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Research Article

Pharmacognostic Standardization and Quantitative Estimation of some Isolated Phytoconstituents from *Croton oblongifolius* Roxb.

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Abstract

Croton oblongifolius Roxb. (Euphorbiaceae) is a weed available all over in the agricultural fields of West Bengal villages. Traditionally this plant is used as wound healing drug in the Bengal villages. As there is less number of scientific information present, an attempt has been taken to search some important phytoconstituents and their amount which may act as marker compounds. Simultaneously, a preliminary standardization of this plant was performed by pharmacognostic, morphological and microscopical investigations. Here flavonoids, terpenoids and tannins were isolated and estimated to identify the marker compounds and different standardization parameters and their results were recorded for future requirements.

Keywords: Pharmacognostic standardization, phytoconstituents, *Croton oblongifolius*

Introduction

In India, the use of different parts of several medicinal plants to cure specific ailments has been in vague from ancient times. The indigenous system of medicine namely Ayurvedic, Siddha and Unani have been in existence for several centuries. These systems of medicine cater to the needs of nearly seventy percent of our population residing in the villages. In Homeopathy system, 70% of the medicines are prepared from plants. As Homeopathy originated in Europe, naturally, majority of the drugs prepared from plants are of exotic origin. Our is a vast country where wide variations in climate, soil, altitude and latitude are available. Nature has bestowed on us a agro climatic conditions permitting the growth of almost 20,000 plants species, of which about 2,500 are of medicinal very rich botanical wealth and a large number of diverse types of plants grow wild in different parts of the country. India is a country rich in indigenous herbal resources which grow on their varied topography and under changing value.[1] In Indian scenario, it has been recognized that 2,500 plants have been found to be have medicinal values out of 17,000 plants.[2] The world is now looking towards India for new drugs to manage various challenging diseases because of its rich biodiversity of medicinal plants and abundance of traditional know-how such as Siddha, Ayurveda etc., to cure different diseases.[3-5] From over 3, 00,000 species of higher plants to occur in nature, only about 2% have been screened so far. Extract of plants from 157 families have been reported to be active against microorganisms.[6] *Croton oblongifolius* Roxb. is extensively used in herbal medicine in South-East Asia. Its various uses in traditional medicine are reviewed in Table 1.[21]

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Materials and Methods

Morphological study of *Croton oblongifolius*[21]

Croton is a middle sized tree, deciduous; bark brownish, branches lepidote while young. Leaves are alternate, crowded towards the ends of the branchlets, simple; stipules deciduous; petioles cylindrical, lepidote; laminae elliptic-oblong or oblong-lanceolate, the bases acute or obtuse, the margins serrate, the tips acute to acuminate, unicostate, reticulate, the surfaces glabrescent, the upper dark green. Its inflorescences in terminal paniculate cymes, the cymules fascicled at the axils of minute bracts, monoecious, the staminate flowers at the upper portion, more numerous than the pistillates, the pistillates at the lower; peduncles erect, lepidote; bracts lanceolate to ovat. Flowers of Croton are ebracteolate, pedicellate, unisexual, actinomorphic, pentamerous, hypogynous. Staminate flowers: Calyx synsepalous, 5-partite, lepidote, persistent. Corolla apopetalous, the petals 5, ovate, villous within, glabrous without, pale yellowish green. Androecium polyandrous, stamens 12, inserted on a villous receptacle, the filaments long, slender, inflexed in

in bud, more hairy at the bases, the anthers dithecous, oblongoid, adnate, introrse, dehiscence longitudinal. Pistillode absent. Pistillate flowers: Calyx synsepalous, 5-partite, the lobes broadly ellitptic, lepidote, persistent. Corolla obsolete. Androecium nil. Pistil 1, ovary oblongoid, glabrous, 3-carpelled, syncarpous, 3-loculed, the placentation axile, the ovule one in each locule, the styles 3, the stigmas 2-fid for more than half the length. Fruit a regma of three, 2-valved 1-seeded cocci, globose, 3-lobed, depressed at the top, with persistent calyx, pale greenm lepidote; seeds 3, oblongoid, pale green, smooth, caruncle small, endosperm copious, fleshy. Flowering period of Croton are from December to February and the fruiting period are from June to April.

Generally Croton is a small deciduous tree, branches rather stout, leaves oblong, elliptic-oblong or ovate or lanceolate acute more or less repand, toothed or serrate penninerved, very pale green when dry, nerves 12-26 pairs, racemes often fascicled, erect, pedicels long or short.

Table 1: Review of the various medicinal uses of the study plant

Species	Family	Traditional use
C. oblongifolius Roxb.	Euphorbiaceae	Root bark, leaves and seeds. Root bark- Pneumonitis; Hepatitis; Hepatomegaly; Arthritis. Bark-Oedema; Hepatitis; Hepatomegaly; Best antidote for snake bite; Pyexia. Seeds- Diarrhoea; Oedema; Very useful for inflammations either taken orally or as an external application

Determination of ash values[16]

Total ash

Total ash is designed to measure the total amount of material produced after complete incineration of the ground drug (2 g) at above 500^{0}C temperature to remove all the carbons.

Acid insoluble ash

The total ash was boiled with 25 ml of 2M hydrochloric acid for 5 min and then the insoluble matter was collected on an ashless filter paper in a Gooch crucible and washed with hot water, ignited and cooled in a dessicator and weighed. The percentage of acid-insoluble ash was calculated with reference to the air dried drug.

Water soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 min. The insoluble matter was collected on an ashless filter paper in a crucible and washed with hot water and ignited in a crucible for 15 min at a temperature not exceeding 450^{0}C. The weight of this residue in mg was subtracted from the weight of total ash. The content of water soluble ash in mg/g of the air dried material was then calculated.

Sulphated ash

Total ash was treated with dilute H_2SO_4 and further re-dried to constant weight which gives the sulphated ash. The percentage of sulphated ash was calculated with reference to the air dried drug.

Determination of solvent Extractive values[16]

5 g of the air-dried, coarsely powdered drug was macerated with 100 ml of different solvents in the closed flasks for 24 hours, shacked frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, filtered rapidly taking precautions against loss of solvent and 25 ml of the filtrate were evaporated to dryness in a tared flat-bottomed shallow dish, dried at 1050C and weighed. The percentage of solvent-soluble extractive with reference to the air dried drug was calculated.

Microscopical study

Transverse section of leaf, stem and root were performed and analyzed the tissue arrangements. The above sections were stained by phloroglucinol solution.

Phytochemical study

Preparation of extract

Whole plant of Croton Oblongifolius were collected from Police line area, BURDWAN, West Bengal during the month of July-August, 2008 at 5.30pm and authenticated by Botanical Survey of India, Shibpur Botanical Gurden, Howrah. A specimen herbarium of Croton oblongifolius was submitted to Botanical Survey of India (Herberium No. CNH/1-1/(201)/2009/Tech. III/12).

The material was shade dried, powdered and was extracted with hydro-alcohol (80:20) by maceration process. The solvent in the crude extract was then evaporated by desiccators.

Qualitative phytochemical analysis

The presence of alkaloids, flavonoids, glycosides, reducing sugars, saponins, tannins and terpenoids were tested qualitatively using the standard procedures to identify the constituents.

Test for Alkaloids

To 2ml of test solution, added 2 N HCl, aqueous layer formed was decanted and to that added few drops of Mayer's reagent. Cream ppt. was observed indicating the presence of alkaloid.[17]

Test for carbohydrate

Molisch's test

To 2 ml of test solution, added few added few drops of alcoholic α- napthol, and then added few drops of concentrated sulphuric acid through sides of test tube. Purple colour ring was appeared at the junction indicating the presence of carbohydrate.[18]

Barfoed's test

1 ml of test solution is heated with 1 ml of Barfoed's reagent on water bath. Red cupric oxide was formed indicating the presence of monosaccharide. If disaccharides were present, the solution was required to prolong heat (about 10 min).[18]

Test for glycoside

To 2 ml test solution, added equal quantity of Fehling's solution A and B. After heating brick red precipitate was obtained indicating the presence of glycoside.[18]

Test for flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H2S04. A yellow colouration was observed indicating the presence of flavonoids. The yellow colouration disappeared on standing. Few drops of 1% aluminium solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids.

A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids.[15,17]

Test for Phenols

To 2ml of test solution, added alcohol and then few drops of neutral ferric chloride solution was added. The test result was observed.[17]

Test for Saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion[17]

Test for Steroids

To 2ml test solution, added minimum quantity of chloroform. Then 3-4 drops of acetic anhydride and 3 drops of concentrated sulphuric acid were added. The colour changed from violet to blue or green in some samples indicating the presence of steroids.[15]

Test for terpenoids (Salkowski test)

Five ml of extract was mixed in 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids.[18]

Test for Tannins

The presence of tannins was confirmed by the following test. About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.[18]

Thin layer chromatography (TLC) profile of the *Croton oblongifolius*

Qualitative analysis for flavonoid

Thin-layer chromatography (TLC) was performed on precoated 20 x 20 cm TLC plates coated with 0.25 mm layers of silica gel 60 F254 (Merck). After application of the extract and standard solutions (1L), the plates was developed for 19 cm in paper-lined all glass chambers (Desaga, Germany) previously left to equilibrate for at least 30 min. Two chromatography solvents were used: ethyl acetate/formic acid/acetic acid/water, 100:11:11:26 (V/V) and ethyl acetate/formic acid/water, 8:1:1 (V/V).7,8 Visualization of the flavonoids was achieved by spraying the sheets with natural products polyethylene glycol reagent (NP/PEG)(Fluka Chemie, Switzerland). Typical intense fluorescence in UV light at 365 nm was produced immediately on spraying (flavonoids appeared as orange-yellow bands whereas phenolic acids formed blue fluorescent zones). Addition of polyethylene glycol solution lowered the detection limit and intensified fluorescence.[7]

Quantitative analysis for flavonoid

The content of flavonoids calculated as quercetin in the plant samples was determined by the method of Christ and Müller.[9]

After acid hydrolysis (with 25% hydrochloric acid in acetone for 30 minutes at 100°C), the liberated aglycones were spectrometrically determined at 425 nm by forming a complex with AlCl3 in a methanol/ethyl acetate/acetic acid medium.[10,11]

Qualitative analysis for diterpenoid and triterpenoid

The dried plant powder was extracted with two solvents dichloromethane (DCM), dichloroethane (DCE).[19]

The 10 × 5 cm TLC plates were (stationary phase) prepared with homogenous suspension of silica gel G and silica gel GF254. The TLC plates were activated by heating at 110°C for at least an hour and protected from moisture. The solvent extracts of Croton oblogifolius were run with various solvent systems like like ethylacetate, ethanol, water (75:15:10); toluene, ethylacetate (95:05) and hexane, ethylacetate (4:1). The different spots developed in various mobile phase and the spots were identified by vanillin sulphuric acid reagent.[12,14]

Quantitative analysis for diterpenoid

The dried plant material was extracted with CH_3OH, CH_3OH-CH_2Cl_2 (1:1) for one week at 60-70^{0}C. The combined methanolic extract is concentrated in vacuo. The concentrated methanolic extract was successively washed with hexane, CH_2Cl_2 and EtOAc. Each portion is concentrated and then subjected to Si-gel column chromatography to get diterpenes.[20]

UV spectra studies

UV spectral studies of diterpenes are helpful in detecting the presence of a particular type of chromophore.

Molecule	Absorption band (λ_{max}) nm
Clerodanes	215
α, β unsaturated carbonyls	250
γ, δ unsaturated ketones	210-239, 214 244, 300

Tannin determination by Van-Burden and Robinson (1981) method

500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.I N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.[13]

Results and Discussion

Croton oblongifolius is traditionally used as wound healing and antidote of snake venom in most of the villages of West Bengal. A Burmese-Myanmar transcript Agriculture Department 2000 has reported the scientific information of the same.

We have performed the different parameters of pharmacognostic investigation (Table 2) to standardize this herbal drug to some extent. In phytochemical analysis as alkaloid, flavonoid, carbohydrate, glycoside, terpenoid, tannin, and phenolare present in the methanoilc extract of whole plant (Table 3), we have isolated and estimated the amount of flavonoid, terpenoid (Table 4) and tannin present in the whole plant

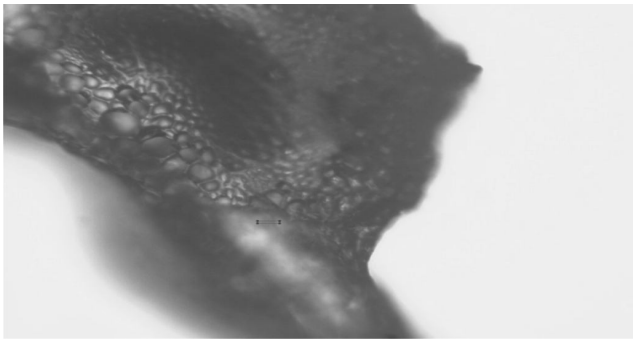

Fig. 1: Transverse Section of Leaf of *C. oblongifolius*

In the transverse section of leaf and stem of *Croton oblongifolius*, the vascular bundle was lignified and given purple colour after treating with phloroglucinol (Fig. 1 and Fig. 2) and on the other hand all the cells of root section was lignified

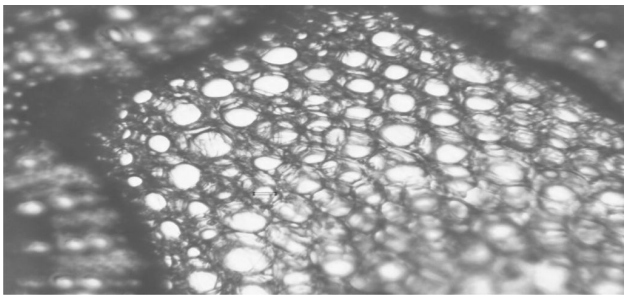

Fig. 2: Transverse Section of stem of *C. oblongifolius*

and given purple colour after treating with phloroglucinol (Fig. 3). The tissue arrangements of all three sections (Leaf, stem and root) were standard like a dicot plant. TLC separation of flavonoids and phenolic acids from methanolic extracts of *Croton oblongifolius* indicated the presence of Quercetin (R_f =0.97), Anthocyanin R_f= (0.95) and chlorogenic acid (R_f =0.50). The amount of Quercetin was found to be 1.15 ±

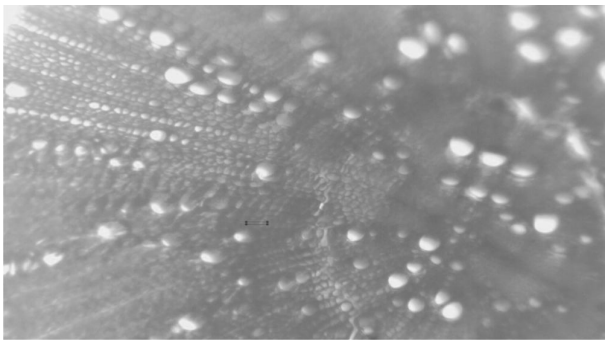

Fig. 3: Transverse section of root of *C. oblongifolius*

0.05%. So here the amount of diterpenoid was found to be 0.04 ± 0.02 % and the amount of tannin was found to be 0.73 ± 0.30%.

Conclusion

After performing this work, it has been concluded that a good amount of tannin and flavonoid and a moderate amount of diterpenoid are present in this plant. Thus, *Croton oblongifolius* may be an important herbal drug with some important marker useful to treat some challenging diseases to mankind in future life.

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Table 2: Pharmacognostic study

	Ash value	Extractive Value
Total ash	6.67%	Water soluble 29.6% w/w extractive values
Acid insoluble ash	0.72%	Alcohol soluble 22.433 % w/w extractive values
Water soluble ash	1.21%	Ethyl acetate 14.2% w/w extractive values
Sulfated ash	0.63%	Petroleum benzene 9.66% w/w soluble extractive values

Table 3: Qualitative analysis of the phytochemicals of the medicinal plant

Alkaloid	Carbohydrate	Flavonoid	Phenol	Saponin	Steroid	Terpenoid
Present	Present	Present	Present	Absent	Absent	Present

Table 4: TLC separation of diterpenoid and triterpenoid

Extract	Solvent system	Visualizing agent	Detection colour	R_f value	Component
DCE extract dissolved in DCE	$CH_3COOC_2H_5$: C_2H_5OH: H_2O (75:15:10)	Vanillin, sulphuric acid reagent	Violet	0.79	Terpene
DCM extract dissolved in Toluene	$CH_3COOC_2H_5$: $C_6H_5CH_3$ (95:5)	Vanillin, sulphuric acid reagent	Violet	0.85	Terpene
DCM extract dissolved in Toluene	$CH_3COOC_2H_5$: $C_6H_5CH_3$ (95:5)	Iodine chamber	Dark yellow	0.85	Terpene

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