COMPARATIVE EVALUATION OF UN-PURIFIED AND PURIFIED FORM OF NERVAALAM (CROTON SEEDS) BY PHARMACOGNOSTICAL, PHYSICOCHEMICAL AND FT-IR ANALYSIS.

J. Kanmozhi*, S. Vanathi, A.M. Dharsana and P. Shanmugapriya

Post Graduate Department of Nanju Noolum Maruthuva Neethi Noolum, National Institute of Siddha, Tambaram Sanatorium, Chennai 600047, Tamilnadu, India.

ABSTRACT

The World Health Organization (WHO) has now imparting an greater effort regarding the need and importance of medicinal plants for public health care in developing countries like India and has evolved with specific guidelines to support the member states in their capacity to formulate national policies on traditional medicine and to study their potential usefulness including standardization, evaluation, safety and efficacy. Standardization of herbs is an essential factor in order to assess the quality, purity, safety and efficacy of drugs based on the concentration of their active principles. It is very important to establish a system of standardization for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous. Still now there is no proper documentary evidence available on purification aspect of this medicinal herb and hence this prompted us to peruse systematic characterization of purification process of the plant Nervaalam (Croton tiglium). Croton seeds (CS) are traditionally used in abdominal disorders constipation and dyspepsia. Croton seeds were subjected to microscopical, physicochemical and FT-IR analysis in order to ascertain its purity. Results of pharmacognostical evaluation of CS show the presence of well distinguished seed coat, pericarp, cotyledon and section of seed coat reveals visible meso and endocarp. Further presence of calcium oxalate crystals was confirmed in the endocarp. The results obtained from physicochemical analysis of un purified CS reveals that total ash value of CS is about 2.74% in which acid insoluble ash is 1.44%. Extraction value of drugs renders most significant information about partition of the active component the results of extract...
value study shows that alcohol soluble extract of CS is 11.50% and water soluble extract is 8.49%. The loss on drying value of CS is about 11.35%. Physicochemical analysis of Purified CS reveals that total ash value of CS is about 2.28% in which acid insoluble ash is 1.08%. The results of extract value study shows that alcohol soluble extract of CS is 9.87% and water soluble extract is 2.36%. The loss on drying value of purified CS is about 10.80%. Result obtained from the FT-IR analysis reveals that raw unpurified CS shows the presence of functional groups like alcohol, alkane, nitro, acid and aldehyde ,whereas the purified sample indicates the presence of alkenes and amines in addition to existing functional groups.

KEYWORDS: Nervaalam, Croton tiglium, Croton seeds, Pharmacognostical evaluation Physicochemical, FTIR.

INTRODUCTION
In Ayurveda, siddha and unani (ASU) system of medicine plants, minerals, and animal products are used as main drugs to cure various ailments.[1] There is a global resurgence in the use of these medicines along with a growing scientific interest in them as a source of new drugs.[2] There has been a boom in the usage of ASU drugs and export is appreciably high in the last two decades.[3]

The usage of plants as medicines goes back to early man. Certainly the great civilization of the ancient chinese, indians and north africans provided written evidence of man's ingenuity in utilizing plants for the treatment of a wide variety of diseases. Herbal preparations against human ailments are gaining importance due to the partial rejection of synthetic drugs because of their side effects. Complementary and alternative medicines may be appealing to patients due to their seemingly low side effects profile and optimistic evidence especially when more treatments that are conventional have high failure rates or numerous side effects. The earliest mention of the medicinal use of plants is to be found in the Rig-Veda which dates back as early as 3500 BC.

Currently about 80% of the world population depends on herbs, herbo-minerals and other plant-derived medicine for the first line of primary health care for human alleviation because it has very minimal side effect. Herbal medicine will be broadly classified into various basic systems: Traditional indian herbalism, which is part of traditional oriental medicine, siddha herbalism, which is derived from siddha system of medicine.[4,5]
Nervaalam (Croton tiglium) is an important medicinal plant of the family Euphorbiaceae which is used for the treatment of constipation, dyspepsia, dysenteriae, gastrointestinal disorders, intestinal inflammation, rheumatism, peptic ulcer, visceral pain and headache.\[^6,7,8,9\] C. tiglium seeds oil is reported to contain phorbol esters and crotonic acid along with the fatty acids.\[^{10}\] C. tiglium, commonly known as Kumbhinī is well known for its severe purgative action.\[^{11}\]

Croton tiglium (CT) belongs to the family Euphorbiaceae is widely used in siddha formulation like soolai kudaram, Agasthiyar kuzhambu, Thazhamboo Mathirai, Meganatha Kuligai, etc after purification process. Croton seeds are traditionally used in abdominal disorders constipation and dyspepsia.\[^{12,13,14}\]

Several studies are being carried towards its activities genotoxicity, M\(^3\) muscarinic receptor and Ca\(^{2+}\) influx mediated muscle contraction, Anti-HIV-1 phorbol ester activity, purgative and inflammatory activity.\[^{15}\]

It consists majorly phorbol ester which has tumor enhancing property. Seeds are reported to contains croton, a toxalbumin and crotonoside. Major known chemical constituents are glyceryl crotonate, crotonic acid, crotonic resin. The amino acid composition of purified lectin from seeds was reported as aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, histidine, lysine, arginine and cysteine. Siddha system of medicine recommends the administration of Nervaalam only after purification. Standardisation of drug means confirmation of its identity, quality and purity throughout all phases of its cycle.\[^{16}\]

Drug standardization and way forwards for indian system of medicine drug manufacturing industry as the national authorities like national drug regulatory authorities should ensures that all ASU pharmaceutical product subjected to their control are in conformity with quality, safety, efficacy and that all premises employed the manufacturing and distribution of these preoducts comply with god manufacturing process (GMP) standards, so as to ensure the continued conformity of the products with these requirement until such time as they are delivered to the end user. By understanding the need of current scenario with respect to herbal drug purification and standardization the current work was undertake to evaluate the purity of croton seed by systematic purification process as per siddha principles and further study drug is subjected to microscopical, physicochemical and FT-IR analysis.
MATERIALS AND METHODS

Purification Process

Test drug collection

Nervaalam (Croton tiglium) was obtained from reputed drug store in Chennai.

Identification and Authentication

The drug was identified and authenticated by Prof. Dr. Jayaraman, Ph.D. (voucher no PARC/2016/3221), Plant Anatomy Research Centre, West Tambaram.

Method of Purification

3 Liters of Buffalo dung juice was taken in a mud pot. 650gms of Croton seeds were knotted in a cotton cloth and soaked in the dung juice by hanging in the above pot as Thulayanthiram. The mud pot was heated using firewood under low flame until the dung juice was reduced to 1 lit. The knotted seeds were taken out and cloth over the seeds were removed. Then washed with water, remove the outer seed coat and inner cotyledon were removed. Remaining 350gms of seeds are again subjected to thulayanthiram by using Raw rice with water for 3 hours. After 3hrs boiling the knotted seeds were taken out and cloth over the seeds were removed, the seeds were washed with water. Again the seeds were subjected to thulayanthiram now by using Milk for 3 hours. After 3hrs, knotted seeds were taken out and the cloth over the seeds were removed, then washed with water and dried it. After drying, it was fried with Castor oil. After complete of frying purified Croton seeds was collected and stored in an air tight container (Book name- Sigicha rathna deepam).

Pharmacognostical Analysis

Macroscopical Investigation

The macroscopical evaluation of CS was performed as per the methods of Khandelwal.[17] Various organoleptic characters such as color, shape, size, odour, taste and texture were studied and further width of the raphe and hilum were carefully observed using simple microscope.

Microscopical Investigation

The seeds were cut into thin transverse section using a sharp blade and the sections were stained with saffranine. Transverse sections were photographed using LEICA trinocular microscope attached with camera under bright field light.[18]
For the microscopical evaluation, the fresh samples were cut free handed and immersed in clearing reagent (chloral hydrate). The sections were dehydrated with varying strength of absolute alcohol and then stained with the mixture of phloroglucinol and conc. HCl (1:1, v/v).\textsuperscript{[19]} Anatomical characteristics were described from the samples which were fixed and embedded in plastic resin.\textsuperscript{[20]} The blocks were sectioned at 10 µm on a rotary microtome with steel knives type C. The sections were stained with Toluidine Blue 0.05% in acetate buffer with pH 4.7.\textsuperscript{[21]}

**Physico-chemical analysis**

**Percentage Loss on Drying\textsuperscript{[22]}**

10gm of CS was accurately weighed in evaporating dish and was air dried at 105°C for 5 hours and then weighed.

**Determination of Total Ash\textsuperscript{[23]}**

3 g of CS was accurately weighed in silica dish and incinerated at the furnace a temperature 400 ºC until it turns white in color which indicates absence of carbon. Percentage of total ash will be calculated with reference to the weight of air-dried drug.

**Determination of Acid Insoluble Ash**

About 0.5gm of the ash obtained by total ash test will be boiled with 25 ml of dilute hydrochloric acid for 6mins. Then the insoluble matter is collected in crucible and will be washed with hot water and ignited to constant weight. Percentage of acid insoluble ash will be calculated with reference to the weight of air-dried ash.

**Determination of Alcohol Soluble Extractive**

About 5 g of the air dried CS will be macerated with 100 ml of Alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowing to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105°C, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.
**Determination of Water Soluble Extractive**

About 5 g of the air dried CS will be macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowing tost and for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105°C, to constant weight and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug.

**Determination of pH**

About 5 g of CS will be dissolved in 25ml of distilled water and filtered the resultant solution is allowed to stand for 30 mins and the subjected to pH evaluation using pH meter.

**Fourier Transform – Infra Red Spectroscopy**

Fourier Transform – Infra Red Spectroscopy Study (FTIR) IR data acquired with FT-IR spectrometer FT/IR-4100 –Jascoasia portal. About 20 mg of the CS before and after purification was taken on a microspatula and grounded well with required quantity of KBr salt. Sample admixed with KBr with trituration aided by mortar and pestle until to get a uniform fine powder of sample- KBr mixture. Further mixture was loaded in pellet die and subjected to 5000-10,000 psi in pelletizer. Resulting pellet was placed in FTIR sample holder and expose to IR radiation to get the spectra.

**RESULTS**

**Macroscopic Evaluation of CS**

Macroscopic examination of CS appeared as oblong, oval, approximately 12-15mm in length, 5-8 mm in width, ventrally shows longitudinally running centrally located ridge of raphe. A small circular point located at the narrow end is the hilum. It is brown and some seeds are black in colour. Hence the drug was confirmed as seed of *Nervalam (Croton tiglium)*.

**Microscopic Evaluation of CS**

Results of pharmacognostical microscopic evaluation reveals that the seed is spherical capsule, three valued or three deciduous, two valved coeei, seeds three, smooth, carunculate; Testa, crustaceous; albumin copious. Cotyledon flat, ovary covered with stellate tomanatum. Further seed shows wide and thick, broadly elliptical body. It also consists of outer seed coat and inner pericarp. These two cotyledons which are flat, long and parallel to each other
(Figure 1). The cotyledons have thin layers of epidermis and circular thin walled ground parenchyma cells.

Figure 1: Vertical section of SC

The outer seed coat includes thick layer palisade like vertically elongated compact macrosclereids; this zone in the epicarp of the seed coat (Figure 2).

Figure 2: Sectional view of the seed coat of SC

Inner to the epicarp, is a thin portion of three or four layers of vertically elongated, spindle shaped thick walled cells (Figure 3).
Figure 3: Slide view of Endocarp and Mesocarp of SC

The endocarp portion is wide and thick. It consists of epidermal layer of squamous cells and circular, thin walled compact parenchyma cells (Figure 4).

Figure 4: Sectional view of cotyledon and endocarp

The cells possess dense starch grains (Figure 5). These are also sparsely distributed calcium oxalate dense type of crystals.
Physico-chemical Evaluation of CS

The results obtained from physicochemical analysis of un purified CS reveals that total ash value of CS is about 2.74% in which acid insoluble ash is 1.44%. Extractive values are representative of the presence of polar and non-polar compounds in a finished product. The water soluble extractive value can be used to indicate poor quality, adulteration with any unwanted material or incorrect processing of the crude drug during the process of drying; storage etc. Extraction value of drugs renders most significant information about partition of the active component the results of extract value study shows that alcohol soluble extract of CM is 11.50% and water soluble extract is 8.49%. The loss on drying value of CM is about 11.35%. Physicochemical analysis of Purified CS reveals that total ash value of CS is about 2.28% in which acid insoluble ash is 1.08%. The results of extract value study shows that alcohol soluble extract of CM is 9.87% and water soluble extract is 2.36%. The loss on drying value of purified CM is about 10.80%. The results of physicochemical analysis were tabulated in Table 01.

Table 1: Physico-chemical Evaluation of CS

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Physico-chemical Parameter</th>
<th>Before Purification % in W/W (mg/g)</th>
<th>After Purification % in W/W (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Loss on drying at 105°C</td>
<td>11.35 %</td>
<td>10.80 %</td>
</tr>
<tr>
<td>2.</td>
<td>Ash Values</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Ash</td>
<td>2.74 %</td>
<td>2.28 %</td>
</tr>
<tr>
<td></td>
<td>Acid Insoluble Ash</td>
<td>1.44 %</td>
<td>1.08 %</td>
</tr>
<tr>
<td>3.</td>
<td>Extract Values</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water soluble</td>
<td>8.49 %</td>
<td>2.36 %</td>
</tr>
<tr>
<td></td>
<td>Alcohol soluble</td>
<td>2.36 %</td>
<td>9.87 %</td>
</tr>
<tr>
<td>4.</td>
<td>pH</td>
<td>5.2</td>
<td>6</td>
</tr>
</tbody>
</table>
FT-IR Analysis of CS

FT-IR analysis of un-purified croton seeds shows the presence of most significant functional groups such as alcohol, alkane, nitro, acid and aldehyde and while compared to un-purified sample, the FT-IR spectra of purified sample shows the addition to two more functional groups, that is alkene and amine in addition of these functional groups are might be importance of the purification process. The FT-IR spectrum with corresponding stretching and bending were shown in the figure 6 and 7.

Figure 6: FT-IR spectrum of CS before purification

Figure 7: FT-IR spectrum of CS after purification
DISCUSSION

Majority of pharmaceutical companies are presently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value. Traditional psychopharmacology has innumerable models to study the pharmacodynamic activities. But the extrapolation of the findings of these models to clinical indication is not easy and can be misleading too. Many medicinal plants from India have been shown to have activity by the conventional methods of psycho neuropharmacology.

According to WHO, the macroscopical and microscopical description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such plant. Macroscopic identity of medicinal plant material is based on sensory evaluation parameters like, shape, size, color, texture and odour. Here the macroscopic examination it appeared as oblong, oval, approximately 12-15mm in length, 5-8 mm in width, ventrally shows longitudinally running centrally located ridge of raphe. A small circular point located at the narrow end is the hilum. It is brown and some seeds are black in colour. Hence the drug was confirmed as seed of Nervalam (Croton tiglium).

Transverse section of the sample CS showed, seed is elliptical in shape, thin layer of nucleus, centrally located cotyledons embedded in a narrow cavity and encircled by wide oily endosperm. TS of seed coat shows on outer row of palisade like cells of testa with deposits of carbonated and covered with thin cuticle. The cells are thick walled.

On morphological examination it was revealed that there were considerable changes in Organoleptic character. The colour of un-purified Croton seeds was brownish black in colour, when compared to this Purified Sample which was brown in colour. At the same time odour of Unpurified Sample was strong irrient in nature, but in purified Sample had castor oil in smell.

Calcium (Ca) oxalate crystals are prevalent in fungi and many higher plants, including legumes. The shapes, locations and hydration forms of these crystals are specific for a species and developmentally determined by the type of cell, tissue and organ in which they occur. Presence of CaO crystals determines the nature and purity of the herbs. Microscopical evaluation of the present investigation reveals the presence of CaO crystals which determine its purity.
WHO and AYUSH insisted many guidelines to be followed for quality control for a better standardization of the drugs.\cite{31} Siddha system of medicines comprises many numbers of safe and valuable herbal medicines have better therapeutic efficiency either at its raw state or purified form and are clinically used by the Siddha practitioners.

The total ash value of un-purified croton was found to be 2.74 and finally it reaches 2.28% in purified samples which is very minimal level. It indicates the purity of drug. Acid insoluble is designed to measure the amount of ash insoluble to diluted hydrochloric acid. Acid insoluble ash of un-purified CS was found to be 1.44% and it reduced to 1.08% after purification.

Strongly acidic nature of the drug may cause the harmful effects to the body and poorly absorbed from gut, so the screening for pH is important for drug. It represents the chemical nature of the drug and the site of absorption.\cite{32} The pH of un-purified CS was 5.2, but after purifying with buffalo dung, raw rice, milk and castor oil it was observed that the pH of 6. It is weekly acidic and it may aids in quicker absorption.

Loss on drying test is designed to measure the amount of water and volatile matter in a sample when sample is dried under specified conditions. Moisture is one of the major factor that responsible for the deterioration of the drugs. Low moisture content is always desirable for higher stability of drug. The percentage of loss on drying of CS before and after purification (11.35% to 10.80%) was found within acceptable limit (1- 20%).

Water soluble extractive value of un-purified CS is 8.49% and in purified sample it was 2.36% it shows the possibility of water soluble constituents such as tannins, sugar and alcohol soluble substance such as alkaloids may be present in the drug. Alcohol soluble extractive value of un-purified CS is 11.50% and in purified sample it was 9.87%.

FTI-R analysis of un-purified croton seeds shows the presence of bio-active functional groups such as alcohol, alkane, nitro, acid and aldehydes. Whereas the report of purified CS shows the presence of two functional groups, that is alkene and amine along with the existing ones. Addition of these functional groups justifies the importance of the purification process.

The quality and therapeutic efficacy of herbal drugs is dependent on the active constituents which are present in the plant cell. Survey denotes that newly approved drugs reported between 1983 and 1994, drugs of natural origin predominated (78%) in the antibacterial area, while 61% of the 31 anticancer drugs approved in the same period were either natural
products, nature derived products or compounds modeled on natural product parents or “leads”. In addition, 50% of the bestselling pharmaceuticals in 1991 were either natural products or their derivatives.[33,34]

CONCLUSION
Quality control for efficacy and safety of herbal products is of paramount importance. Quality can be defined as the status of a drug that is determined by identity, purity, content and other chemical, physical, or biological properties, or by the manufacturing processes. From the data’s of the present investigation it was concluded that the siddha drug Nervaalam (Croton seeds) was purified and analyzed according to the standard procedures. Results of the study generated an evidence based data with respect to macroscopical, microscopical, physicochemical and functional group parameters of the study drug CS. There were notable changes was found between un-purified and purified form of CS. Further studies have to be carried with special emphasis on phytochemical and characterization aspect of the drug and its future.

ACKNOWLEDGEMENTS
I wish to acknowledge my thanks to Dr. Annop Austin and IIT Madras for their analytical support in this research work and The Noble research solutions, Chennai for their technical support for preparing this manuscript.

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