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Evaluation of the role of *Shodhana* (Purification) process in *Croton tiglium* seeds for reduction of toxic content

Research Article

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Abstract

In Ayurvedic texts, *Jayapala* or *croton tiglium* seeds are well known as *khumbini* for its toxicity (severe purgative action) and are used for the treatment of constipation after shodhana (detoxification) of the seed with *godugdha* (cow milk). The oil content of the seeds was responsible for the purgative property, and its reduction enhances its medicinal usage. The presence of Crotonoside or iso-guanosine in seed extract was identified in HPTLC and quantified using high pressure liquid chromatography technique both before and after the purification process of the seed. The phytochemicals and physicochemical parameters of seeds were analyzed to find the level of changes in the processed seeds. The other chemical constituents of the seed extracts are studied using different techniques like HPTLC, HPLC, LC-MS, and GC-MS. The study has revealed that the detoxification process, as per classical texts, has shown an effective depletion in the quantity of crotonoside in processed seeds.

Key Words: *Ayurveda,* Purification, Crotonoside, LC-MS, GC-MS.

Introduction

Croton tiglium L. (Euphorbiaceae) is an important medicinal plant which is commonly known as *Jayapala*, or Croton oil plant. It is an erect, evergreen shrub/small tree growing up to 7 meters tall and has been grown for these uses for beyond 2,000 years. It is still often cultivated nowadays and also sometimes grown as an ornamental. The roots, seeds, and seed oil are traded within India & South-East Asia, and the oil is exported to Europe. (1-2)

The plant has a very long tradition of herbal use, being employed as a powerful laxative and oil to treat a wide range of skin problems. It is used to treat constipation, dyspepsia, dysentery, gastrointestinal disorders, intestinal inflammation, rheumatism, peptic ulcer, visceral pain, and headache. (3-5)

All parts of the plant are poisonous. The seeds are recognized to be very poisonous, and are used as a fish poison and criminal activities, four seeds can be a lethal dose for a human adult. Symptoms of croton oil poisoning are at pain starts at the back of the throat and then in the anal canal. A dose of bismuth is know as an immediate antidote. Extreme caution must be taken with all medicinal applications of this plant, given its extreme toxicity. (6-9)

Particularly the seeds have been chemically analysed only. Major constituent is a fixed oil named croton oil (30 - 45%) and protein (20% approx). The oil comprises the fatty acids - myristic acid, oleic acid, linoleic acid, arachidic acid, palmitic acid, formic acid, stearic acid, acetic acid, and smaller amounts of tiglic acid, butyric acid, lauric acid, and valeric acid. Severe inflammation on the skin is caused by Croton oil. The toxic principles are a group of proteins called 'crotin', about 3.5% croton resin ('crotonol'), a glucoside called crotonoside (iso-guanosine), and a non-volatile unsaturated fatty acid responsible for the purgative properties. The mixture of toxic proteins croton globulin and croton albumin Crotin. (9)

The plant is known as *Kumbhinī* in Ayurvedic texts and is used to treat constipation after *Śhodhana* (detoxification process) with *Godugdha* (cow milk) of the seeds. (10-11)

In the present study, *Croton tiglium* seeds were purified with cow milk as reported in Ayurvedic classics. The phytochemical constituents of the seed are analyzed to identify the biologically active compounds. The standard Crotonoside (Sigma Aldrich) was purchased and identified using the HPTLC technique and quantified using the HPLC technique for raw and purified seeds and other physicochemical parameters. GC-MS and LC-MS are also performed to extract the seeds to establish the significant reduction of Crotonoside and other fatty acids responsible for the purgative nature after purification.

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Materials and Methods

Sample collection

The *Jayapala* (Croton tiglium Linn) seeds were procured from the local crude drug market Chennai, Tamilnadu, and authenticated at Botany Department of Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute, Chennai, with the help of flora. A voucher specimen was submitted in the department of Botany of the Institute.

Purification/Detoxification

500g of the dry raw *Jayapala* seeds are cleaned, weighed, and taken. The outer coat of the testa of the sample was removed with the help of iron mortar and pestle. It is then tied in a muslin cloth and hanged inside the mud pot without touching the bottom. *Swedana* is performed by *dola-yantra* in cow's milk for three hours. Cooled, removed and washed with warm water and removed the cotyledon of the seeds with the help of the knife. Dry, powder, and then soak the sample in lemon juice (*nimbu swarasa*) for three days. Fresh juice was added every day. Then the paste was applied to the outside of the mud pot and dried in sunlight to remove the excess oil content of the seed. Once dried, the powder is collected and used for analysis. (12)

Standardisation of *Jayapala* seeds

To ascertain the quality of the procured *Jayapala* seeds used in the study physico-chemical analysis was done. Qualitative analysis of phytochemicals like alkaloids, tannins, flavonoids, steroids, saponins, phenols, coumarins, glycosides, acids, proteins were analysed. Physico-chemical parameters like Alcohol and Water-soluble extractive values, pH, Total Ash, Acid- insoluble Ash, and Loss on Drying were quantitatively analyzed based on the standard procedures in Ayurvedic pharmacopeia of India (API) methods. (13-15, 9)

Sample/Extract preparation

10g of both raw and purified samples are taken in thimble along with 250ml of ethanol in Round Bottom flask. Soxhlet extraction was performed for 6-8 hours, and the extracts are collected. The same procedure was repeated with chloroform and hexane; the extracts are organized and labeled properly. Known quantities of these extracts are taken to prepare the test solution for HPTLC, HPLC, GC-MS, and LC-MS analysis.

Reference standard preparation

Crotonoside or iso-guanosine was taken as the reference standard and was quantified in HPLC. The standard was purchased from Sigma Aldrich, and HPLC grade water was used. 260 ppm of Crotonoside standard was prepared by dissolving in methanol and used for analysis. All other chemicals & solvents used were of AR grade.

Identification of Bio-marker by HPTLC:

The residues obtained from ethanol and chloroform extracts were weighed and dissolved in methanol. 2.6mg of the standard was weighed and prepared in a 10 ml volumetric flask. It was then filtered through 0.22 µ membrane filters and used to identify reference standard *Crotonoside* - biomarker compound. 15l of each test solution of chloroform, ethanol extract, and Crotonoside reference standard solution were spotted in different tracks on a precoated silica gel 60 F_{254} TLC plate (E. Merck) of 0.2 mm thickness. The plate was developed in the suitable solvent system of *N-butanol: Methanol: Water: Ammonia Solution (25%)* (4:4:1:0.5) and rises to a distance of 8 cm. The plate was observed through CAMAG TLC Visualizer under UV at 254 nm and 366 nm, and photos were documented. Finally, the plate was dipped in vanillin-sulphuric acid reagent and heated in a hot air oven at 105°C until the color of the spots appeared, and the photo was documentation under white light.

Chemical profiling of *Croton tiglium* seeds

The chromatographic profiling was performed using four different chromatographic techniques to study the effect of the shodhana process on the *Croton tiglium* seeds.

HPTLC chromatographic profiling of *Croton tiglium* seeds

The Fingerprint profile was performed for the above-developed plate (Figure 1) used for identification. Before derivatization, the plate was scanned under UV at 254 nm and 366 nm using deuterium and mercury lamp, respectively. After derivatization plate was scanned at 540 nm using a tungsten lamp.

GC-MS chromatographic profiling of *Croton tiglium* seeds

The test solutions were prepared by dissolving the dried extracts of hexane and chloroform in chloroform and methanol solvents of the desired volume. It is filtered and sent for GC-MS analysis. Here interpretation on mass spectrum GC-MS was conducted by using the database of the National Standard and Technology (NIST) library. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The hexane and chloroform extracts of the *Croton tiglium* seeds before and after the shodhana process, the processed and unprocessed samples are compared under the same chromatographic conditions.

LC-MS chromatographic profiling of *Croton tiglium* seeds

The test solution was prepared by dissolving ethanol extracts of *Croton tiglium* seeds before (20.8mg) and after (20.7 mg) shodhana process in HPLC grade methanol up to 1.0ml volume. It is filtered and sent for LC-MS analysis. The ethanol extracts of the *Croton tiglium* seeds before and after the shodhana process, the processed and unprocessed samples are compared under the same chromatographic conditions.

Estimation of Crotonoside

Ten µl each of the extract test solutions was injected into the HPLC system. The chromatogram was recorded, the area of peak of test solution was determined corresponding to that of crotonoside, described above from the calibration curve.

Results and Discussion

Preliminary analysis

Preliminary phytochemicals screening results showed the presence or absence of certain phytochemicals in the Croton tiglium seeds. The comparative preliminary phytochemicals screening results of Croton tiglium seeds before and after the *shodhana* process are tabulated in Table 1.

Table 1: Preliminary phytochemicals screening of Croton tiglium seeds before and after *shodhana* process

S. No.	Parameters	*Croton tiglium*	
		Before *Shodhana*	After *Shodhana*
1	Alkaloids	+	+
2	Coumarins	+	+
3	Flavanoids	+	+
4	Glycosides	+	+
5	Phenols	+	+
6	Proteins	+	+
7	Saponins	+	+
8	Steroids	+	+
9	Sugar	-	-
10	Tannins	+	++

Physico-chemical analysis

The comparative analysis results of physicochemical parameters for *Croton tiglium* seeds samples before and after shodhana processes are tabulated in Table 2. The results of all parameters are complying the Ayurvedic Pharmacopeia of India (API) standards.

Table 2: Physicochemical parameters of *Croton tiglium* seeds before and after *shodhana* process

S. No.	Parameters	*Croton tiglium*	
		Before *Shodhana*	After *Shodhana*
1	pH (10% w/v aqueous solution)	5.66	3.24
2	Loss on drying at 105°C (% w/w)	6.82	9.49
3	Water soluble extractive (% w/w)	8.20	12.41
4	Alcohol soluble extractive (% w/w)	39.52	45.77
5	Ash content (% w/w)	2.68	2.58
6	Acid-insoluble ash (% w/w)	0.21	0.20

The percentage of water-soluble extractive, alcohol soluble extractive, and loss on drying at 105°C were found to increase ash content, acid-insoluble ash, and pH value reduced in the *shodhit* (processed) as compare to *ashodhit* (unprocessed) *Croton tiglium* seeds sample.

Extraction procedure

The total Ethanol and Chloroform extracts of *Croton tiglium* seeds are extracted using the Soxhlet method, and the obtained values are tabulated and given below:

Table 3: Extractive values of *Croton tiglium* seeds before and after *shodhana* process

S. No.	Name of extracts	Weight of sample (g)		Weight of extract (g)		Extractive value % (w/w)	
		Before	After	Before	After	Before	After
1	Chloroform extract	10.0432	12.2184	4.4538	7.4086	44.3443	60.6348
2	Ethanol extract	10.0582	12.049	4.7999	7.2345	47.7212	60.0423

Identification of Bio-marker by HPTLC:

The calculated R_f value details were given in Tables 4 to 6. A band at 254 nm (Blue, R_f 0.54) corresponding crotonoside is visible in both the reference standard and test solution tracks of chloroform and ethanol extracts of *Croton tiglium* seeds before and after the *shodhana* process.

Table 4: R_f values of *Croton tiglium* seeds chloroform & ethanol extracts before and after *shodhana* process and Crotonoside reference standard at UV 254 nm

Tracks	Before *Shodhana*			After *Shodhana*		
	Peak	R_f	Color	Peak	R_f	Color
Track 1 & 5 Chloroform extract	1	0.02	Green	-	-	-
	2	**0.54**	**Green**	-	-	-
	3	0.82	Green	1	0.89	Green
Track 2 & 4 Ethanol extract	1	0.02	Green	-	-	-
	2	0.15	Green	-	-	-
	3	0.18	Green	-	-	-
	4	**0.54**	**Green**	-	-	-
	5	0.62	Green	-	-	-
	6	0.77	Green	-	-	-
	7	0.96	Green	1	0.89	Green
Track-3 Crotonoside standard	**1**	**0.54**	**Green**	-	-	-

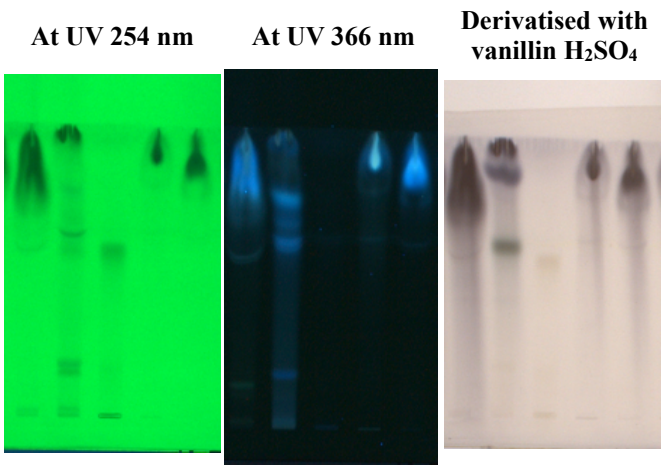
Table 5: R_f values of *Croton tiglium* seeds chloroform & ethanol extracts before and after *shodhana* process at UV 366 nm

Tracks	Before shodhana			After shodhana		
	Peak	R_f	Color	Peak	R_f	Color
Track 1 & 5 Chloroform extract	1	0.15	Pale yellow	-	-	-
	2	0.57	Blue	1	0.63	Blue
	-	-	-	2	0.79	Blue
	3	0.85	Blue	3	0.89	Blue
Track 2 & 4 Ethanol extract	1	0.18	Blue	-	-	-
	2	0.59	Blue	-	-	-
	3	0.62	Blue	1	0.63	Blue
	4	0.69	Blue	-	-	-
	5	0.78	Blue	2	0.79	Blue
	6	0.96	Blue	3	0.89	Blue

Table 6: R_f values of *Croton tiglium* seeds chloroform & ethanol extracts before and after shodhana process at 540 nm

Tracks	Before shodhana			After shodhana		
	Peak	R_f	Color	Peak	R_f	Color
Track 1 & 5 Chloroform extract	1	0.58	Grey	1	0.63	Grey
	2	0.77	Grey	2	0.88	Grey
Track 2 & 4 Ethanol extract	1	0.17	Brown	-	-	-
	2	0.56	Brown	-	-	-
	3	0.60	Grey	1	0.63	Grey
	4	0.86	Grey	2	0.88	Grey
	5	0.96	Grey	-	-	-

Figure 1: HPTLC fingerprint Profiling of *Croton tiglium* seeds chloroform & ethanol extracts before and after *shodhana* process and Crotonoside reference standard



Chloroform extract: **Track-1**: Before *Shodhana*; **Track-5**: After *Shodhana*;
Reference standard**: Track-3**: Crotonoside Ethanol extract: **Track-2**: Before *Shodhana*; **Track-4**: After *Shodhana*

Chemical profiling of *Croton tiglium* seeds

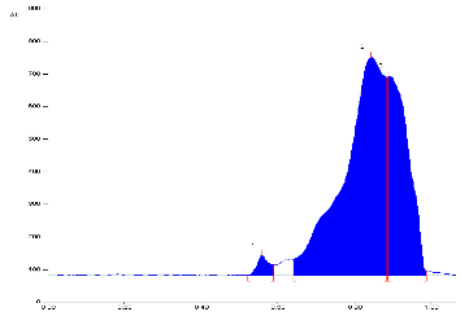
The details of the chromatographic profiling were given below:

HPTLC chromatographic profiling of *Croton tiglium* seeds

The Fingerprint profile was performed for the above-developed plate (Figure 1) used for identification. The R_f values and fingerprint data were recorded by WIN CATS software. Details of HPTLC fingerprint profiling are given in Figures 2 & 3.

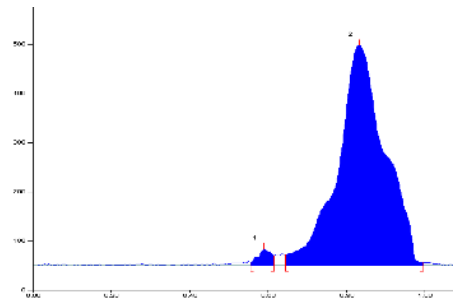
Figure 2: HPTLC fingerprint profile of *Croton tiglium* seeds chloroform & ethanol extracts before and after *shodhana* process and Crotonoside reference standard at UV 254 nm

Chloroform extract before *Shodhana*



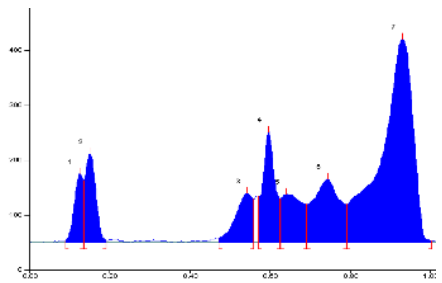
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.50 Rf	3.4 AU	0.55 Rf	65.8 AU	10.22 %	0.57 Rf	42.2 AU	1733.8 AU	1.94 %
2	0.62 Rf	64.2 AU	0.66 Rf	577.3 AU	89.78 %	0.99 Rf	0.1 AU	87526.7 AU	98.06 %

Chloroform extract after *Shodhana*



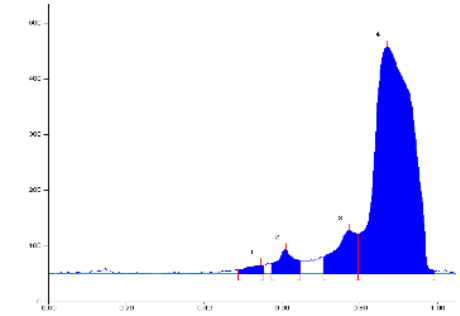
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.56 Rf	5.8 AU	0.59 Rf	32.8 AU	6.88 %	0.62 Rf	19.0 AU	875.5 AU	2.06 %
2	0.64 Rf	20.0 AU	0.83 Rf	444.3 AU	93.12 %	1.00 Rf	0.2 AU	41659.5 AU	97.94 %

Ethanol extract before *Shodhana*



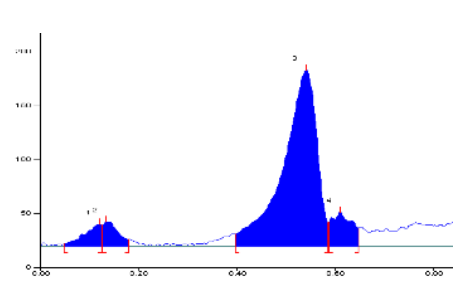
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.09 Rf	3.4 AU	0.13 Rf	125.2 AU	10.96 %	0.13 Rf	13.2 AU	2141.0 AU	4.73 %
2	0.14 Rf	113.4 AU	0.15 Rf	160.8 AU	14.05 %	0.19 Rf	3.4 AU	3340.8 AU	7.38 %
3	0.47 Rf	9.2 AU	0.54 Rf	88.2 AU	7.69 %	0.56 Rf	77.6 AU	2983.2 AU	6.60 %
4	0.57 Rf	83.9 AU	0.60 Rf	200.7 AU	17.54 %	0.65 Rf	78.8 AU	4781.8 AU	10.53 %
5	0.63 Rf	79.1 AU	0.64 Rf	86.9 AU	7.55 %	0.69 Rf	30.0 AU	3558.5 AU	7.87 %
6	0.69 Rf	68.0 AU	0.74 Rf	114.1 AU	9.97 %	0.79 Rf	37.1 AU	5886.8 AU	13.01 %
7	0.79 Rf	67.2 AU	0.93 Rf	367.8 AU	32.14 %	1.00 Rf	0.7 AU	22560.1 AU	49.88 %

Ethanol extract after *Shodhana*



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.49 Rf	5.2 AU	0.55 Rf	14.3 AU	2.68 %	0.58 Rf	13.2 AU	495.5 AU	1.29 %
2	0.57 Rf	17.2 AU	0.61 Rf	41.8 AU	7.84 %	0.65 Rf	20.5 AU	1392.6 AU	3.64 %
3	0.71 Rf	27.3 AU	0.77 Rf	74.2 AU	13.91 %	0.80 Rf	38.5 AU	3217.7 AU	8.10 %
4	0.80 Rf	88.8 AU	0.87 Rf	403.1 AU	75.57 %	0.99 Rf	0.5 AU	30311.3 AU	85.68 %

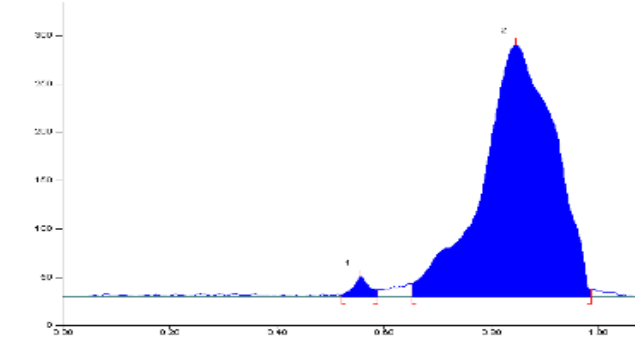
Crotonoside reference standard



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.09 Rf	9.4 AU	0.14 Rf	22.0 AU	7.35 %	0.18 Rf	6.2 AU	934.9 AU	6.13 %
2	0.42 Rf	16.6 AU	0.54 Rf	161.5 AU	53.98 %	0.59 Rf	20.1 AU	8407.3 AU	55.16 %

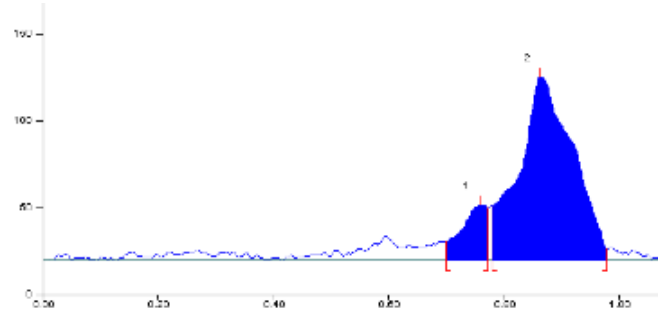
Figure 3: HPTLC fingerprint profile of *Croton tiglium* seeds chloroform & ethanol extracts before and after *shodhana* process at UV 366nmHPLC chromatographic profiling of Croton tiglium seeds

Chloroform extract before *Shodhana*



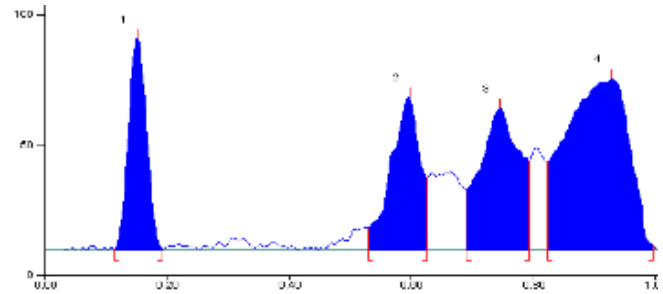
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.51 Rf	2.5 AU	0.55 Rf	22.1 AU	9.58 %	0.58 Rf	8.3 AU	629.4 AU	2.11 %
2	0.62 Rf	13.5 AU	0.78 Rf	208.3 AU	90.42 %	1.00 Rf	0.4 AU	29177.4 AU	97.89 %

Chloroform extract after *Shodhana*



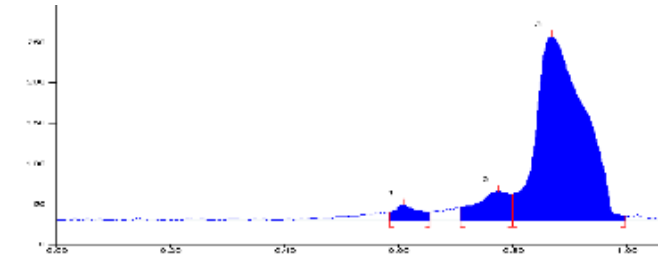
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.69 Rf	10.3 AU	0.84 Rf	166.0 AU	100.00 %	0.99 Rf	1.0 AU	13934.6 AU	100.00 %

Ethanol extract before *Shodhana*



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.11 Rf	0.2 AU	0.15 Rf	81.3 AU	31.36 %	0.19 Rf	0.3 AU	1884.3 AU	15.42 %
2	0.53 Rf	8.3 AU	0.60 Rf	58.5 AU	22.59 %	0.63 Rf	27.6 AU	2199.8 AU	18.01 %
3	0.69 Rf	22.8 AU	0.75 Rf	54.0 AU	20.85 %	0.79 Rf	33.9 AU	2795.3 AU	22.88 %
4	0.82 Rf	33.4 AU	0.93 Rf	65.3 AU	25.20 %	1.00 Rf	1.4 AU	5336.5 AU	43.69 %

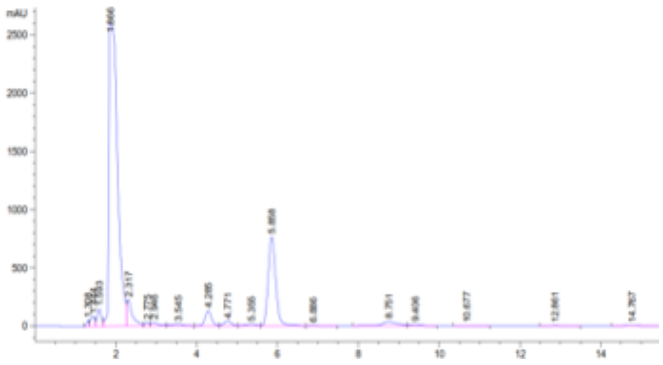
Ethanol extract after *Shodhana*



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.58 Rf	8.7 AU	0.61 Rf	17.6 AU	6.42 %	0.65 Rf	7.7 AU	582.8 AU	3.52 %
2	0.71 Rf	14.2 AU	0.78 Rf	32.9 AU	12.00 %	0.80 Rf	29.2 AU	1488.4 AU	8.99 %
3	0.80 Rf	29.3 AU	0.87 Rf	223.4 AU	81.58 %	1.00 Rf	0.1 AU	14493.1 AU	87.50 %

Figure 4: HPLC Profiling Chromatogram of *Croton tiglium* seeds chloroform extracts before and after *shodhana* process

Unprocessed sample



Processed sample

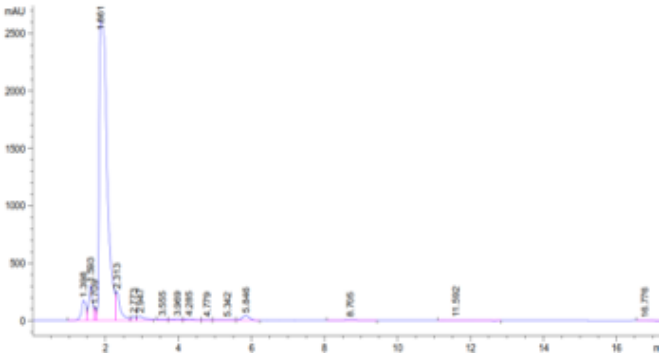
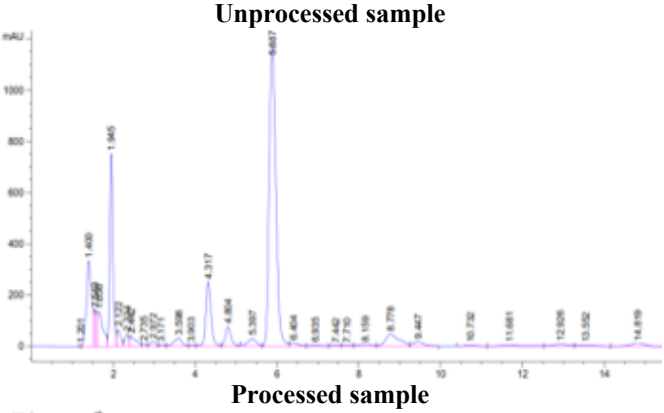
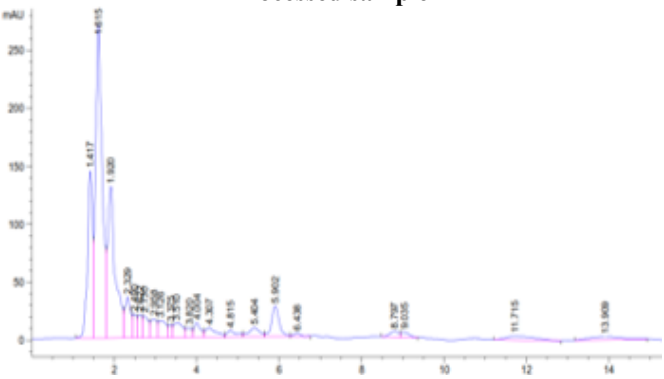


Figure 5: HPLC Profiling Chromatogram of *Croton tiglium* seeds ethanol extracts before and after *shodhana* process

Unprocessed sample



Processed sample



In comparing HPLC Chromatographic profiling of chloroform extracts, 18 peaks in unprocessed and 16 peaks in processed samples were detected. Twenty-nine peaks in processed and 22 peaks in unprocessed samples of each ethanol extract were detected. It is observed that the peak area of all peaks of processed samples was reduced as compared to unprocessed samples. The detailed peak identification and peak area results are shown in Figures 4 & 5 and Table 7 & 8. The remarkable changes have been observed in the chloroform and ethanol extracts HPLC profiling chromatograms of the *Croton tiglium* seeds before and after the *shodhana* process.

Table 7: HPLC peak details of *Croton tiglium* seeds chloroform extracts before and after *shodhana* process

Unprocessed sample				Processed sample			
Peak No.	**Ret. Time [min]**	**Area [mAU*s]**	**Area %**	**Peak No.**	**Ret. Time [min]**	**Area [mAU*s]**	**Area %**
1	1.308	168.35727	0.2924	1	1.398	1564.19421	3.1961
2	1.444	602.68860	1.0466	2	1.593	2100.35010	4.2916
3	1.593	1066.77148	1.8525	3	1.709	437.55542	0.8940
4	1.866	38122.7	66.2013	4	1.861	40008.1	81.7473
5	2.317	2230.60718	3.8735	5	2.313	2240.00977	4.5769
6	2.775	244.66501	0.4249	6	2.773	291.11786	0.5948
7	2.946	446.94354	0.7761	7	2.947	579.47070	1.1840
8	3.545	407.13733	0.7070	8	3.555	180.02390	0.3678
9	4.285	1525.03955	2.6483	9	3.969	210.16249	0.4294
10	4.771	574.77069	0.9981	10	4.285	218.60977	0.4467
11	5.355	331.66455	0.5759	11	4.779	55.14981	0.1127
12	5.858	10185.4	17.6873	12	5.342	118.92033	0.2430
13	6.886	88.24789	0.1532	13	5.846	557.70709	1.1395
14	8.751	1003.82599	1.7432	14	8.705	223.64122	0.4570
15	9.406	236.68639	0.4110	15	11.592	135.29448	0.2764
16	10.677	30.00565	0.0521	16	16.776	20.86800	0.0426
17	12.861	64.25497	0.1116	-	-	-	-
18	14.767	256.25562	0.4450	-	-	-	-
Total	**57586**	**100.0000**	**Total**	**48941.2**	**100.0000**		

Table 8: HPLC peak details of *Croton tiglium* seeds ethanol extracts before and after *shodhana* process

Unprocessed sample				Processed sample			
Peak No.	**Ret. Time [min]**	**Area [mAU*s]**	**Area %**	**Peak No.**	**Ret. Time [min]**	**Area [mAU*s]**	**Area %**
1	1.201	6.83685	0.0221	1	1.417	1212.14758	13.7826
2	1.400	3057.50342	9.9052	2	1.615	3033.32080	34.4900
3	1.549	592.55927	1.9197	3	1.920	1679.63477	19.0981
4	1.656	1340.63806	4.3432	4	2.329	331.62753	3.7707
5	1.945	3969.21338	12.8588	5	2.490	139.77296	1.5893
6	2.122	438.00662	1.4190	6	2.622	138.78291	1.5780
7	2.334	310.46707	1.0058	7	2.730	182.34494	2.0733
8	2.442	441.22461	1.4294	8	2.958	175.94273	2.0005
9	2.235	80.55502	0.2610	9	3.126	191.78812	2.1807
10	2.972	189.42357	0.6137	10	3.375	71.05652	0.8079
11	3.171	64.68430	0.2096	11	3.510	191.54918	2.1780
12	3.598	468.20312	1.5168	12	3.820	82.56252	0.9388
13	3.903	45.03476	0.1459	13	4.004	137.13824	1.5593
14	4.317	2235.47900	7.2421	14	4.307	149.76952	1.7029
15	4.804	750.93158	2.4327	15	4.815	91.74092	1.0431
16	5.397	465.05450	1.5066	16	5.404	143.11061	1.6272
17	5.887	13258.5	42.9525	17	5.902	339.80380	3.8637
18	6.404	209.98225	0.6803	18	6.438	32.85186	0.3735
19	6.935	99.92309	0.3237	19	8.797	74.11476	0.8427
20	7.442	56.83461	0.1841	20	9.035	65.72379	0.7473
21	7.710	57.35696	0.1858	21	11.715	159.79659	1.8169
22	8.159	195.26073	0.6326	22	13.909	170.20543	1.9353

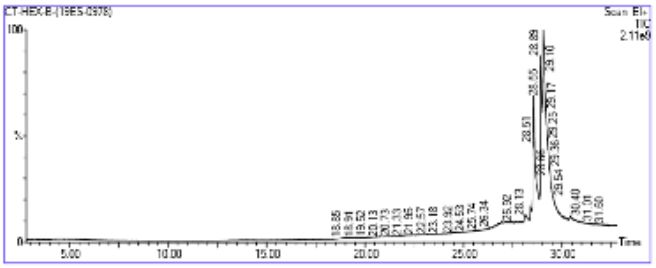
23	8.778	1106.25854	3.5839	-	-	-	-
24	9.447	326.18021	1.0567	-	-	-	-
25	10.732	84.68050	0.2743	-	-	-	-
26	11.681	225.43211	0.7303	-	-	-	-
27	12.926	246.15454	0.7974	-	-	-	-
28	13.552	169.09544	0.5478	-	-	-	-
29	14.819	376.28485	1.2190	-	-	-	-
Total	**30867.7**	**100.0000**	**Total**	**8794.78609**	**100.0000**		

GC-MS chromatographic profiling of *Croton tiglium* seeds

GC-MS analysis of hexane extracts of *Croton tiglium* seeds shows the presence of 6 peaks in the unprocessed sample and eight peaks in the processed sample. The detailed peak identification is shown in Figure 6, and retention time, peak area, area percentage, compound name, and molecular weight are given in Table 9. The chloroform extracts of *Croton tiglium* seeds show ten peaks in the unprocessed sample and 12 peaks in the processed sample. The detailed peak identification is shown in Figure 7, and retention time, peak area, area percentage, compound name, and molecular weight are given in Table 10. The remarkable changes have been observed in the hexane and chloroform extracts, GC-MS profiling chromatograms of the *Croton tiglium* seeds before and after the *shodhana* process.

Figure 6: GC-MS Profiling Chromatogram of *Croton tiglium* seeds hexane extracts before and after *shodhana* process

Unprocessed sample



Processed sample

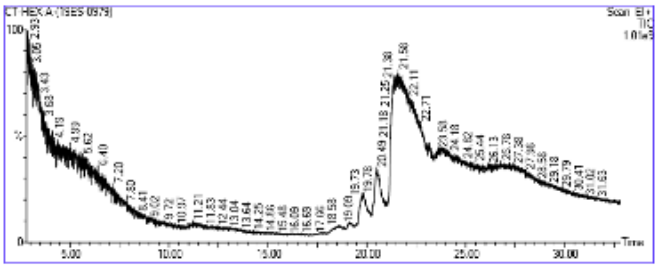
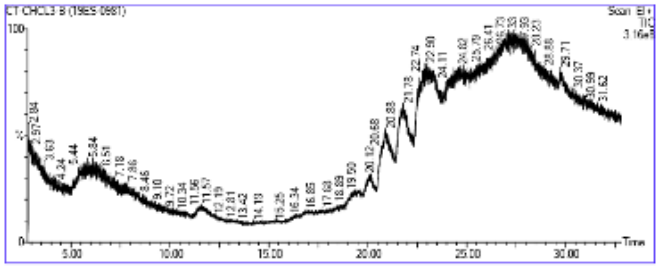


Figure 7: GC-MS Profiling Chromatogram of *Croton tiglium* seeds chloroform extracts before and after *shodhana* process

Unprocessed sample



Processed sample

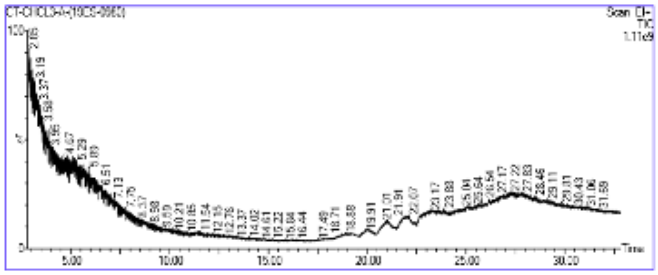


Table 9: GC-MS Peak details of *Croton tiglium* seeds hexane extracts before and after *shodhana* process

Unprocessed sample					
Peak	**RT**	**Area**	**Area %**	**Name of the compound**	**Molecular weight**
1	28.099	1,51,89,568	1.583	2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl -but-2-enyl)-Cyclohexene	222
2	28.399	1,31,91,537	1.375	1,5-Cyclodecadiene, 1,5-dimethyl-8- (1-methylethenyl)-,[S-(Z,E)]-	204
3	28.549	19,86,00,032	20.698	4,4,6A,6B,8A,11,11,14B-octamethyl-1,4,4A,5, 6,6A, 6B,7,8,8A,9,10,11,12, 12A, 14,14A,	424
4	28.909	18,89,37,664	19.691	2R-Acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl -2-buten-1-yl)-1t-cyclohexane	282
5	29.039	52,89,20,512	55.123		282
6	30.430	1,46,84,390	1.530	2,4,4-Trimethyl-3-hydroxymethyl-5a- (3-methyl-but-2-enyl)-cyclohexene	222

Processed sample					
Peak	**RT**	**Area**	**Area %**	**Name of the compound**	**Molecular weight**
1	19.806	5,87,19,072	2.480	Tetradecanoic acid	228
2	20.491	10,25,38,400	4.330	N-Hexadecanoic acid	256
3	21.581	1,02,92,25,664	43.462	6-Heptadecyne, 1-chloro-	270
4	23.137	9,26,23,368	3.911	9,12Octadecadienoyl chloride, (Z,Z)	298
5	23.767	56,00,32,768	23.649	17-Octadecynoic Acid	280
6	25.828	5,47,76,220	2.313	Z,Z-6,13-Octadecadien-1-Ol acetate	308
7	26.208	5,00,04,384	2.112	2-Methyl-6-methylene-octa-1,7-dien-3-ol	152
8	26.783	42,02,07,360	17.744	Z,Z-6,13-octadecadien-1-ol acetate	308

Table 10: GC-MS Peak details of *Croton tiglium* seeds chloroform extracts before and after *shodhana* process

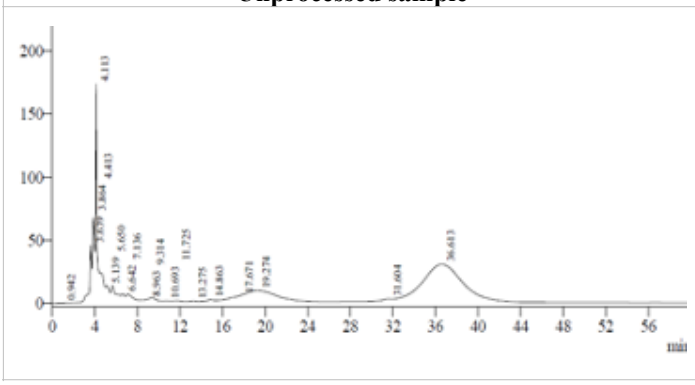
Unprocessed sample					
Peak	**RT**	**Area**	**Area %**	**Name of the compound**	**Molecular weight**
1	20.676	48,76,207.0	0.985	Tetradecanoic acid	228
2	20.881	48,84,822.5	0.987	Octadecanoic acid	284
3	21.776	2,56,05,386.0	5.173	Eicosanoic acid	312
4	22.847	3,33,04,378.0	6.729	Oleic acid	282
5	22.987	6,61,82,716.0	13.372	9-Octadecenal	266
6	24.337	3,68,69,356.0	7.449	1-Hexyl-2-nitrocyclohexane	213
7	24.823	3,46,45,632.0	7.000		213
8	27.118	19,53,49,632.0	39.469	4-Pentadecyne, 15-chloro-	242
9	27.519	8,80,95,824.0	17.799	2,6-Pyrazinediamine	110
10	29.710	51,30,300.5	1.037	Pregnan-3,11-diol-20-one	334
Processed sample					
Peak	**RT**	**Area**	**Area %**	**Name of the compound**	**Molecular weight**
1	20.946	12,95,488.9	3.000	Tetradecanoic acid	228
2	24.823	12,68,241.5	2.936	Oleic acid	282
3	25.073	31,64,184.2	7.326	2-Piperidinone, N-[4-bromo-N-butyl]-	233
4	26.168	25,08,068.8	5.807	2,7-Octadiene-1,6-diol, 2,6-dimethyl-, (Z)-	170
5	26.678	18,02,641.9	4.174	Oleic acid	282
6	26.893	41,10,459.0	9.517	D-mannitol, 1-O-(22-hydroxydocosyl)-	506
7	27.299	62,32,076.0	14.429	Estran-3-one, 17-(acetyloxy)-2-methyl-, (2.alpha.,5.alpha.,17.beta.)-	332
8	27.459	14,46,128.2	3.348	2-isopropyl-5-methylcyclohexyl 3-(1-(4-chlorophenyl)-3-oxobutyl)-coumarin	524
9	27.834	1,65,16,810.0	38.242	Di-N-decylsulfone	346
10	28.464	12,07,268.4	2.795	2,6-Lutidine 3,5-dichloro-4-dodecylthio-	375
11	28.599	22,21,505.5	5.144	Cholesta-8,24-dien-3-ol, 4-methyl-, (3.beta.,4.alpha.)-	398
12	28.909	14,17,292.9	3.282	Pseduosarsasapogenin-5,20-Dien methyl ether	428

LC-MS chromatographic profiling of *Croton tiglium* seeds

LC-MS Chromatographic profiling of ethanol extracts of *Croton tiglium* seeds shows 19 peaks in the unprocessed sample and 17 peaks in the processed sample. The detailed peak identification is shown in Figure 8, and retention time, peak area, and area percentage are given in Table 11. The remarkable changes have been observed in the ethanol extracts LC-MS profiling chromatograms of the *Croton tiglium* seeds before and after the *shodhana* process.

Figure 8: LC-MS Chromatogram of *Croton tiglium* seeds ethanol extracts before and after *shodhana* process

Unprocessed sample



Processed sample

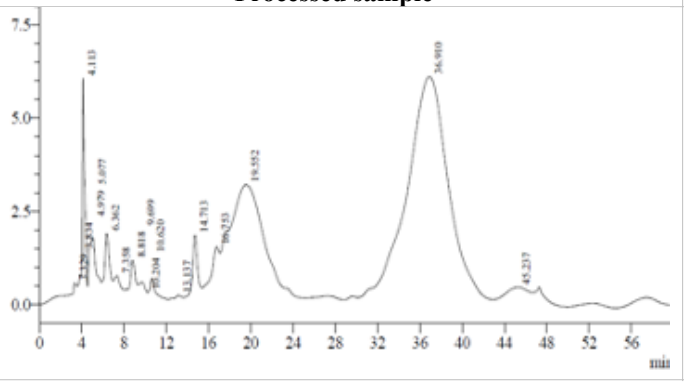


Table 11: LC-MS Peak details of *Croton tiglium* seeds ethanol extracts before and after *shodhana* process

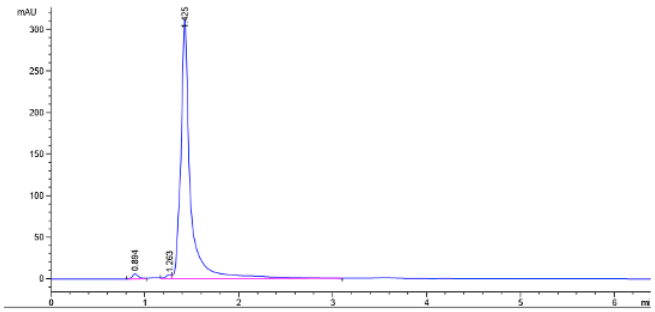
Unprocessed sample				Processed sample			
Peak	**Ret. Time**	**Area**	**Area %**	**Peak**	**Ret. Time**	**Area**	**Area %**
1	0.942	2010	0.019	1	3.329	2448	0.124
2	3.639	170426	1.647	2	3.834	887	0.045
3	3.864	279746	2.704	3	4.113	71707	3.646
4	4.113	664843	6.425	4	4.979	21523	1.094
5	4.413	64347	0.622	5	5.077	16188	0.823
6	5.139	36854	0.356	6	6.362	29261	1.488
7	5.650	75609	0.731	7	7.358	5694	0.289
8	6.642	15949	0.154	8	8.818	16927	0.861
9	7.136	70283	0.679	9	9.699	4266	0.217
10	8.963	8341	0.081	10	10.204	0	0.000
11	9.314	91690	0.886	11	10.620	8833	0.449
12	10.693	2037	0.020	12	13.137	1684	0.086
13	11.725	8596	0.083	13	14.713	42841	2.178
14	13.275	7857	0.076	14	16.753	10959	0.557
15	14.863	26000	0.251	15	19.552	271312	13.795
16	17.671	186732	1.805	16	36.910	1437398	73.085
17	19.274	1562917	15.104	17	45.237	24822	1.262
18	31.604	20352	0.197	-	-	-	-
19	36.613	7052972	68.161	-	-	-	-
Total	**10347561**	**100.000**	**Total**	**1966749**	**100.000**		

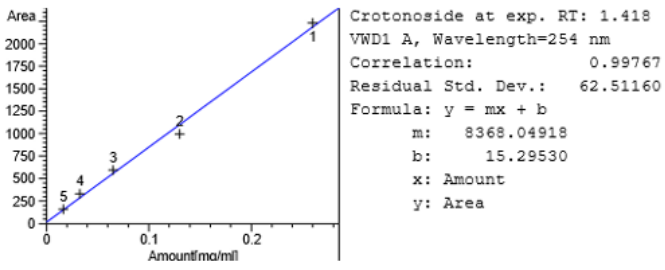
Quantitative estimation of Crotonoside in *Croton tiglium* seeds by HPLC

Standard calibration curve for *Crotonoside*

The calibration curve was established for peak area Vs. the concentration of Crotonoside applied is shown in Figure 9.

Figure 9: HPLC Chromatogram of Crotonoside standard and calibration curve



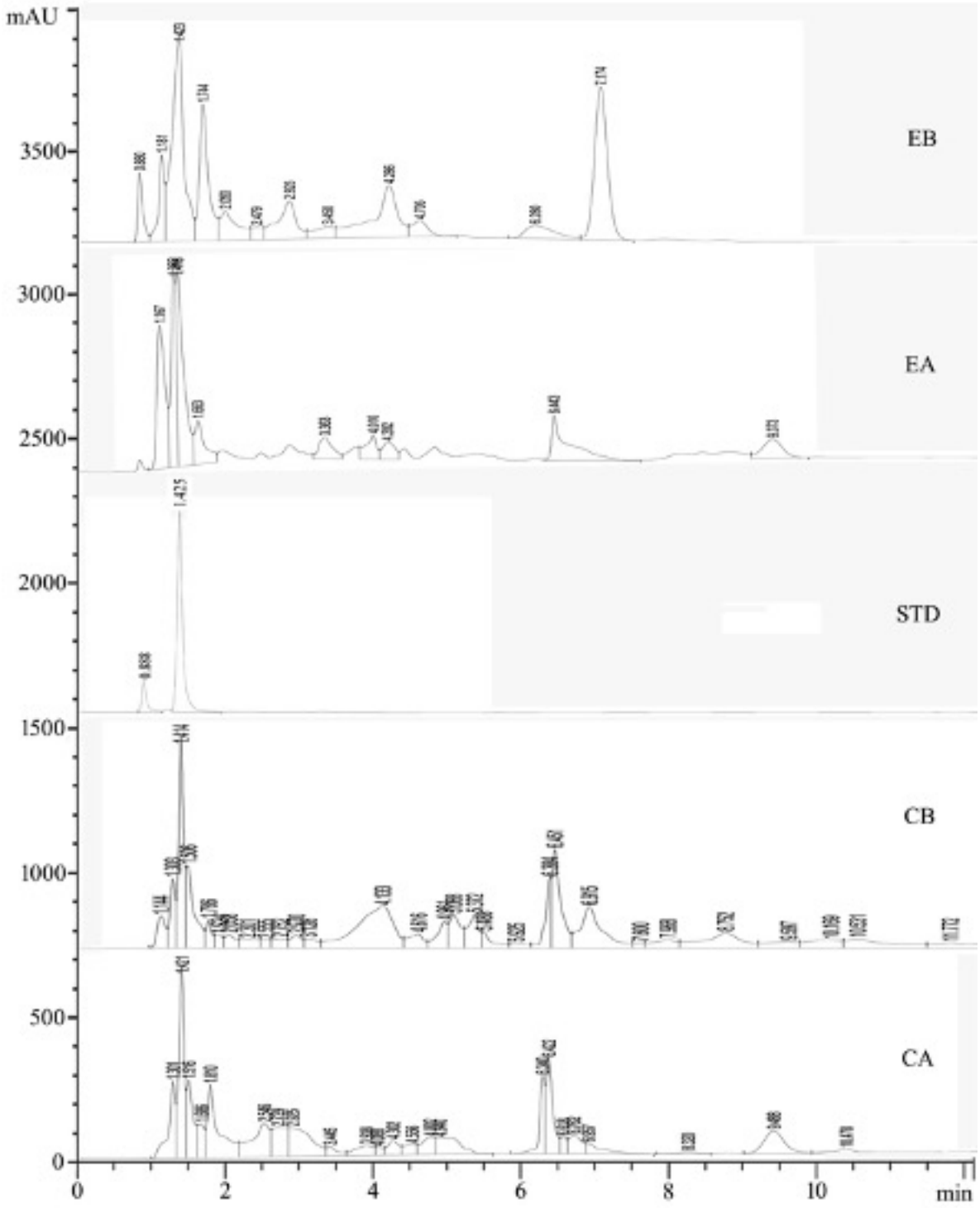


Estimation of *Crotonoside*

The calculated amount of crotonoside present in the residues extracted in ethanol and chloroform for each test sample obtained from before and after *shodhana* process samples of *Croton tiglium* seeds is given in Figure 10 and Table 12.

The results obtained from HPLC analysis revealed that the depletion in the level of crotonoside in ethanol and chloroform extracts after the shodhana process of *Croton tiglium* seeds compared to unprocessed seeds. HPLC analysis showed that the percentage of crotonoside was reduced by 2.43% in chloroform extract and 21.26% in ethanol extract after the *shodhana* process.

Figure 10: Estimation of Crotonoside in the of *Croton tiglium* seeds chloroform and ethanol extracts of before and after *shodhana* process



[EB-Ethanol before shodhana extract; EA- Ethanol after shodhana extract; STD- Crotonoside Standard; CB-Chloroform before shodhana extract; CA- Chloroform after shodhana extract]

Table 12: Estimation of Crotonoside in the of *Croton tiglium* chloroform and ethanol extracts of before and after *shodhana* process

S. No.	Name of extracts	Crotonoside (% w/w)				
		Before *Shodhana*		After *Shodhana*		Percentage reduced
		Results	Mean	Results	Mean	
1	Chloroform extract	0.0623	0.0623	0.0606	0.0608	2.43%
		0.0623		0.0610		
		0.0624		0.0608		
2	Ethanol extract	0.0816	0.0875	0.0694	0.0689	21.26%
		0.0943		0.0690		
		0.0866		0.0684		

*Percentage of results was given from the means of triplicates for both before and after *shodhana* samples of optimized two solvent extracts of ethanol and chloroform.

Conclusion

In this study, an attempt has been made to purify the *Croton tiglium* seeds by the classical Ayurvedic shodhana process. Preliminary phytochemical screening and physicochemical parameters were determined before and after the shodhana process of *Croton tiglium* seeds sample.

The percentage of water-soluble extractive, alcohol soluble extractive, and loss on drying at 105°C were found to increase ash content, acid-insoluble ash, and pH value reduced in the *shodhit* (processed) *Croton tiglium* seeds as compare to *ashodhit* (unprocessed) sample. The remarkable changes have been observed in different physicochemical parameters, HPTLC, HPLC, GC-MS, and LC-MS chromatographic profiling before and after the shodhana process of *Croton tiglium* seeds. In the HPLC chromatographic profiling, the peak area of all processed samples peak area was reduced compared to unprocessed samples.

The results obtained from HPLC analysis revealed that the depletion in the level of crotonoside in ethanol and chloroform extracts after the shodhana process of *Croton tiglium* seeds compared to unprocessed seeds. HPLC analysis showed that the percentage of crotonoside was reduced 2.43% in chloroform extract and 21.26% in ethanol extract after the *shodhana* process, respectively.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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