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Research Article

STANDARDIZATION OF *VELLARUGU CHOORANAM*: A SIDDHA HERBAL DRUG

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ABSTRACT

Pharmacognostical standardization of herbal formulation is essential in order to assess the quality of drugs, based on the concentration of their active principles. *Vellarugu chooranam* is a reputed drug mentioned in the ancient books of Siddha Medicine for the treatment of *Vata* diseases, arthritis, constipation, and diabetes mellitus. The main component of *Vellarugu chooranam* is whole plant of *Enicostemma littorale* Blume (Siddha Name: Vellarugu) (Gentianaceae). In the present study an attempt has been made to standardize the *Vellarugu chooranam*. For the standardization of this drug Organoleptic properties, Phytochemical screening, Fluorescence analysis, Elemental analysis, Physicochemical parameters such as moisture content, ash values, extractability in water and ethanol were carried out. TLC and HPLC fingerprints of the *Vellarugu chooranam* were also prepared to evaluate its quality. These set of parameters were found to be sufficient to evaluate authenticity of the *Vellarugu chooranam* and can be used as reference standards for the preparation of a standardized pharmaceutical product and further quality control researches.

KEYWORDS: Herbal Drug, Parameters, Siddha Medicine, Standardization, *Vellarugu chooranam*.

INTRODUCTION

Plant materials are used throughout developed and developing countries as home remedies, over the counter drug products and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market [1]. Herbal medicines as the major remedy in traditional system of medicine have been used in medical practices since antiquity. The practices continue today because of its biomedical benefits as well as place in cultural beliefs in many parts of the world and have made a great contribution towards maintaining human health. Approximately 60% of the population use herbal medicines to treat medical illnesses. Therefore, reproducible standards of each plant are necessary for effective quality control to prevent adulteration [2].

The subject of herbal drug standardization is massively wide and deep. Standardization is an essential factor for herbal formulation in order to assess the quality of the drugs based on the concentration of their active principle and to ensure that every packet of medicine that is sold has the correct amount and will induce its therapeutic effect [3].

Thus, the present study deals with standardization of Siddha herbal preparation, *Vellarugu chooranam* is mentioned in the ancient books of Siddha Medicine for the treatment of *Vata* diseases, arthritis, constipation, and diabetes mellitus [4-5]. The term *Chooranam* (Powdered herbal preparation for internal use) is applied to the powder prepared by a single or a combination of two or more herbal ingredients [6-7].

Systematic protocols for standardization of *Vellarugu chooranam* is not available, hence it was decided to evaluate the qualitative and quantitative analysis for *Vellarugu chooranam* scientifically to prevent its adulteration. For the standardization of this drug Organoleptic properties, Phytochemical screening, Fluorescence analysis, Elemental analysis, and Physicochemical parameters, were carried out. TLC and HPLC fingerprints of the *Vellarugu chooranam* were also prepared to evaluate its quality.

MATERIALS AND METHODS

Plant material

Vellarugu chooranam consist of the whole plant of *Enicostemma littorale* Blume. For the preparation of *Vellarugu chooranam*, whole plants of *E. littorale* were collected from the natural habitats during the month of October 2011 to January 2012 in and around Jaffna District. The plant was botanically authenticated and voucher specimen (No: 2454) was deposited in the Bandaranayaka, Memorial Ayurvedic Research Institute (BMARI), Navinna, Maharagama, Sri Lanka.

Preparation of *Vellarugu chooranam*

The *Vellarugu chooranam* was prepared according to the procedure given in Siddha text^[4].

PHARMACOGNOSTIC STUDIES

Organoleptic evaluation

Organoleptic evaluation was used for identification of sensory characteristics like colour, odour, taste, texture, etc^[8].

Physicochemical evaluation

Physicochemical parameters such as ash values, Loss on drying, extractive values and pH^[9] of *Vellarugu chooranam* were determined as per guidelines of World Health Organization^[1].

Fluorescence analysis

Fluorescence analysis was carried out in accordance with the procedure reported by Kokoshi *et al* as described by Sriwastava *et al*, 2010^[11].

Preliminary phytochemical screening

The powder sample, extracted with ethanol, methanol and water using hot and cold extraction technique (ratio 1:5). The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per the standard procedures^[12-16].

Quantitative estimations

Total alkaloids content (20% acetic acid extract) and flavonoids (80 % aqueous methanol extract) were determined using the procedure according to the methods described Edeoga *et al*^[17]. Saponins (hot water extract) was determined according to the method described by World Health Organization^[1].

Determination of heavy metal and other elements

Vellarugu chooranam was subjected to heavy metal analysis by a graphite furnace Atomic Absorption Spectrophotometer (AAS; GBC 932 plus, Australia). Heavy metal and other

elements analysis were determined as per the AOAC guidelines^[18].

Analysis of Thin Layer Chromatography profiles

TLC profiles of ethanol, methanol and aqueous extracts of *V. chooranam* were carried out as per the WHO guidelines^[1].

Analysis of High-Performance Liquid Chromatography profiles

For determination of the HPLC profiles, the aqueous extract *Vellarugu chooranam* was subjected to reversed phase column of a High-Performance Liquid Chromatography System (Schimadzu, Kyoto, Japan) connected to a UV-Vis detector (Model SPD- 10AVP). The HPLC analysis was performed using a linear gradient of methanol and water (solvent system) in a ratio of 50: 50 for sample for 20 min, with a flow rate 1 ml/ min, detection at 254 nm^[19].

Statistical analysis

For statistical analysis, six replicates were conducted for each activity and the experiments were repeated twice. The values are represented as mean \pm S.D. (standard deviation).

RESULTS AND DISCUSSION

The field of the herbal drugs and formulations is very vast and there is still lot to explore on the subject of standardization of these. So, while developing an herbal drug formulation it is must to have all the related knowledge of that particular drug including all its organoleptic characters to phytoconstituents to pharmacological action to its standardization in respect to various parameters via various techniques^[20].

Organoleptic parameters

The Organoleptic characters of the *Vellarugu chooranam* were as shown in Table 1. *Vellarugu chooranam* was light greenish brown smooth fine powder with characteristic odour and high bitter taste. Ethanol, Methanol and Aqueous extracts of *Vellarugu chooranam* had similar organoleptic characters except the colour.

Physicochemical parameters

The Physicochemical parameters of the *Vellarugu chooranam* are tabulated as Table 2.1 and the Extractive values of this *Vellarugu chooranam* are tabulated as Table 2.2.

The loss on drying at 105°C in the *Vellarugu chooranam* was found to be 10.25%. The ash values of *Vellarugu chooranam* total ash, water soluble ash, acid insoluble ash and sulfated ash were found to be $8.16 \pm 0.1\%$, $2.75 \pm 0.1\%$, $1.89 \pm 0.1\%$ and $1.30 \pm 0.1\%$

respectively. The pH of 1% w/ v and 10% w/ v solutions were found to be 05.12 ± 0.02 and 04.87 ± 0.04 respectively.

Table 1: Organoleptic characters of the *Vellarugu chooranam*

Name of the chooranam and its extract	Appearance	Colour	Taste	Touch	Odour
<i>Vellarugu chooranam</i>	Powder	Greenish brown	High bitter	Coarse	Characteristic
Hot aqueous extract	Liquid	Brown	High bitter	Smooth	Characteristic
Cold aqueous extract	Liquid	Golden brown	High bitter	Smooth	Characteristic
Hot ethanol extract	Liquid	Dark green	High bitter	Smooth	Characteristic
Cold ethanol extract	Liquid	Dark green	High bitter	Smooth	Characteristic
Hot methanol extract	Liquid	Dark green	High bitter	Smooth	Characteristic
Cold methanol extract	Liquid	Dark green	High bitter	Smooth	Characteristic

Table 2.1: Physicochemical parameters of the *Vellarugu chooranam*

Physicochemical Parameters	Values (% W/W)
Loss on drying at 105°C	10.25 ± 0.33
Total ash value	08.16 ± 0.09
Water soluble ash	02.75 ± 0.08
Acid insoluble ash	01.89 ± 0.07
Sulfated ash value	01.30 ± 0.10
pH of 1% w/v formulation solution	05.12 ± 0.02
pH of 10% w/v formulation solution	04.87 ± 0.04

Values are expressed as mean% \pm S.D., n=6

Table 2.2: Extractive values of the *Vellarugu chooranam*

Name of the solvents	Extractive values (% w/w)
Hot aqueous extract	37.21 ± 1.27
Hot ethanol extract	24.92 ± 0.64
Cold aqueous extract	28.67 ± 0.58
Cold ethanol extract	12.68 ± 0.99
Cold n-Butanol extract	05.29 ± 0.05
Cold ethyl acetate extract	05.47 ± 0.26
Cold toluene extract	05.03 ± 0.33
Cold hexane extract	04.39 ± 0.11

Values are expressed as mean% \pm S.D., n=6

The solubility percentage of *Vellarugu chooranam* in hot and cold aqueous extract is higher ($37.21 \pm 1.27\%$ & $28.67 \pm 0.58\%$), when compared with hot and cold ethanol extract ($24.92 \pm 0.64\%$ & $12.68 \pm 0.99\%$) respectively. The present yields of cold n-Butanol, ethyl acetate, toluene, and hexane extractive values were found to be very low.

FLUORESCENCE ANALYSIS

The fluorescents analysis of *Vellarugu chooranam* treated with different chemical reagents is tabulated in the Table 3. The colour of the extracts from organic and inorganic solvents were observed both under ordinary and UV light. There is little difference between extracts and the light sources.

Table 3: Powder fluorescence test of *Vellarugu chooranam*

<i>Vellarugu Chooranam</i>	UV 254 nm	UV 366 nm	In day light
Dry powder	Greenish brown	Greenish brown	Greenish brown
Powder as such			
NaOH (1N) in water	Green	Dark green	Yellowish green
P + HCl (1N)	Green	Brown	Yellowish green
P + NaOH (1N) in MeOH	Green	Dark green	Yellowish green
P + 50% KOH	Light brown	Dark green	Yellowish green

P + 50% H ₂ SO ₄	Green	Dark green	Yellowish green
P + 50% HNO ₃	Brown	Dark green	Yellowish green
P + concentrated HNO ₃	Yellowish green	Dark Green	Brownish orange
P + CH ₃ COOH	Green	Dark green	Yellowish green
P + concentrated H ₂ SO ₄	Dark brown	Dark green	Green
P + iodine in water	Dark brown	Dark brown	Yellow

Preliminary phytochemical screening

The phytochemical active compounds of different extracts (Cold and hot ethanol, methanol, and aqueous) of *Vellarugu chooranam* was qualitatively analyzed and the results are presented in Table 4.

The preliminary phytochemical screening of cold and hot ethanol, methanol and

aqueous extracts of *Vellarugu chooranam* indicated the presence of alkaloids, flavonoids, steroids, tannins, quinones, phenols, coumarins, saponins, and nutrition reducing sugar, and proteins.

Table 4: Phytochemical screening of cold and hot alcoholic and aqueous extracts

Components	Cold Ethanol	Hot Ethanol	Cold Methanol	Hot Methanol	Cold Aqueous	Hot Aqueous
Phytochemicals:	+++	+++	+++	+++	+++	+++
Phenolic compounds						
Tannins- Ferric chloride test	+++	+++	+++	+++	+++	+++
Flavonoids- Shinoda test	+++	+++	+++	+++	++	++
Coumarins	+++	+++	+++	+++	++	++
Steroidal glycosides- Libermann Burchard's test	+++	+++	+++	+++	+++	+++
Alkaloids- Mayer's Test	+++	+++	+++	+++	+++	+++
Dragendroff's Test	+++	+++	+++	+++	+++	+++
Quinones	+++	+++	+++	+++	++	++
Anthraquinones	0	0	0	0	0	0
Saponins- Foam test	++	++	++	++	++	++
Fixed oil and Fats	0	0	0	0	++	++
Nutrition:						
Reducing sugars- Fehling's test	+++	+++	+++	+++	+++	+++
Protein- Xanthoproteic Test	+	+	+	+	++	++

+++ = appreciable amount, ++ = average amount, + = trace amount, 0 = absent

Quantitative Estimations

Table 5.1, summarizes the total alkaloid, and flavonoid contents of *Vellarugu chooranam*. The quantified values of the phytoconstituents can be used as a major tool for obtaining a quality control profile of drug.

The total alkaloid and flavonoid content were found to be 2.25±0.01 % and 25.34 ±0.24 %, in the *Vellarugu chooranam*. The saponin content was relatively low in this preparation.

Table 5.1: Quantitative estimation of important phytoconstituents of *Vellarugu chooranam*

Phytoconstituents	Values (% w/w)
Total alkaloid content	02.25 ± 0.01
Total flavonoid content	25.34 ± 0.24
Total saponin content	Foaming index < 100

Value are expressed as mean% ± S.D, n=3

Heavy metal analysis

Heavy metals may be present in crude drugs through atmospheric pollution and through the soil. Moreover minerals and metals are also used in preparing indigenous formulations. However, heavy metals have been associated with various adverse effects.

Hence, heavy metals need to be detected in such preparations [21]. As apparent from

Table 6.1, a result for heavy metals (Pb, As, Cd and Hg) in *Vellarugu chooranam* was compared with the permissible limits (PL) and acceptable daily intake (ADI) as set by World Health Organization (WHO) and Food and Drug Administration (FDA).

Table 6.1: Heavy metal contents of *Vellarugu chooranam*

Heavy metals	<i>Vellarugu chooranam</i> (mg/ Kg)	Permissible limits (ADI / FDA values) {mg of metal per Kg (ppm)}
Lead (Pb)	0.76	10 mg/ Kg
Arsenic (As)	0.44	0.5 ppm
Cadmium (Cd)	< 0.1 / ND	0.3 mg/ Kg
Mercury (Hg)	< 0.05 / ND	1.0 ppm

Values are expressed mg /Kg, N.D. – Not detected

Table 6.2: Other elemental contents of *Vellarugu chooranam*

Other Metals	<i>Vellarugu Chooranam</i> (mg/ kg)
Calcium (Ca)	37.2
Iron (Fe)	< 0.1 / ND
Sodium (Na)	61.1
Magnesium (Mg)	0.2

Values are expressed mg /Kg, N.D. – Not detected

Heavy metal analysis revealed that the *Vellarugu chooranam* was below the WHO/FDA permissible limits. As apparent from Table 6.2, analysis of metals and other elements (Ca, Fe, Na and Mg) showed the detectable levels in *Vellarugu chooranam*. Iron content was not detected in this preparation. Calcium and

sodium levels of *Vellarugu chooranam* were found to be high.

THIN LAYER CHROMATOGRAPHY (TLC)

Table 7, summarizes the Rf values and colour of spots visible in the TLC profiles of the cold and hot methanol and ethanol extracts of *Vellarugu chooranam* in same solvent system.

Table 7: Thin Layer Chromatography analysis (Rf values and colour of the spots) of different extracts of *Vellarugu chooranam*

Solvent system: Methanol: Dichloromethane: Cyclohexane (0.2 : 5.8 : 4 v/ v)			
Solvent front 7.5cm		Solvent front 4.5cm	
Cold Methanol Extract	Hot Methanol Extract	Cold Ethanol Extract	Hot Ethanol Extract
0.08# Grey	0.08# Grey	0.09# Pink	0.09# Pink
0.13\$ Green	0.13\$ Green	0.13# Pink	0.13# Pink
0.27# Grey	0.27# Grey	0.20# Pink	0.20# Pink
0.35# Grey	0.35# Grey	0.31\$ Pink	0.31\$ Pink
0.41*Dark Pink	0.41*Dark Pink	0.36# Brown	0.36# Brown
0.48# Pink	0.48# Pink	0.40# Brown	0.40# Brown
0.55* Dark Pink	0.55*Dark Pink	0.49*Dark Pink	0.49*Dark Pink
0.60\$Green	0.60\$Green	0.60#Green	0.60#Green
0.68# Pink	0.68# Pink	0.69\$ Pink	0.69\$ Pink
0.73# Pink	0.72# Green	0.80*Dark Pink	0.80*Dark Pink
0.80* Dark Pink	0.80# Pink	0.91*Dark Pink	0.91*Dark Pink
0.89\$ Pink	0.89*Dark Pink	-	-

* - intense, \$ - Moderately intense, # - Faint

The best separation of the methanol and ethanol (cold and hot) extracts occurred in the solvent system comprised of Methanol: Dichloromethane: Cyclohexane (0.2: 5.8: 4 v/v) as the mobile phase.

Visualization was attempted by spraying vanillin sulphate reagent, TLC fingerprints of Cold and Hot methanol and ethanol extracts of chooranam (Figure 1 & 2) revealed the differences of chemical constituents in these extracts. In different extracts of *Vellarugu chooranam* analyzed by TLC, the number of spots with the Rf values were observed not remarkable difference in between Cold and Hot methanol and ethanol extracts of *Vellarugu chooranam*. Figure 4 showed that densitometer fingerprint profile for cold and hot methanol extracts of *Vellarugu chooranam*.

Table 8: Retention time of main peaks in HPLC profile of *Vellarugu chooranam*

Peak no	Retention time (min)
1	3.528
2	4.138
3	9.087
4	11.163

CONCLUSION

Standardization of *Vellarugu chooranam* (single herbal formulation) has been carried out according to WHO guidelines and standard procedures. Heavy metal analysis revealed that the *Vellarugu chooranam* was below the WHO/FDA permissible limits and safe for consumption. On the basis of observations and experimental results, these set of parameters were found to be sufficient to evaluate authenticity of the *Vellarugu chooranam* and can be used as reference standards for the preparation of a standardized pharmaceutical product and further quality control researches. Further studies may be carried out based on identification and separation of active ingredients with the help of bio markers.

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At the same time, Cold and Hot aqueous extracts of *Vellarugu chooranam* (figure 3) did not get mobile phase or colour of spots in the same solvent system. Because, the aqueous extract has higher polar content than other solvents.

High performance liquid chromatography (HPLC)

Aqueous extract of *Vellarugu chooranam* was subjected to reverse phase chromatography as described in the Methodology section. As apparent in Figure 5 and Table 8, most of the major peaks (4 areas) in the aqueous extract of *Vellarugu chooranam* appeared in time between 20 min. These were subjected to reverse phase chromatography, as most of the major peaks were appeared within 20 minutes.

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TLC FINGER PRINTS OF VELLARUGU CHOORANAM

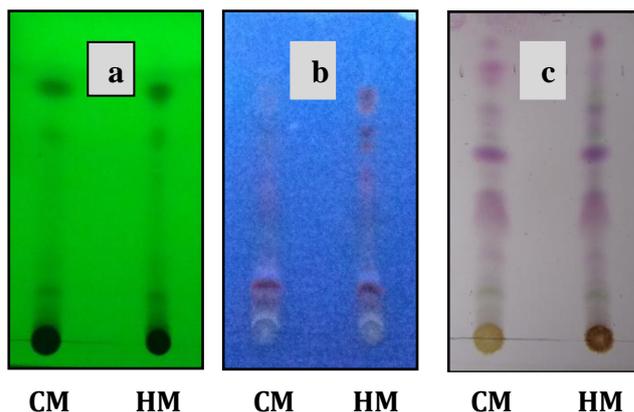


Figure 1: TLC finger prints for cold methanol {CM} and hot methanol {HM} extracts of Vellarugu chooranam (a) at 254 nm, (b) 366 nm, and (c) day light after spray with Vanillin sulphuric acid reagent.

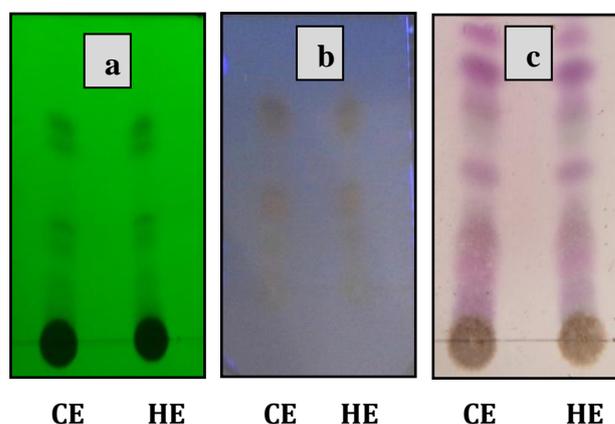


Figure 2: TLC finger prints for cold ethanol {CE} and hot ethanol {HE} extracts of Vellarugu chooranam (a) at 254 nm, (b) 366 nm, and (c) day light after spray with Vanillin sulphuric acid reagent.

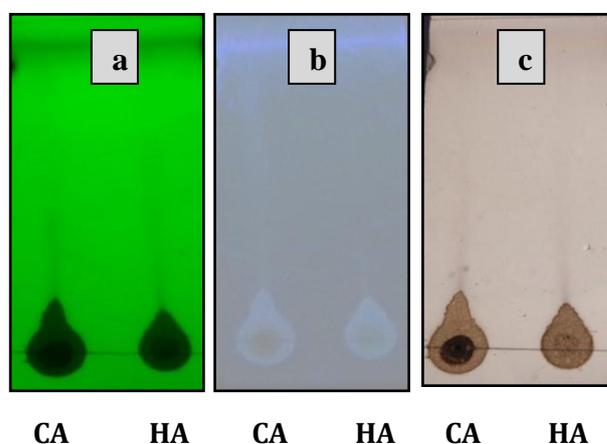


Figure 3: TLC finger prints for Cold aqueous {CA} and Hot aqueous (HA) extracts of Vellarugu chooranam in same solvent mixture a) at 254 nm, b) 366 nm, and c) day light after spray.

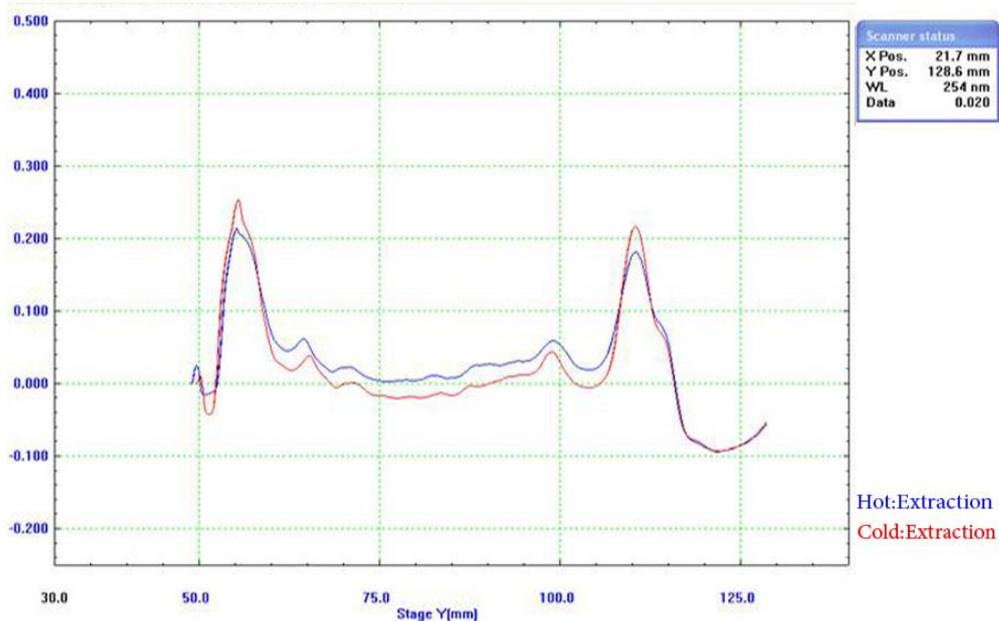


Figure 4: Densitometer finger prints for cold and hot methanol extracts of Vellarugu chooranam

HPLC PROFILE OF VELLARUGU CHOORANAM

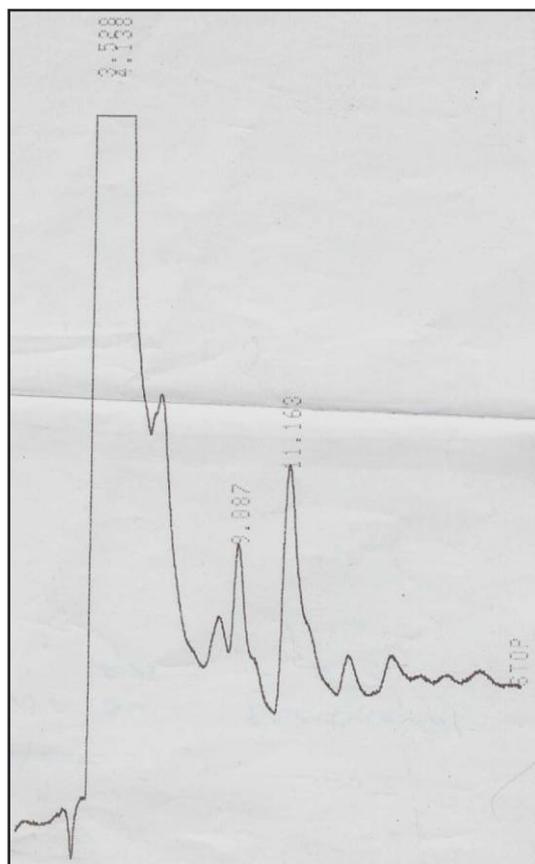


Figure 5: HPLC profiles of aqueous extracts of Vellarugu chooranam

PHOTOGRAPHS
Enicostemma littorale



Figure 6: (a) Fresh plant



Figure 6: (b) Dried Plant



Figure 7: Voucher specimen of Vellarugu plant

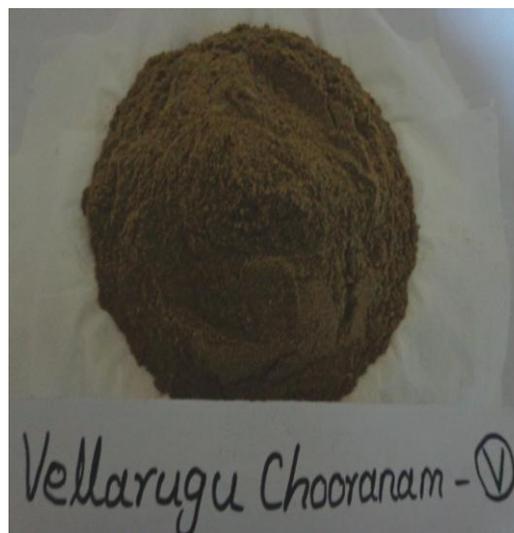


Figure 8: Vellarugu Chooranam