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Shanmugapriya P
Department of Nanju Maruthuvam, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India

Anoop Austin
Research and Development, Cholayil Private Limited, Chennai, Tamil Nadu, India

Thamodharan S
Assistant, Medical officer Salavakkam PHC, Kanchipuram, Tamil Nadu, India

Jiji Mol VC
Medical officer, Govt. Siddha Hospital, Vallakadavu, Kerala, India

Jeeva Gladys R
Lecturer, Velumailu Siddha Medical College, Sriperumbudur, Tamil Nadu, India

Ramamurthy M
Lecturer, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India

Christian GJ
Professor, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India

Correspondence
Shanmugapriya P
Department of Nanju Maruthuvam, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India

Standardization and validation of traditional siddha purification process for detoxifying *Croton tiglium* seeds by modern analytical methods

Shanmugapriya P, Anoop Austin, Thamodharan S, Jiji Mol VC, Jeeva Gladys R, Ramamurthy M and Christian GJ

Abstract
Modern analytical equipment's offers a tremendous advantage among validation of drugs from herbal origin. Gas chromatography mass spectrometry (GCMS) and High-performance liquid chromatography (HPLC) is one such instrumental method that can be utilized for quantitative and qualitative identification of biologically active phytocomponents present in various herbs and its extracts. *Croton tiglium* (CT) seeds are widely used in the traditional system of Indian medicine like Siddha, Ayurveda and Chinese medicine as a Purgative. Seeds of CT possess certain toxic principles which have to be detoxified and purified before inclusion of the same for therapeutic ailments. Siddha system of medicine has unique method of purification without compromising the biologically active components present in the drugs. To evaluate the chemical changes being which occur due to the detoxification process to elucidate the changes which would help us to understand the difference and the scientific rationale involved in such traditional practice. Hence the main aim of the present investigation is to carry out the detailed phytochemical and sophisticated instrumental analysis of the croton seeds with HPLC and GC-MS incorporating all the stages of purification procedures as listed in the Vedic literature. The results of the study has revealed that there was a significant reduction of Phorbol, a toxic organic compound from 5.18 to 3.86% and also the saturated fatty acids like, Arachidic acid, Behenic acid, Stearic acid and Palmitic acid whereas all the un saturated fatty acids like Oleic acid, Linoleic acid seems unaltered. In conclusion the present research finding would be highly beneficial for the researchers to understand the compositional verification that happens in the process of purification and its impact on biological activity.

Keywords: Siddha, *Croton tiglium*, croton sees, detoxification, Phorbol, fatty acid, HPTLC, GCMS

1. Introduction
Siddha is a special traditional art that heals the body through regulation balance among five fundamental elements. Even before several centuries Siddhar's had practiced the traditional medicine as an ailment for treating several dreadful disorders. Siddha formulation commonly relies on herbs, metals, minerals, animals and other marine derived products. Being a medicine of several biological origins it become mandatory for the practitioners to ensure the purity and safety of such medicines before clinical usage in humans. Siddha system of medicine has several techniques for purification, extraction, isolation and detoxification procedures.

Herbs have integrated power of healing though their versatile biologically activity components such as alkaloids, flavonoids, terpenoids, saponins etc. Gas chromatography and High-performance liquid chromatography are widely used method for quantitative and qualitative analysis. It can be applied in analysis of the gas, fluid, or solid samples. This includes herbal medicine, raw materials and other finished products. In GCMS analytes are volatile or able to be converted to volatile with good thermal stability, such as volatile oils as well as pesticide residues in herbs or herbal products.

It was evident through several researches that most of the times the active components present with in the herb possess significant pharmacological activity. *Croton tiglium*, L belonging to the family Euphorbiaceae is indigenous to China, Tibet and Northern parts of India. The seeds are extensively used as a purgative in Siddha, Ayurveda and Chinese medicine. It is a drastic purgative. It is widely used in various formulations used for arthritis, delirium, body ache, stomach troubles and constipation [1]. In small doses it is purgative and emetic in large doses [2]. Still now there is no proper documentary evidence available on the series of chemical changes that is happening in the stepwise purification procedure of *Croton tiglium.* Hence the present investigation aimed at analytical evaluation and validation of traditional siddha purification process of *Croton tiglium* seeds by four sequential analysis methods.

2. Materials and Methods

2.1 Collection and Authentication of Raw materials

Commercially available croton seeds were procured from Chennai and authenticated through microscopic and macroscopic studies at National Institute of Siddha, Tambaram, Chennai. After authentication the seeds were subjected to purification process according to prescribed texts.

2.2 Purification process of *Croton tiglium* seeds

500 gm of Croton seeds were weighed and tied in a clean white cloth to form a bolus. It was soaked in 2 liters of cow dung solution in a mud-pot and boiled for 2 hrs, after that washed with water. Then the seeds were treated with cow's urine for 2 hrs in a similar manner as mentioned above followed by lemon juice for the next 2 hrs. Subsequently, the seeds were washed with water and removed the outer skin and cotyledons and finally fried with ghee.

Samples were collected from each step of the purification process and analyzed both qualitatively and quantitatively.

Collected samples are as follows

Sample 1- unpurified (raw) croton seeds

Sample 2-Croton seeds processed with Cow dung solution.

Sample 3- Croton seeds processed with Cow's urine

Sample 4- Croton seeds processed with lemon juice

Sample 5- Croton seeds fried with ghee (Purified seeds)

2.3 Physicochemical analysis of Croton seeds

Seeds and oils of the above processed samples were subjected to various physico-chemical parameters such as loss on drying, total ash, pH and solubility, specific gravity, oil yield, refractive index, saponification value and Iodine number by following the methods of Ayurvedic pharmacopoeia of India [3]

2.4 Extraction of Croton oil

200 g of sun-dried powdered seeds were extracted with hexane using soxhlet apparatus. Then the extract was filtered and evaporated at 35 °C to yield the extract which is hexane-soluble.

2.5 Phytochemical analysis of croton seeds

Preliminary phytochemical analyses were carried to screen the presence of alkaloids, glycosides, steroids, sugars, tannins, saponins, proteins, phenol and fixed oil [4]

2.6 Quantitative analysis by HPLC [5]

Quantitative analyses of tannin and phorbol ester were carried out using spectrophotometer and HPLC methods. The analytical column used was a Zorbax, C18 (4 µm particle size and 150 mm × 3.9 mm internal diameter) column. The column was thermally controlled at 25°C. A mixture of acetonitrile and deionized water (80:20, v:v) was used as the mobile phase at a flow rate of 1 ml/min. The diode array detector wavelength was set at 254 nm. The phorbol-12myristate 13-acetate (PMA) was used as an external standard.

2.7. GCMS Validation

GCMS Specification

Agilent 7890B GC connected to 5977A MSD, NIST Ver.2.1 MS data library

Column Name

- HP_5MS 5% Phenyl Methyl Silox -60 °C -325 °C (325 °C) 30m×250µm×0.25µm
- Split less mode injection
- 1µL injection volume

Oven program

- 50 °C for 2 min then ramp 5 °C per minute till 270 °C, then 270 °C maintained for 2min, total run time 42 min
- Detector temperature 275 °C
- Injector temperature 250 °C
- Solvent delay 2min
- m/z Scan range 50-600amu

Start ime(min)	End Time(min)	Start m/z	End m/z Scan	Speed
2.50	18.00	50.00	650.00	2000

GC-MS Plays a key role in the analysis of unknown components of plant origin. GC-MS ionizes compound and measures their mass numbers. Ionization method includes EI (Electron Ionization). The EI method produces ions by colliding thermal electrons emitted from a filament with sample gas molecules. This method provides high stability in ionization and obtained mass spectra show good reproducibility. The EI method provides good result for quantitative analysis as well. Quantitative analysis with GC-MS, in which only ions specific to the compounds are measured, is highly selective method without interfering components. Gas chromatography Technique involves the separation of volatile components in a test sample using suitable capillary column coated with polar or non-polar or intermediate polar chemicals. Elite-1 column (100% Dimethyl polysiloxane) is a non-polar column used for analysis of phyto-components. Elite -5 column (5% phenyl and 95% methyl polysiloxane) is an intermediate column and also used for the estimation of Phytochemical. An inert gas such as hydrogen or nitrogen or helium is used as a carrier gas. The compounds of test sample croton oil is evaporated in the injection port of the GC equipment and segregated in the column by absorption and adsorption technique with suitable GC programme [6, 7].

3. Results

3.1. Preliminary phytochemical analysis of croton seed

The preliminary phytochemical analysis was carried out for both aqueous and ethanol extracts of various samples on each step and is illustrated in Table-1. Preliminary phytochemical analysis of croton seeds in its unpurified state (Sample1) showed the presence of glycosides (aqueous extract), steroids (ethanolic extract), sugars (aqueous and ethanolic extract), tannins (aqueous and ethanolic extract), phenol (aqueous extract) and fixed oils (ethanolic extract). Upon purifying the seeds in each step the toxic principles such as glycosides and phenols were removed (Sample-3) and the phytochemicals such as the steroids, tannins and fixed oil were retained (Sample 5).

Table 1: Preliminary phytochemical analysis of croton seed

S. No	Test	Sample 1		sample 2		Sample 3		Sample 4		Sample 5	
		A	E	A	E	A	E	A	E	A	E
1	Alkaloids	-	-	-	-	-	-	-	-	-	-
2	Glycosides	+ +	-	+ +	-	+ +	-	-	-	-	-
3	Steroids	-	+ +	-	+ +	-	+ +	-	+ +	-	+ +
4	Sugars	+ ++	+	+ +	+	+	+	+ +	+	+	-
5	Tannins	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
6	Saponins	-	-	-	-	-	-	-	-	-	-
7	Proteins	-	++	-	+	-	-	-	-	-	-
8	Phenol	+ +	-	+ +	-	-	-	-	-	-	-
9	Fixed oil	-	+ +	-	+ +	-	+ +	-	+ +	-	++

+ - Indicates Presence and – indicates absence of phytocomponents.

3.2 Result Analysis on Qualitative Analysis of croton seeds and Croton oil

Results of qualitative analysis revealed that the loss on drying of each sample increased from 5.79% to 10.32%, showed in Table- 2. Specific gravity, saponification value, RI at 40°C and iodine number of extracted croton oil of all the 5 samples were retained through out the purification process (Table -3) which is almost similar to the standard. Oil yield varied in each samples, in that the final sample yielded maximum amount of oil as compared to the Sample-1.

Table 2: Qualitative analysis of croton seeds

S. No	Process	LOD	Total ash
1	Sample 1	5.89%	1.20%
2	Sample 2	13.44%	0.834%
3	Sample 3	17.15%	0.778%
4	Sample 4	20.18%	0.96%
6	Sample 5	10.32%	2.35%

Table 3: Qualitative parameters of croton oil

S. No	Sample	Specific gravity (wt/ml)	Oil yield (%)	RI at 40°C	Saponification value	Iodine number
1	Sample1	0.952	11.01	1.471	213	105
2	Sample2	0.952	12.08	1.471	213	105
3	Sample3	0.952	8.06	1.471	213	105
4	Sample4	0.952	8.75	1.471	213	105
5	Sample5	0.954	17.51	1.473	213	105

3.3 Result Analysis on Esterified fatty acid present in croton oil

GCMS analysis of croton oil samples reveals the presence of various acid esters at each stage of purification was indicated in table 4 and represented in figure 1 to 5. Favorably, all the un saturated fatty acids like linoleic acid, oleic acid remained unaltered as such and saturated fatty acids such Myristic acid, Palmitic acid, Linoleic acid, Arachidic acid and Behenic acid were reduced considerably at final stage of purification (Sample 5).

Table 4: Analysis of various acid esters of croton oil

Fatty Acid	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Myristic acid	10.17	0.15	0.59	0.62	1.62
Palmitic acid	8.6	3.75	2.58	3.48	7.49
Stearic acid	0.274	0.52	3.89	0.19	0.49
Oleic acid	10.06	5.44	5.44	7.8	9.7
Linoleic acid	44.83	37.72	36.11	38.49	45.56
Arachidic acid	1.26	1.1	1.52	0.98	0.31
Behenic acid	0.44	3.51	2.12	2.46	0.26

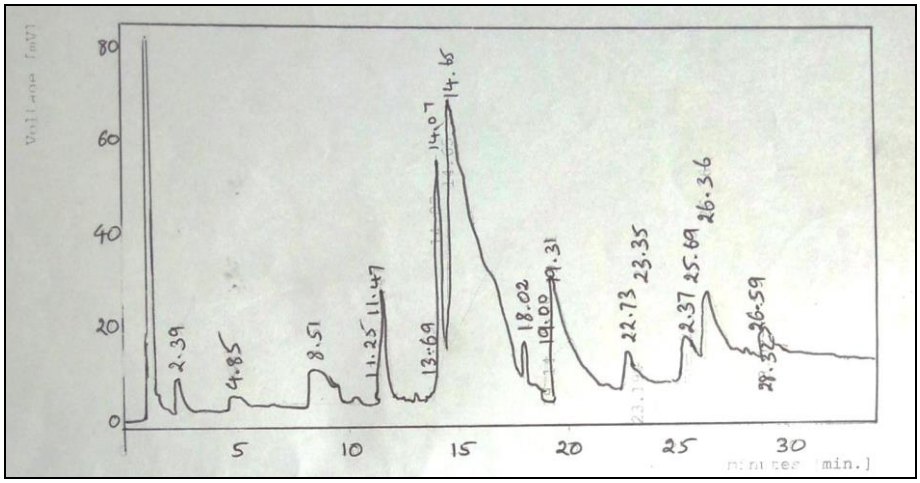

Fig 1: GCMS chromatogram of Croton seed oil – Sample 1

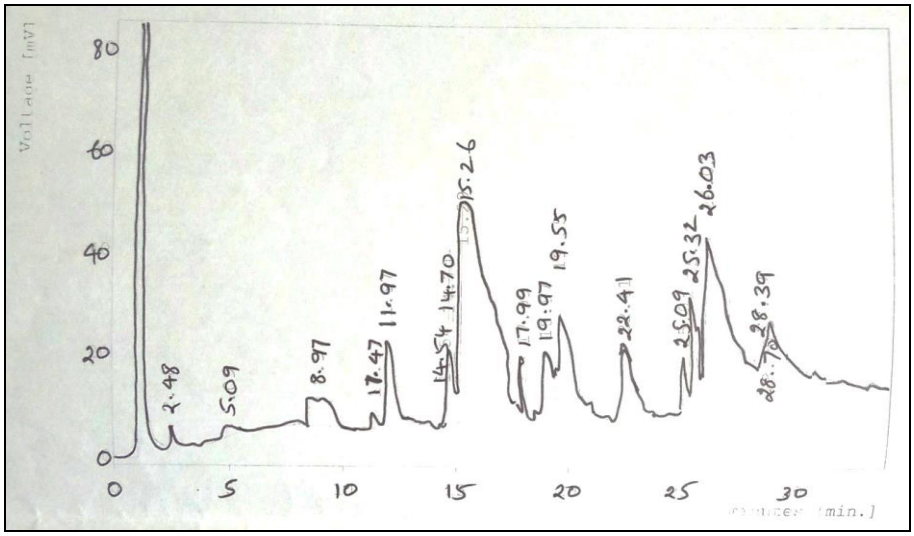

Fig 2: GCMS chromatogram of Croton seed oil – Sample 2

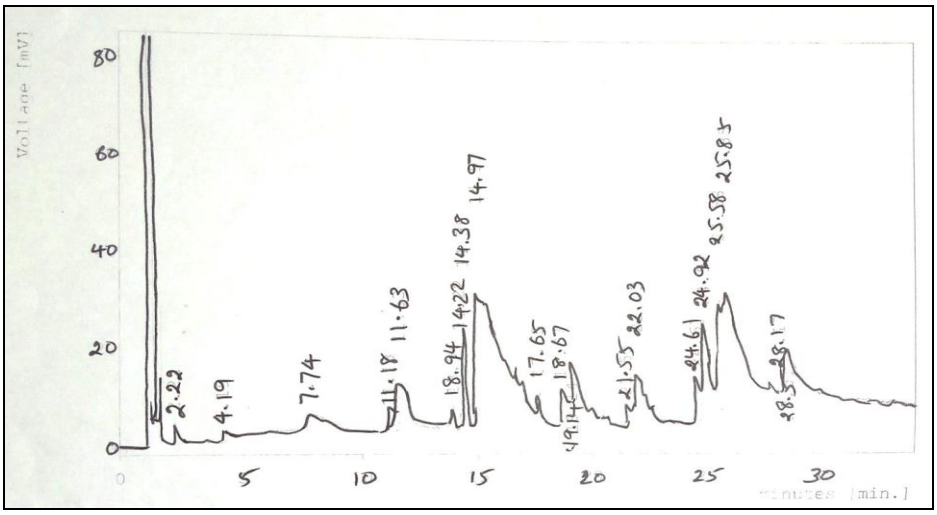

Fig 3: GCMS chromatogram of Croton seed oil – Sample 3

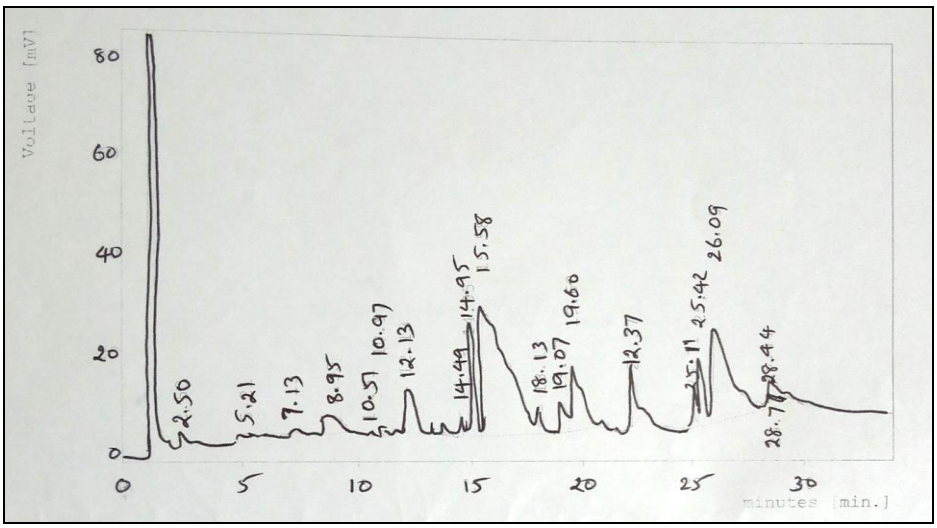

Fig 4: GCMS chromatogram of Croton seed oil – Sample 4

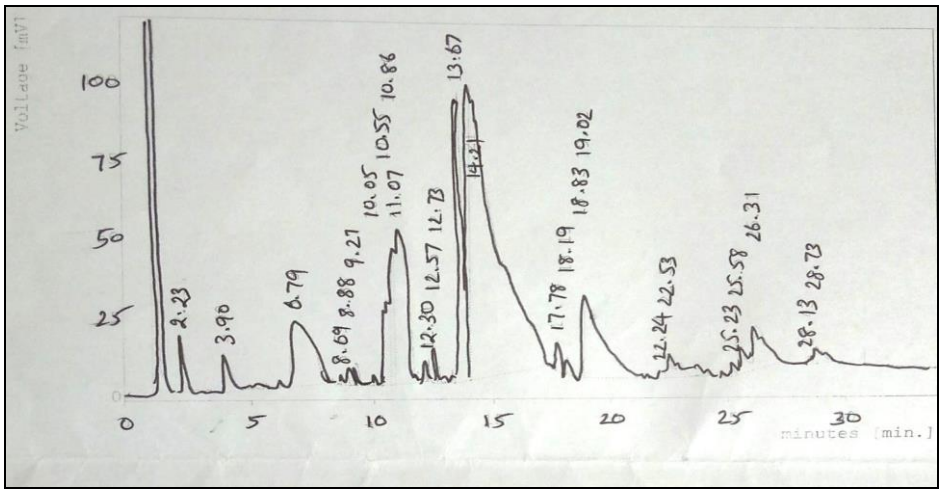

Fig 5: GCMS chromatogram of Croton seed oil – Sample 5

3.4 Result Analysis on Quantitative estimation of Tannins and Phorbol in croton oil

Results of HPLC analysis of croton oil reveals that there was a significant reduction in the level of phorbol content from 5.18% to 3.96%. Further, Tannin content was also most reduced from 31.5% to 12.6%. Results were tabulated in table 5.

Table 5: Quantitative Estimation of Tannins and Phorbol

S. No	Sample	Tannins (mg%)	Phorbol (mg%)
1	Sample 1	31.5	5.18
2	Sample 2	30.7	5.12
3	Sample 3	18.6	4.92
4	Sample 4	24.6	4.82
5	Sample 5	12.6	3.96

4. Discussion

Global usage of herbal medicine has achieved a remarkable hike in recent time due to enormous advantage of using it as a first line treatment towards several disease conditions. Herbs are considerably safe and offer greater protection and rejuvenation in treating communicable and non-communicable disease as well. Preliminary phytochemical analysis of croton seeds in its unpurified state showed the presence of glycosides, steroids, sugars, tannins, phenol and fixed oils Croton seeds are said to contain various compounds such as trypsin inhibitor, lectins, phytates, saponins besides the major toxic compound Phorbol esters [8]. It was observed that the trypsin inhibitors and lectins are heat labile and can be destroyed by heat while Phorbol esters and phytates were insensitive to heat [9]. Phorbol esters are compounds with fatty acid moieties esterified to the hydroxyl groups of tiglaine diterpenes [10]. The report of a previous study reveals that the enzymes produced by microbes could degrade the tiglaine and diterpene esters [11]. In the present investigation it was observed that the loss on drying of each samples increased from 5.79% to 10.32%.

The kernel of *Croton tiglium* Seeds contain 55-57% croton oil [12]. The purgative property of *Croton tiglium* is due to the presence of crotonoside (is oguanosine) which is a nonvolatile, unsaturated fatty acid. Crotonoside also shows cytotoxic effects against several tumor cell lines [13]. The vesicant and irritant properties of seed oil is due to croton resin which contain esters of long chain fatty acids and diterpene called phorbol. In addition to the vesicant and purgative principle which pass into the oil, the seed kernels contain 2 toxic proteins, croton-globuline and croton albumin, sucrose-glycoside and a glycoside-crotonoside. Fixed oil such as Tiglic acid is non-purgative but corrosive for skin. It also contains some volatile oil and fatty acids [14]. GCMS analysis of croton seed oils reveals the presence of un saturated fatty acids like linoleic acid, oleic acid and saturated fatty acids such Myristic acid, Palmitic acid, Linoleic acid, Arachidic acid and Behenic acid. Most of these fatty acids have vital role in management of numerous biological activities in humans and animals.

Siddha system of traditional medicine has several purification processes in which each methodology has a unique scientific rationale. One such method of detoxification is purification using cow dung and urine. Since in this traditional method of purification, the raw croton seeds are boiled with cow dung followed by cow's urine, the presence of microbes, water and heat favors the chemical reaction hydrolysis thereby breaking the toxic protein and ester bonds. This procedure is further repeated using lime juice which in turn provides an alkaline medium for further detoxification. Moreover, treatment with lime juice detoxifies the action of phytates, as the heat labile phytates antinutrients which inhibits the absorption of iron, calcium and zinc are overcome by the presence of rich source of ascorbic acid in lime juice. Results of HPLC analysis of croton oil reveals that there was a significant reduction in the level of toxic component called phorbol from 5.18% to 3.96% and it is almost equivalent to the estimated value of phorbol reduction from 5.12% to 3.85% by chemical purification. Further, Tannin content was also most reduced from 31.5% to 12.6%.

As cow dung, cow's urine, lemon juice act as alkalizing agent, further boiling the content in those solutions consequently favors the detoxification of the croton seeds. Previous study indicates that sodium bicarbonate moist combined with heat treatment and water wash removed 76.48% of the phorbol esters [15]. Distillate of cow urine also acts as anti-cancerous agent and bio pesticide which in turn may also nullify the hidden toxic principle of croton seeds [16]. In siddha system of medicine croton seed is one of the ingredients in many of the formulations and are commonly indicated for the uterine complaints and purgation. The possession of oleic acid in the purified seeds may be responsible for the reduction of insulin resistance which may lead to its desired action on uterine complaints [17]. Frying in cow ghee enhances the site of action of croton and also helps in the retention of essential fatty acid as such in the drug, there by favor the therapeutic efficacy of drug as well.

5. Conclusion

In this present study *Croton tigilum* seeds were purified by various substances such as cow's dung, cow's urine, lemon juice and ghee. In a view of exploring some scientific evidence for one of the Siddha traditional purification process of *Croton tiglium* seeds, the reduction in the level of phorbol content and the changes underwent by and during the purification process indicate the detoxification process of croton seeds. While various techniques such as chemical, biological and radiation treatment has been adopted in the past to remove the anti-nutrients and toxic components, still all of them have been commented unfriendly, time consuming and expensive. These findings strongly confirm the effectiveness of Siddha purification process in nullifying the alleged toxicity of drugs at a simpler and economical way.

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