extraction method leads to more leaching of the phytochemical.
15	crotonoside	283.24	10,564	26,353	17,265	ND	Shodhana increases leaching of Phytochemical in aqueous extract.
16	phorbol 13-acetate 12-myristate	634.42	1083	ND	ND	ND	Shodhana decreases leaching of Phytochemical in aqueous extract

In short, cytotoxicity studies were performed by seeding CHO cells at a density of 3×10^3 cells per well of 96 well plates in triplicates. Three technical and three biological replicates were performed. Cells were maintained in Dulbecco minimum essential medium (DMEM) + Fetal bovine serum + Glutamine at 37 °C in the 5% CO_2 incubator. Cells from the 96 well plates were observed under inverted microscope for growth and confluency. The medium was decanted and cells were treated with 5, 10, 20, 40, 60, 80, 100, 150, 180, 200 μg/mL drug for 24 h prepared in fresh media. Control well was maintained only with the media. After 24 h of drug treatment 50 μl of MTT solution was added and the plate was incubated for 4 h at 37 °C, 5% CO_2. After 4 h, the plate was wrapped with foil and spined down for 5 min-3500 rpm, supernatant solution was decanted and formazan crystals solubilized using DMSO (100μl/well). The absorbance (570 nm) was measured using a plate reader. The readings were recorded; graphs plotted for the same and analysis of data was done. Blank was subtracted from all the readings and percent cell proliferation was calculated as compared to control. The IC_{50} was calculated using an online calculator tool by AAT Bioquest [18].

2.4. Mutagenicity testing by Ames test

The Ames test was performed using methods described by Ames (1971) [19] and details as given by Mortelmans and Zeiger (2015 [20,21]. *Salmonella typhi* TA-98, *S. typhi* TA-100 and *S. typhi* TA-102 strain were obtained from Krishgen Biosystems were inoculated in Nutrient Broth and incubated at 37 °C for 12 h in the rotary shaker to obtain primary culture. Loopful of primary culture was inoculated in fresh Nutrient Broth to obtain secondary culture, on day 2. Different doses of extracts were distributed in sterile 15 mL centrifuge tubes, to it 100 μl of secondary bacterial culture was added and incubated on a shaker incubator at 37 °C for 30 min. The strain specific positive control was used. After 30 min incubation, Top Agar was added to these tubes and immediately poured on Minimal Glucose Agar (MGA) plates to form a uniform layer. Plates were incubated at 37 °C for 48−72 h. Three technical and two biological replicates were performed. Results in the form of colony counting were obtained after 72 h and were subjected to statistical analysis.

3. Results

Croton tiglium L. (Jaypal) seed aqueous and hydro-alcoholic extracts were prepared before *Shodhana*. The seeds obtained passed the API standards (Table 1). Further, *Shodhana* was performed as per the procedure described in Ayurvedic Pharmacopoeia of India and aqueous and hydro-alcoholic extracts were prepared. Both the extracts were lyophilized and percent yield was calculated. Percent yield increased after *Shodhana* from 3.37 to 3.91 to 9.06 and 8.86 percent of aqueous and hydro-alcoholic extracts respectively.

4. LCMS analysis

LC-MS analysis results are depicted in Table 2 and Figs. 2 and 3. Concentration of few plant metabolites increased (eg.

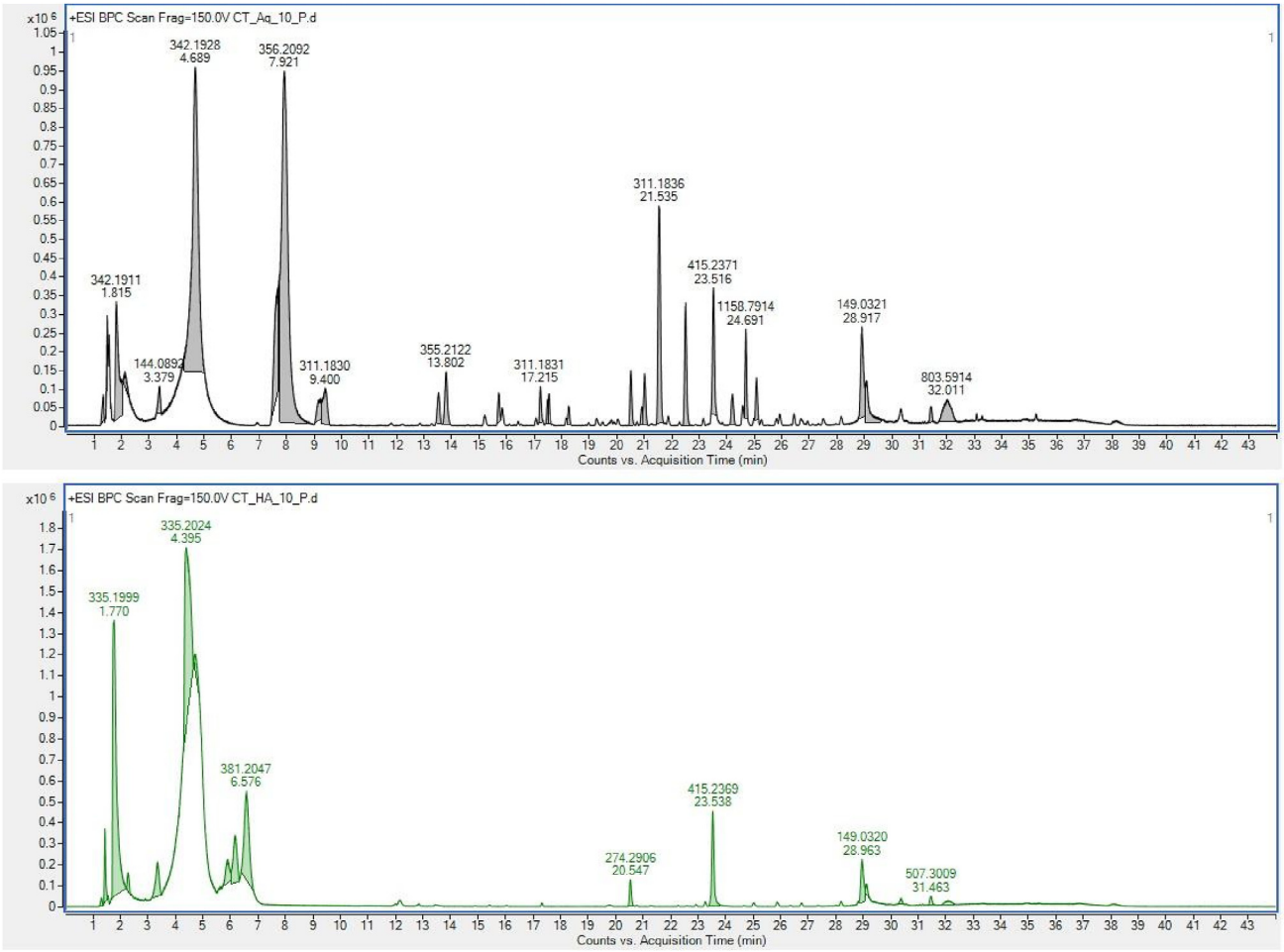

Fig. 2. LC-MS Analysis of *Croton tiglium Linn in aqueous and hydroalcoholic extracts before Shodhana*.

Sphinghanine, Myristic acid) or decreased (1-Aminocyclohexane-carboxylic acid). Some phytochemicals which were not detected initially were detected after *Shodhana* eg. Palmitic acid, Myricanol. Few chemicals which were initially detected vanished after purification process eg. Crotonoside., Sinapic acid. Extraction method also had impact on concentration of phytochemicals. eg. Palmitic Acid was detected only in Hydro-alcoholic extraction process after *Shodhana*.

5. Cytotoxicity studies

Cytotoxicity of *Croton tiglium Linn* was studied in the CHO cell line before and after purification (Fig. 4). In both aqueous and hydro-alcoholic extracts, there was decrease in cytotoxic dose of *Croton tiglium Linn* indicating increase in cytotoxic property after purification of the seeds by classical ayurvedic treatment.

6. Mutagenicity studies

Ames test in Auxotrophic strains of *S. typhi* TA 98, 100 and 102 strains (Figs. 5–7 respectively) was performed indicating no evidence of mutagenicity. In aqueous extract, dose 2000 mg/plate caused significant increase in the number of revertant colonies as compared to Negative control when studied in *S. typhi* TA 98 strain. However, this was within biological limits as spontaneous mutation frequency of *S. typhi* TA 98 is 20–50 colonies.

7. Discussion

Cytotoxicity of *Croton tiglium* Linn was studied in the CHO cell line before and after *Shodhana*. In both aqueous and hydro-alcoholic extracts, there was decrease in cytotoxic dose of *Croton tiglium* indicating increase in cytotoxic property after *Shodhana*. Cytotoxic dose of *Croton tiglium* Aqueous Extract was 3.026 mg/mL and 0.9090 mg/mL before and after Shodhana and the same for hydroalcoholic extract was 18.6 mg/mL which decreased to 5.45 mg/mL. Increase in cytotoxic potential indicated increase in potency of the drug due to purification processes i.e. *Shodhana*.

LC-MS Analysis helped to determine specific metabolites from seed extracts. Purification process increases leaching of Myristic acid, 2S-2-hydroxyphytanic acid, Sinapic acid and Phosphatidyl glycerol. However, Myristic acid concentration was more in hydro-

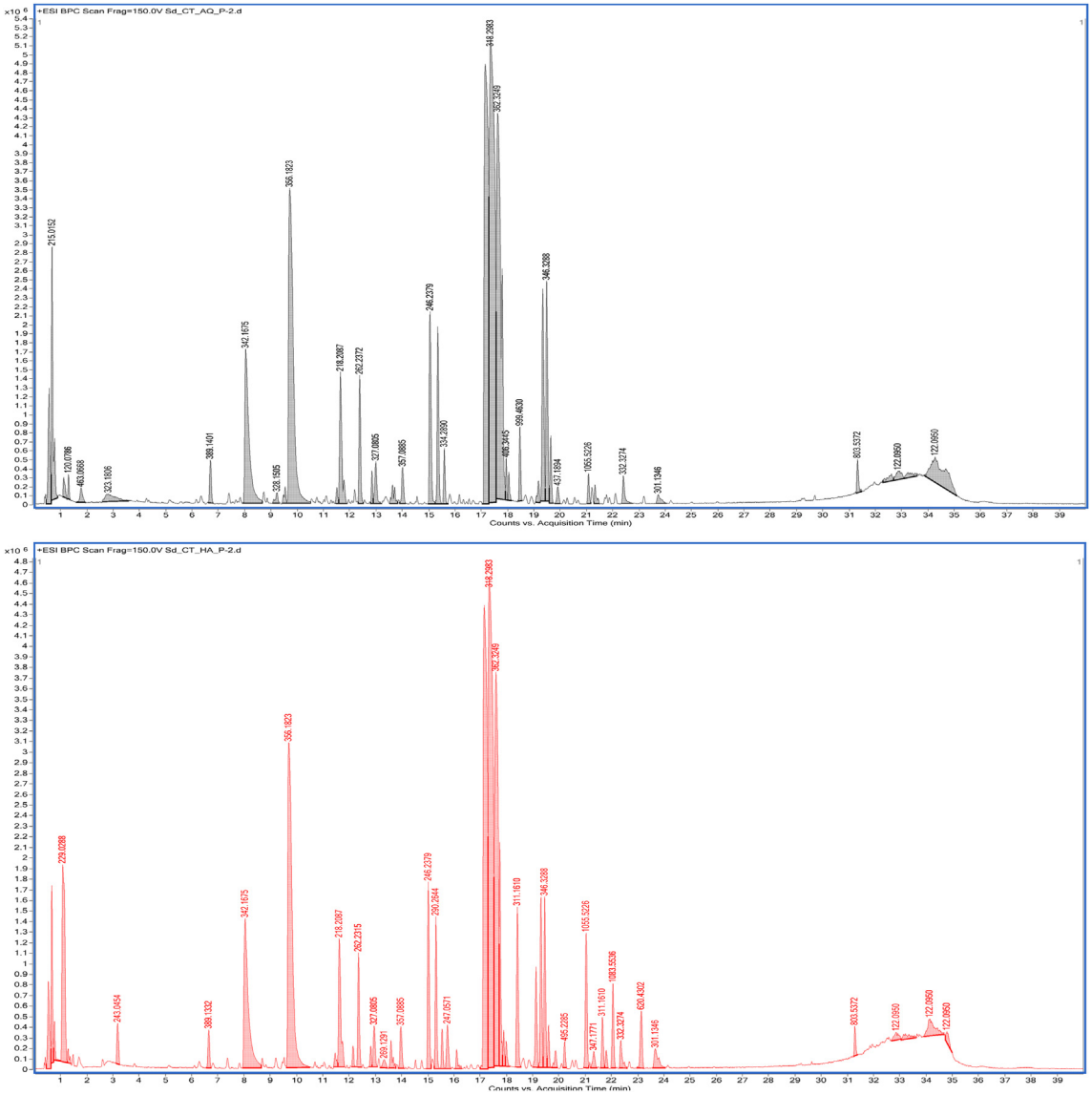

Fig. 3. LC-MS Analysis of *Croton tiglium Linn in aqueous and hydroalcoholic extracts after Shodhana.*

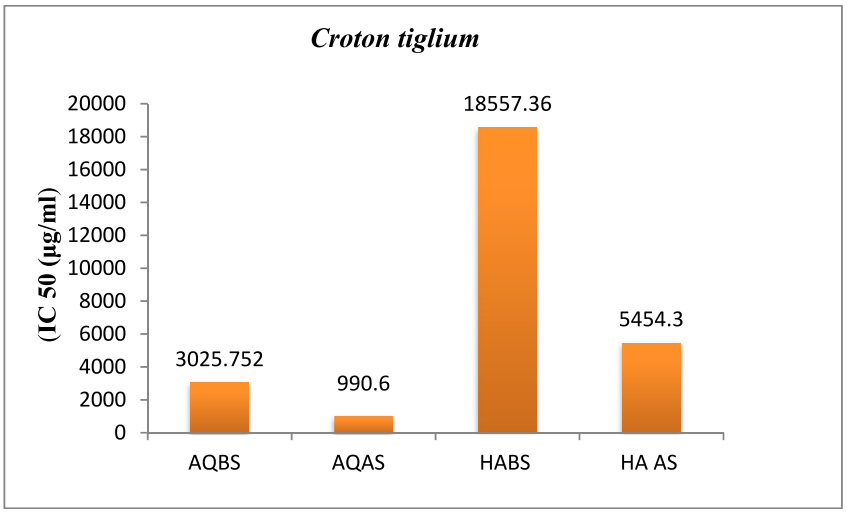

Fig. 4. IC50 values of *Croton tiglium Linn* seed extracts before and after purification (*Shodhana*) in Chinese Hamster Ovary Cell line.

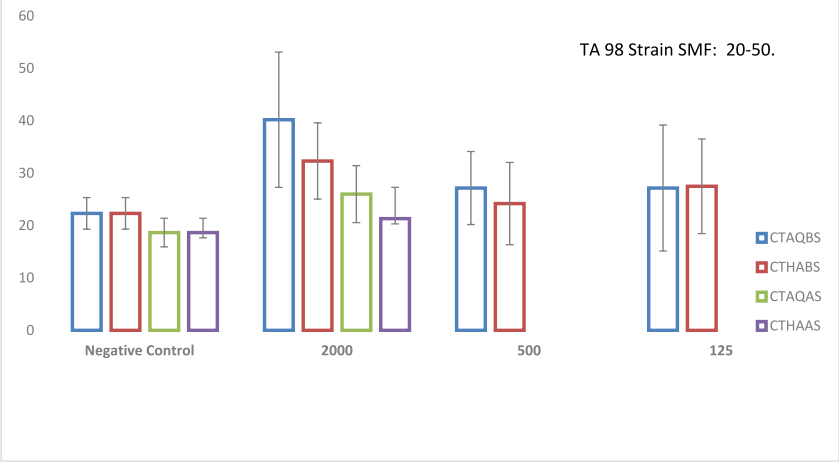

Fig. 5. Ames test results of *Croton tiglium* Linn. seed extract before and after purification on *Salmonella typhi* TA 98. CT: *Croton tiglium*, AQ: Aqueous Extract, HA: Hydroalcoholic Extract BS: Before Shodhana, AS: After Shodhana SMF: Spontaneous Mutation Frequency. Positive control: 4NOPQ 2.5 µg/plate SMF of positive control was 215 ± 38.2 colonies/plate.

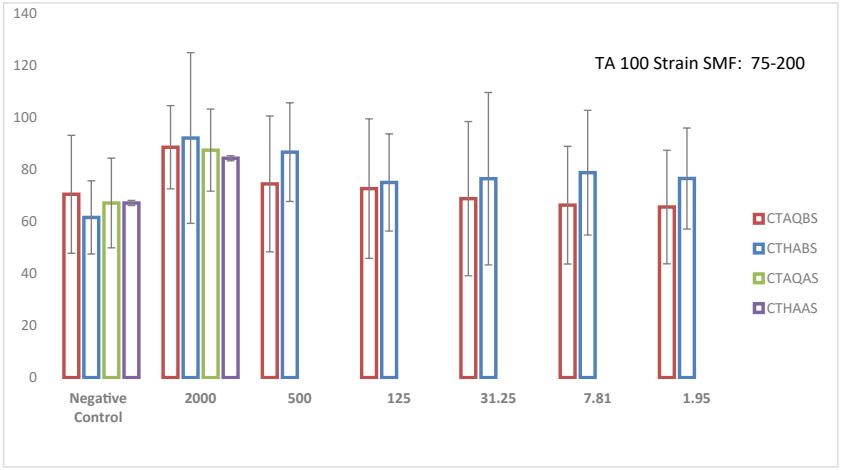

Fig. 6. Ames test results of *Croton tiglium* Linn. seed extract before and after purification on *Salmonella typhi* TA 100. CT: *Croton tiglium*, AQ: Aqueous Extract, HA: Hydroalcoholic Extract BS: Before Shodhana, AS: After Shodhana, SMF: Spontaneous Mutation Frequency. Positive control: Sodium azide 5 µg/plate SMF of positive control was 535.78 ± 139 colonies/plate.

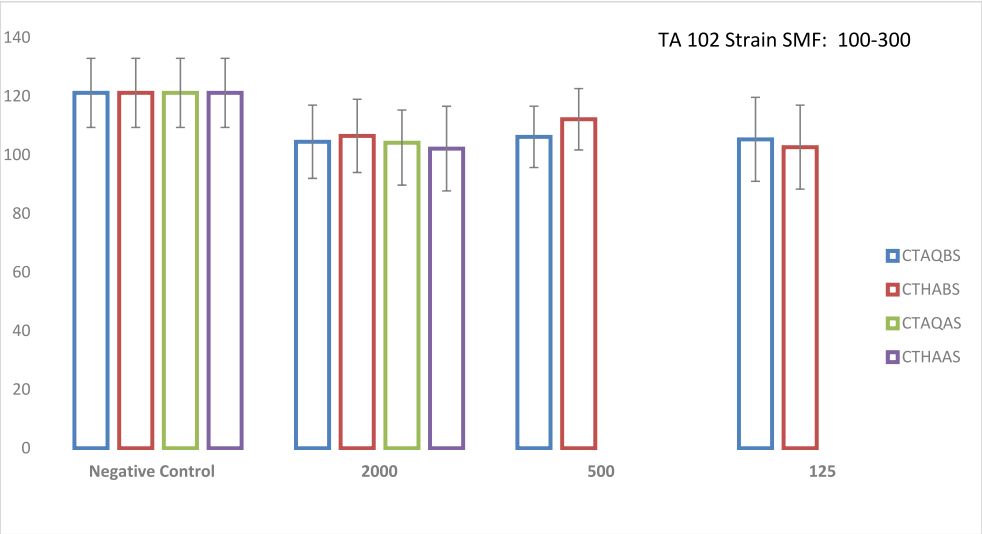

Fig. 7. Ames Test Results of *Croton tiglium* Linn seed Extracts before and after purification on *Salmonella typhi* TA 102. CT: *Croton tiglium*, AQ: Aqueous Extract, HA: Hydroalcoholic Extract BS: Before Shodhana, AS: After Shodhana SMF: Spontaneous Mutation Frequency Positive control: Mitomycin C 0.5 μg/plate SMF of positive control was 550 + 18.97 colonies/plate.

alcoholic extract and 2S-2-hydroxyphytanic acid and Phosphatidyl Glycerol were more eluted in Aqueous extract. Myristic acid, which leached out more after purification process, is also reported to be cytotoxic and irritant to the gastrointestinal tract [22,23]. Sinapic acid was not at all detected in aqueous or hydro-alcoholic extracts before purification. The process improved extractability of Sinapic Acid. The other phytochemical, Palmitic acid which was also solely detected in hydroalcoholic extract of Purified *Croton tiglium* Linn. is reported to be cytotoxic [24].

Purification caused decrease in Cyclic-3,20-bis (1,2-ethanediyl acetal)-11alpha-(acetyloxy)-5alpha, 6alpha-epoxypregnane-3,20-dione, 1-Aminocyclohexanecarboxylic acid and (3S,5R,6R, 6′S)-6,7-Didehydro-5,6-dihydro-3,5,6′-trihydroxy-13,14,20-trinor-3′-oxo-beta, epsilon-caroten-19′,11′-olide 3-acetate. However, these phytoconstituents were first time reported in *Croton tiglium* Linn Seeds. Phorbol ester myristate which is a known tumor promoting agent [25] was present in aqueous extract before purification process. Although it might be present in minute quantity, purification process helped to get rid of it. However, the reason for increase in cytotoxic potential can be attributed to the presence of Myristic acid and Palmitic acid and other similar compounds which showed increased abundance after the purification process. Chinese Hamster Ovary Cell line was chosen for the cytotoxicity study as it has HPRT gene which makes this cell line suitable for HPRT assay which is a forward mutation assay [26]. This study will help to decide the dose for further genotoxicity and cytotoxicity studies [27].

In mutagenicity studies, positive mutagenicity can be attributed to a two-fold increase in the colonies due to treatment. The same was observed in positive control which indicated that the test conditions were adequate. The Ames test indicated there is no evidence of mutagenicity and although significantly increased colonies were observed in TA 98 strain, which was within the biological limits, it can be commented that *Croton tiglium* Linn. is non-mutagenic. However, works published by Yumnamcha et al. (2022) [28] have indicated that *Croton tiglium* Linn. may cause genotoxic effects. The study was performed in the Zebrafish model which indicated some evidence of embryo-toxicity and genotoxicity when studied by micronucleus test and comet assay. *Croton tiglium* Linn. may lead to genotoxic effect but not by base pair substitution or frameshift mechanism which are exhibited in Ames Test. Similarly, the results of this study are not in agreement with et al. Kim et al. (2015) [12], which showed positive mutagenic effect in all the strains indicating frame shift (TA98 and TA1537), base-pair substitution (TA100 and TA1535), or oxidative and cross-linking (TA102) which is surprising. However, the extraction method was totally different from the present work. Kim et al. (2015) performed fractional extraction whereas in present study aqueous and hydroalcoholic extracts were prepared considering its similarity to ayurvedic preparations. It is possible that the concentration of toxic chemicals differs with the extraction process [29].

8. Conclusion

The Ayurvedic purification process definitely has an impact on the cytotoxicity of *Croton tiglium* Linn. as it caused alteration in phytoconstituents concentration and significant decrease in the cytotoxic dose in CHO cell lines. However, *Croton tiglium* Linn. was not found to be genotoxic up to 2000 μg/mL, hence considered safe for consumption as a drug.

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Declaration of competing interest

None.

Author contribution

PJ: Research idea, execution of the work, writing of the manuscript; AR: Ayurveda expert contributed in Shodhana; SB: Execution of the work and Assistance to PJ; SC: LCMS analysis; SJ: Research Idea and result analysis; SP: Execution of the work, manuscript review; GP: Manuscript preparation and administrative part of the project; SG: Project review, article preparation and result interpretation; AG: Manuscript review.

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