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Phytochemical profiling and histochemical localization in leaf and stem of *Trichosanthes cucumerina* (L) var. *cucumerina* with reference to the influence of plant age and geographical variations

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ABSTRACT

The study report comprises the results of an investigation on phytochemical evaluation, and histochemical localization in leaf and stem of medicinal species - *T. cucumerina* (L) var. *cucumerina*. This medicinal herb has wide application in folkloric medicine and Ayurveda owing to its antiinflammatory, antioxidant, hepatoprotective and antihelmintic potential. It is procured on a range of 35 -40 tonnes per year by various Ayurvedic pharmaceutical firms in Kerala for preparing herbal formulations. Parameters studied are the assessment of the efficacy of different extractant solvents in release of plant secondary metabolites, preliminary qualitative and quantitative analysis and evaluation of the occurrence and pattern of distibution of phytochemicals in tissues of leaf and stem through histochemical localization. The stem samples from two localities were compared for the influence of climatic and edaphic conditions on quantity of phytochemicals. Besides, these a comparison on stem of different maturity level (1 year and 2 years old) was also evaluated to assess the impact, if any, of the metabolic state of plant on quantity of secondary metabolites. Extraction of dried and powdered plant parts was carried out successively in five different solvents– petroleum ether, chloroform, ethyl acetate, methanol and water. The results of the study suggested that among the solvents, methanol and water were effective extractants in which most of the common secondary metabolites –alkaloids, flavonoids, phenolics, tannins, terpenoids and saponins – were released. Quantitative analysis of the methanolic and aqueous extracts concluded that both stem and leaf contain appreciable quantity (mg/g tissue) of alkaloids, phenolics and tannins in comparison to flavonoids and terpenoids. Studies on histochemical localization carried out using Wagner's reagent concluded that alkaloids are mostly located in the parenchyma cells bordering the vascular bundles of stem and petiole. Flavonoids, localized with NaOH revealed that they were marked as a distinct yellow band in the sub hypodermal layer of stem. Tannin localization with $FeCl_3$ also suggested their storage in parenchyma cells.

KEY WORDS: *Trichosanthes*, phytochemicals, histochemical localization

1. INTRODUCTION

The genus *Trichosanthes*, belonging to Cucurbitaceae is native to Southern and Eastern Asia, Australia and islands of the Western Pacific. Over 20 species of Trichosanthes are recorded in India of which two namely *T. anguina* and *T. dioica* are cultivated as vegetable. Other important species found in the world are *T. palmata, T. cordata, T. nervifolia, T. cucumerina, T. wallichiana, T. cuspida, T. incisa, T. laciniosa, T. kirilowii etc. Trichosanthes cucumerina var. cucumerna* (L), the wild bitter snake gourd, selected for the present study is a monoecious annual herb climbing by tendrils up to 5 to 6 meters high. The entire plant parts are extremely bitter and are not consumed as a vegetable. It is exploited on a largescale for preparing Ayurvedic formulation- 'padavaladi kashayam' and is also one of the ingredients in several other herbal medicines prepared by 'Kottakkal' and 'Oushadhi' in Kerala. *Trichosanthes cucumerina* is

Figure 1: Habit of *Trichosanthes cucumerina* (L) var. *cucumerina*

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used in the treatment of headache, alopecia, fever, abdominal tumors, bilious, boils, diarrohea and skin allergy. It is used as an abortifacient, vermifuge, stomachic, refrigerant purgative, malaria, laxative, bronchitis and antihelmintic. The leaves and stems are used for bilious disorders and skin diseases and as an emmenagogue. Leaf is alexiteric, astringent, diuretic and emetic[1].

The present study is an attempt to analyse the phytochemical constituents of stem and leaf of *T. cucumerina* (L) var. *cucumerina* by successive extraction of plant parts in solvents with increasing polarity and to assess the pattern of occurrence and cellular distribution of the secondary metabolites, a comparative evaluation of plants from two localities in Kerala– Marayoor (Idukki District) and Trivandrum with different climatic and edaphic conditions to elucidate the influence of these variations on level of phytoconstituents.

2. MATERIALS AND METHODS

2.1. Collection of Plant material and Extract Preparation

The plant material *Trichosanthes cucumerina* (L) var. *cucumerina* (Figure 1) was collected from two localities of kerala - Marayoor–Idukki District, where this plant is cultivated on a large scale for supply to Kottakkal and Oushadhi for preparation of Ayurvedic formulations and from Plants grown in College Botanic Garden, Trivandrum. Leaf, one year old stem (designated as stem1, from Marayoor), two year old stem (designated as stem 2, from Marayoor) and one year aged stem of plants reared in Trivandrum (designated as stem tvm) were procured for a comparative phytochemical analysis to elucidate the influence of climatic and edaphic factors as well as plant age/maturity level on quality and quantity of phytoconstituents. Identification of taxon was carried out with authentic literature. All samples were washed thoroughly in tap water, shade dried and ground into fine powder. 25gm of dried powder of each part were re extracted successively with 100 ml each of petroleumether, chloroform, ethyl acetate, methanol and water. These samples were placed in a water bath at a temperature of 45^{0}C for 10 hours and then placed in a gyratory shaker at 120 rpm for 48 hours. Each type of extract was filtered using whatman No.1 filter paper, dried to attain constant weight.

2.2. Preliminary Qualitative Phytochemical analysis

A stock solution (mg/ml) of extract from each type of solvent was prepared using the respective solvent. These extracts along with blanks were analyzed qualitatively for the presence of various phytochemicals. Phytochemical examinations were carried out as per the standard methods.

2.2. 1.Test for Alkaloids

a) Dragendroff's test: 1ml of Dragendroff's reagent was added to 2ml of the filtrate. Formation of a reddish brown precipitate indicated the test as positive.

b) Mayer's Test: One or two drops of the Mayer's reagent was added to 1ml of test solution or filtrate. A white or a creamy precipitate confirmed the test as positive.

c) Wagner's test: Two drops of Wagner's reagent was added to 1ml of the test solution. The formation of yellow or brown precipitate confirmed the test as positive for alkaloids.

2.2. 2.Test for flavonoids

A small quantity of extract was heated with 10ml of ethyl acetate in boiling water bath for 3 minutes. The mixture was filtered and filtrates are used for the following test.

Shinoda test: A few magnesium turnings and 5 drops of concentrated hydrochloric acid was added drop wise to 1ml of test solution. A pink colour appeared after few minutes confirmed the test.

2.2. 3.Test for Phytosterols/ terpenoids

Liebermann-Burchard's test: 2 mg of the extract was dissolved in 2ml of acetic acid anhydride, heated to boiling, cooled and then 1ml of concentrated sulphuric acid was added along the side test tube. A brown ring formation at the junction confirmed the test for the presence of phytosteols.

2.2. 4.Test for Tannin

Ferric chloride test: A few drops of 5% (w/v) ferric chloride solution was added to 2ml of the test solution. Formation of bluish black colour indicated the presence of hydrolysable tannin.

2.2. 5. Test for Cardiac glycosides

Keller-killiani test: Added 0.4 ml of glacial acetic acid and add afew drops of 5% ferric chloride solution to a little of dry extract. Further 0.5ml of concentrated sulphuric acid was added along with the side of the test tube carefully. The presence of blue colour in acetic acid layer confirmed the test.

2.2. 6.Test for Phenol: Extracts were treated with 3-4 drops of 10% (w/v) ferric chloride solution. Formation of greenish black colour indicated the presence of phenol.

2.2.7. Test for Triterpenoids- Salkowski test: Approximately 2mg of dry extracts was shaken with 1ml of chloroform and a few drops of concentrated sulfuric acid were added along the side of test tube. A red brown color formed at the interface indicated the test as positive for triterpenoids.

2.2. 8. Test for saponin -Foam test: 5ml of the test solution taken in atest tube and shaken well for five minutes. Formation of stable foam confirmed the test.

2.3. Quantitative analysis of Phytochemicals

2.3. 1. Determination of alkaloids[2]

A total of 200 ml of 20% acetic acid was added to 5 g of leaf and stem powders taken in a separate 250 ml beaker and covered to stand for

40 h. This mixture containing solution was filtered and the volume was reduced to one quarter using water bath. To this sample, concentrated ammonium hydroxide was added drop wise until the precipitate was complete. The whole solution was allowed to settle and the precipitated was collected by filtrationand weighed[3]. The percentage of total alkaloid content was calculated as:

$$\text{Percentage of total alkaloids (\%)} = \frac{\text{weight of residue}}{\text{weight of sample taken.}} \times 100$$

2.3.2. Determination of total phenolics

To 0.1ml of the extract add 3.9 ml of distilled water and 0.5 ml of Folin's reagent. Incubate the solution at room temperature for 3 minutes and then add 2 ml of 20% (w/v) sodium carbonate solution to the mixture. The solution was kept in boiling water bath for 1 minute, cooled and the absorbance recorded at 650 nm. Gallic acid was employed as the standard to express the total phenol content [4].

2.3. 3. Determination of total flavonoid

The content of total flavonoids was measured spectrophotometrically by using the aluminium chloride colorimetric assay [5]. 1ml of plant extracts were diluted with 200 µl of distilled water followed by the addition of 150 µl of sodium nitrite (5%) solution. This mixture was incubated for 5 minute and then 150 µl of aluminium chloride (10%) solution was added and allowed to stand for 6 minutes. Then 2 ml of sodium hydroxide (4%) solution was added and made up to 5 ml with distilled water. The mixture was shaken well and kept for 15 minutes at room temperature. The absorbance was measured at 510 nm. Appearance of pink colour showed the presence of flavonoid content.

2.3. 4. Determination of total tannin

a) Reagents

Colouring agent: dissolve 1.6221gm of ferric chloride (o.1M), 0.9 ml of hydrochloric acid (0.1N) and 263.4 mg of potassium ferrocyanide (0.008M) in 100ml of water. Working standard solution contained tannic acid (10%, v/v) in distilled water.

b) Procedure: 1gm of the sample was boiled with 100 ml of water for 30 minutes, cooled and filtered using whatman no.1 filter paper and it made up to 100ml with distilled water. Add 0.5ml of coloring agent to 2 ml sample. Read the blue colour developed at 760nm against reagent blank after 30 minutes at room temperature. Add colouring reagent to the standard (tannic acid at concentrations 20-100µg) and calculate the amount of tannic acid equivalent. Express the value as mg of tannic acid equivalent (TE)/gm of dried sample [6].

2.3.5. Determination of total terpenoid

1gm of each powder dissolved in 10ml methanol and 15 ml water. Mixture was shaken well and centrifuged at 10000 rpm for 10minutes. To 1ml of extracts, added 2 ml of chloroform followed by 3 ml of concentrated sulphuric acid. Formation reddish brown colour indicated the presence of terpenoids. Terpenoids estimated by reading the absorbance at 538 nm against blank of 95% methanol.

2.4. Histochemical Localization

For the histochemical studies, fresh samples of stem, petiole and leaf were cut into small pieces and fixed in FAA (Formalin Acetic acid Alcohol) for 24 hours. After fixation they were washed thoroughly and thin free hand sections were taken and treated with respective reagents used in qualitative analysis to localize specific components, viz alkaloids, flavonoids, phenol and tannin in the tissue.

3. RESULTS AND DISCUSSION

The phytochemical screening indicated that both methanolic and aqueous extracts contain most of the secondary metabolites. The major constituents detected were alkaloids, cardiac glycosides, flavonoids, phenol, saponin, tannin, terpenoids, triterpenes. The results of qualitative analysis of extracts of leaf and stem samples are illustrated in Table 1. Absence of phytochemicals in organic solvents might have resulted from the lack of solubility of active phytoconstituents in non-polar solvents or due to the inefficiency of the non-polar solvents to penetrate the cell wall.

Table 1: Results of qualitative analysis of extracts

Name of Test	Petroleum ether				Chloroform				Ethyl acetate				Methanol				Water			
	L	S1	S2	S tvm	L	S1	S2	S tvm	L	S1	S2	S tvm	L	S1	S2	S tvm	L	S1	S2	S tvm
Alkaloids																				
Dragendroffs	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	++	++		++
Mayer's test	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	++	-		-
Wagner's test	-	-	-	-	-	-	-	-	-	-	-	-	++	+++	++	++	++	++	++	+
Flavonoids																				
Ammonia test	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	++	+	+	+	++
Alkaline test	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++	++	+	+	+	++
Terpenoids	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+	++	++	+	+++	++
Tannin													+++	+++	+	+	++	+++	+	+
$FeCl_3$ test	-	-	-	-	-	-	-	-	-	-	-	-								
Phenol	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	+	+	++	+++	+	+
Triterpenes	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+	+	-	-	-	-
Saponins		-	-	-	-	-	-	-	-	-	-	-	+++	+++	-	+	-	-	-	+
Cardiac glycosides	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+	++	-	+	++	++

'-' = absent: '+' = very low: '++' = moderate level: '+++' = High level **L** - Leaf; **S1**- Stem 1 year; **S2**- Stem 2 year; **S tvm** - Stem from Trivandrum locality

Table 2 : Results of Quantitative analysis of extracts

Name of Part	Flavonoid (mg/g)		Phenol (mg/g)		Tannin (mg/g)		Terpenoid (mg/g)		Alkaloid (mg/g)
	ME	AE	ME	AE	ME	AE	ME	AE	AE
Leaf	5.9	2.4	24.01	8.38	2.54	18.79	1.53	0.175	35.24
Stem 1	1.52	1.38	13.04	16.3	6.51	18.88	1.08	0.003	43.96
Stem 2	0.35	0.2	4.06	7.02	4.63	3.62	1.02	0.068	10.24
Stem tvm	0.61	0.73	9.55	18.1	0.54	5.3	1.1	0.008	21.54

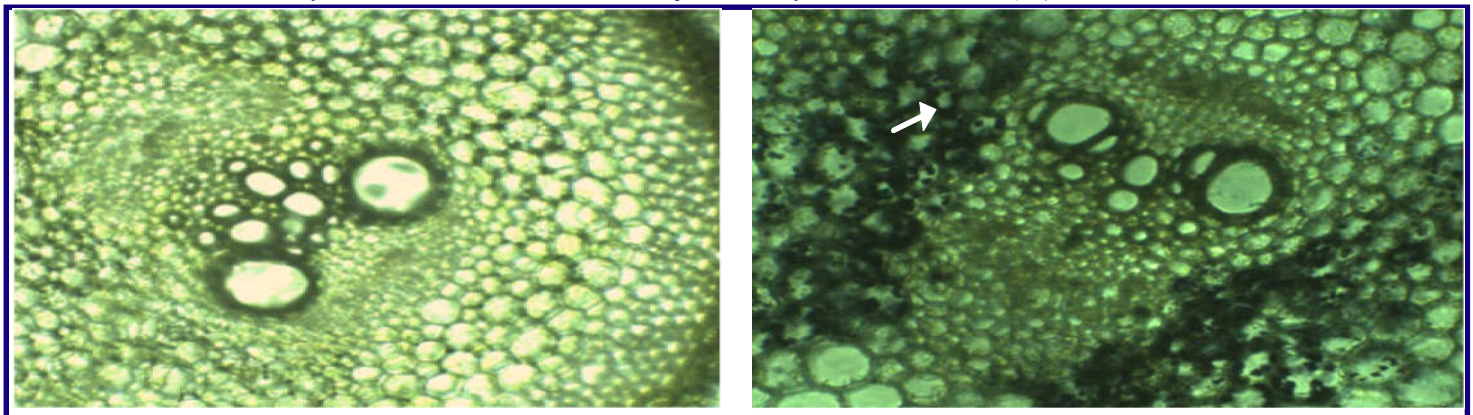
ME - Methanolic Extract; **AE** - Aqueous Extract

In the present study, quantitative analysis of total phenol, flavonoid, tannin and terpenoids indicated that alcoholic extracts possessed higher concentration of phytoconstituents than aqueous extracts (Table 2). All plant parts compared exhibited the same profile suggesting that alcohol can cause metabolites to be released more effectively from cells than water. Besides, alcohol can easily penetrate the cell wall and membranes to release the intracellular ingredients from the plant material. Similar findings were reported where higher antibacterial activity has been reported for alcoholic plant extracts [7,8].

The total alkaloid content in aqueous extracts of various samples is illustrated in Table 2. Variations were noticed in quantity of phytoconstituents in stem samples of different maturity level as well as in samples from different geographical area. The occurrence and distribution of secondary metabolites are dependent to a great extent on age and metabolic level of the plant. During vegetative phase (up to 5 months from seed germination), leaves are abundant and the plant is metabolically active. At this stage the stem possesse only lesser levels of secondary metabolites (as denoted by stem tvm). Towards the end of reproductive phase most of the leaves turn yellowish and dry up and the stem attains considerable thickening and lignification (stem 1 in present study). At this stage the stem also exhibit substantial increase in quantity of phenolics and flavonoids probably due to accumulation and storage of metabolites in stem tissues. The plants harvested at the end of second year (stem 2 in present study) are mostly defoliated with high level of lignification and contained only low levels of alkaloids, flavonoids, phenolics, tannins and terpenoids. The presence of varying concentrations of phytochemicals in stem at different levels of maturity suggests that the accumulation of secondary metabolites in stem requires an active period of vegetative growth. Furthermore, it appears that the level of secondary metabolites gets depleted beyond a certain level of ageing and lignification of plant parts. The leaves during active growth in vegetative phase could be considered as a potent source of antioxidants. This could be attributed to the active metabolism of the plant. This plant is cultivated on a commercial scale by the Eco Development Committees in Tribal areas – Kanthalloor and Marayoor of Idukki district for supply to Pharmaceutical companies. They prefer to harvest stem 2 (defoliated stage) preferably assuming that the plant may have high therapeutic value with ageing. However the present study revaled that the leaves harvested during vegetative phase and the stem harvested after fruit setting (Stem 1) are more potent sources of phytochemicals. The differential distribution of phytocostituent recorded in the present study agrees with the prevoius report on phytochemical studies on edible species- *Trichosanthes anguina* Linn.[9].

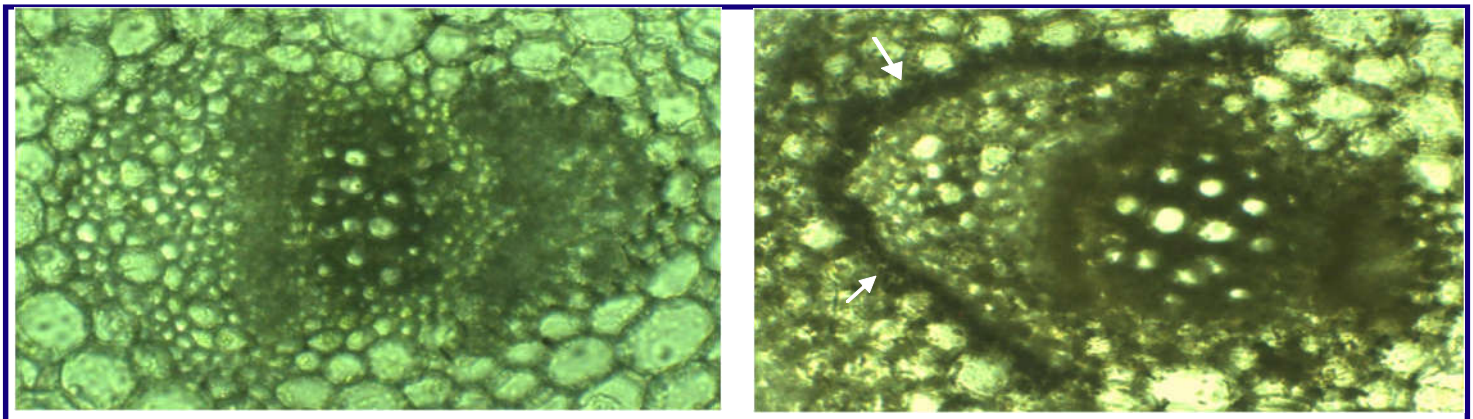
Besides these, the climatic conditions may also influence the level of secondary metabolites in plants. Marayoor and Kanthalloor areas of Idukki are characterized by virtually rain shadow areas, lying in the eastern side of the Western Ghats. Marayoor is a hill station in Kerala with an average altitude ranging from 1600 – 1800m above sea level. The average rain fall varies from 250 to 425 cm. Soil type is forest loam with high organic matter content. The climate in Marayoor is characterized by mild wet winters and hot dry summers. Fluctuation in temperature vary from 8^0C (winter) to 30^0C (summer). Trivandrum has an average *elevation* 4.9 m above sea level. The total *annual average rainfall* is about 150 cm. The temperature varies from 16^0C (winter) to 36^0C (summer) and the soil type is sandy loam. The difference in levels of phytochemicals in stem samples collected from Marayoor and stem from plants reared in Trivandrum could be explained as due to difference in climatic and edaphic conditions of the habitats. The metabolism and accumulation of active ingredients, most of which are secondary metabolites, is the reflection of cumulative impacts of multiple ecological factors on the medicinal plant during their developmental and growth periods in addition to genetic factors. Certain metabolites are only synthesized under specific environments, or their contents significantly increase under specific environments [10]. The effect of ecological conditions influencing the level of phytoconstituents is also documented by Liu *et al*[11].

Studies on histochemical localization indicated that alkaloids are copiously present in stem (Figure 2B) and petiole of leaf (Figure 3B) and were localized as dark brown contents in parenchyma cells bordering the vascular bundles in stem and leaf petiole. In the sub hypodermal region of stem and leaf petiole, flavonoids were located as a distinct yellow layer (Figure 4A). Tannins, detected after localization with $FeCl_3$ suggested its accumulation in medullary ray parenchyma cells of stem (Figure 4B). The presence of alkaloids, flavonoids and tannins confirmed through color developement due to the reaction of the cells with specific reagents provided chemical markers that could be used in identification. Alkaloid localization in tissue sections using Wagner's reagent and their confirmation through characteristic dark coloration have been reported in studies on histochemical localization of *Excocaria agallocha* Linn[12] and in *Barleria lupulina* Lindl.[13]. Localization of flavonoid using 10 % NaOH and its inference on basis of yellow coloration are reported in sections of *Centratherum punctatum* Cass.[14].



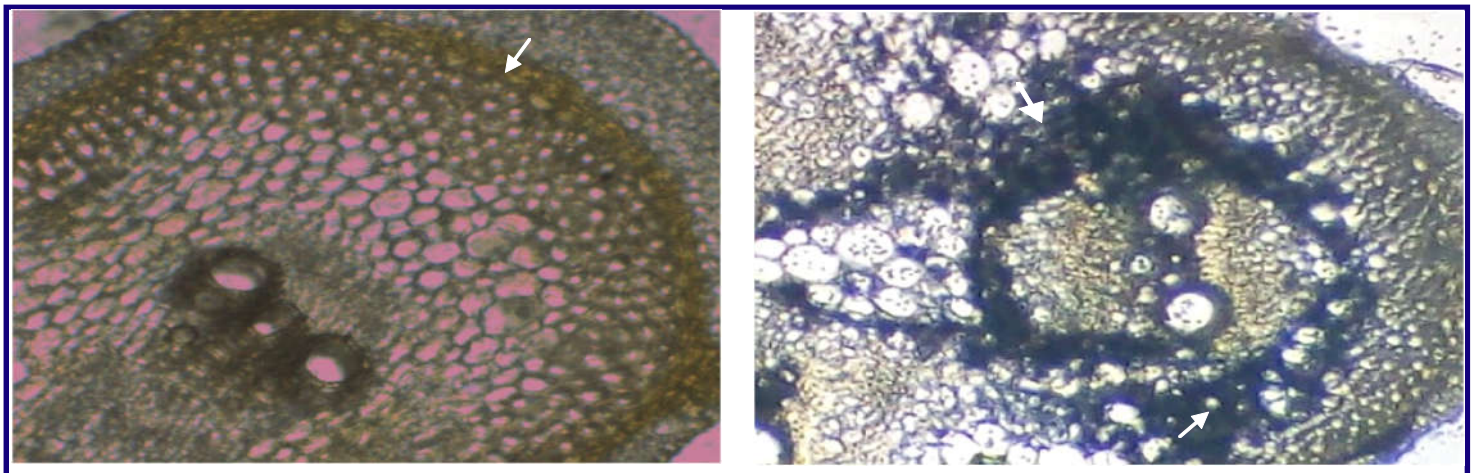
A - Untreated (control); B - Localization after treatment with Wagner's Reagent

Figure 2. Localization of Alkaloid in Stem (40x)



A - Untreated (control) B - Localization after treatment with Wagner's Reagent

Figure 3. Localization of alkaloid in Leaf petiole (40x)



A - T.S of Petiole showing Flavonoid (yellow coloration after NaOH treatment)

B - T.S of stem showing Tannin (Bluish black coloration after $FeCl_3$ treatment)

Figure 4: Localization of Flavonoid and Tannin in Leaf petiole and stem (40x)

4. CONCLUSIONS

The results of the present study suggested the presence of a diverse range of phytochemicals in leaf and stem of *Trichosanthes cucumerina* (L) var. *cucumerina* and these could be effectively extracted in mehanol and water. Leaves and one year old stem were detected as the most potent sources of phytoconstituents. Cultivation

of this valuable medicinal plant in geographical regions of appropriate climatic and edaphic conditions and their harvesting at ideal maturity phase are equally important to exploit its pharmaceutical potential to the optimum level.

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