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Quality Assessment of *Calanthe triplicata* by Using Various Standardization Parameters.

K Mythili*¹, C Umamaheswara Reddy¹, D Chamundeeswari¹, and PK Manna².

¹Faculty of Pharmacy, Sri Ramachandra University, Porur, Chennai, Tamil Nadu, India.

²Department of Pharmacy, Annamalai University, Annamalai Nagar, Tamil Nadu, India.

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*For Correspondence

Faculty of Pharmacy, Sri
Ramachandra University,
Porur, Chennai, Tamil Nadu,
India.

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ABSTRACT

Standardization is the most important factor which stands in the way of wider acceptance of herbal drug and also provides confirmation of its identity and determination of its quality and purity. An attempt for the standardization of ethyl acetate extract of *Calanthe triplicata* was carried out by using various parameters like physical evaluation such as ash values (total, acid insoluble and water soluble), extractive values (alcohol soluble and water soluble), loss on drying and crude fiber content and analyses of heavy metals, pesticides and microbial contaminants. The results of physical evaluation showed good results and the determination of heavy metals; pesticides and microbiological results were complied with the standard specification.

INTRODUCTION

Calanthe triplicata is a species of orchid from the genus *Calanthe* and belongs to the family of Orchidaceae. The herbs are reported to be used in diseases of stomach and intestine; and the root is chewed along with betel nuts or other aromatic substances, in diarrhea. The reported activities of the plant were finger print profile by high performance thin layer chromatography (HPTLC), *in-vitro* anti-oxidant activity, phytochemical and Gas Chromatography –Mass Spectroscopy (GC-MS) analysis [12, 13].

Out of the numerous practical applications of herbal drug, the great importance for the pharmaceutical industry is in the evaluation of the crude drugs. It gives a clear idea about the specific characteristic of crude drugs which involves the determination of identity, purity and quality. Standard specification limits are used to confirm the quality of the herb [2].

Impurities arising from the herbs such as pesticides and heavy metals are normally controlled during the testing of the herbal drug preparation and it is not necessary to test for these in the herbal medicinal product. Suitable validated methods should be used to control all the potential residues. Plant associated microorganisms and their interactions with plant are influencing the plant health. There is a need to specify the Total count of aerobic microorganisms and the absence of specific objectionable bacteria [3,4,5,6,7].

MATERIALS AND METHODS

Plant Material

The plant was collected from Kolli hills, Namakkal district, Tamil Nadu and authenticated by Dr. Sasikala Ethirajulu, Assistant Director (Pharmacognosy), Siddha Central Research Institute Arignar

Anna Govt. Hospital Campus Arumbakkam, Chennai, Tamil Nadu, India.

Chemicals and Reagents

Sodium hydroxide, hydrochloric acid, ethanol, chloroform water, nitric acid, Millipore water, ethyl acetate, acetonitrile, Whatman (No.4) filter paper, phosphate buffered saline, potassium bromide, violet red bile agar, lactose, enrichment broth-mossel, Macconkey broth, tryptophan broth, Indole reagent, sodium sulfate and Primary secondary amine (PSA) sorbent were analytical grade. Methanol and water were HPLC grade.

Physical Evaluation ⁽¹⁾

Crude plant material needs proper assessment and detection based on different physico-chemical parameters like determination of total ash value, acid insoluble ash value, water soluble ash value, loss on drying etc. The evaluation of these parameters gives a clear idea about the specific characteristic of crude drugs.

Determination of total, acid – insoluble and water - soluble ash value

Total ash

Weighed about 2 gm of the powdered drug and transferred it into an accurately weighed and previously ignited tarred silica crucible. The crucible was supported on a stand. Heated with a burner, using a flame about 2 cm high and supporting the crucible about 7 cm above the flame, heated gently till vapors are almost cease to be evolved, then lowered the crucible and heated more strongly until all the carbon is burnt off. The residue was allowed to cool in a suitable dessicator and weighed.

Acid - insoluble ash

The total ash obtained in the above procedure was washed using 25 ml of dilute hydrochloric acid and transferred into a 100 ml beaker. The solution was boiled for 5 min., filtered through an 'ashless' filter paper and washed the residue twice with hot water. An empty silica crucible was ignited in the flame, cooled and weighed. Put the filter paper and residue together into the crucible, heated gently till vapors are almost cease to be evolved, and then more strongly until all the carbon has been removed. The residue was allowed to cool in a suitable dessicator and weighed.

Water - soluble ash

Water soluble ash value was determined in a similar way to acid insoluble ash, using 25 ml of water, in place of dilute hydrochloric acid and the residue of water soluble ash was weighed.

The percentage of ash values were calculated using the following formula

$$\text{Total / acid-insoluble / water soluble ash value of the sample} = \frac{\text{Weight of the residue}}{\text{Weight of the drug taken}} \times 100 \%$$

Determination of alcohol – soluble / water – soluble extractive value

Alcohol – soluble extract

Weighed about 5 gm of the powdered drug in a weighing bottle and transferred it into a dried 250 ml conical flask. About 100 ml of 90 % ethanol was taken in a measuring cylinder. Washed out the weighing bottle and pour the washings, together with the remainder of the solvent into the conical flask. Corked the flask and set aside for 24 h, shaking frequently. Filtered the solution and transferred 25 ml of the filtrate into a weighed porcelain dish. Evaporate to dryness on a water bath and complete the drying in an oven at 100 °C. The residue was allowed to cool in a suitable dessicator and weighed.

Water – soluble extract

Water soluble extractive value was determined in a similar way to alcohol soluble extractive value, using 100 ml of chloroform water, in place of ethanol and the residue of water soluble extract was weighed. The percentage of extractive value was calculated.

Determination of moisture content (Loss on Drying)

Weighed about 1.5 gm of the powdered drug and transferred it into an accurately weighed porcelain dish. Dried in an oven at 100 °C and allowed to cool in a suitable dessicator. The residue was weighed and the percentage of moisture content was calculated.

Determination of crude fiber content by Dutch method

Weighed about 1.5 gm of the powdered drug and transferred it into a beaker. About 50 ml of 10 % v/v nitric acid was added and heated for 30 s after boiling starts, stirred constantly. A fine cotton cloth on a Buchner funnel with suction was used to strain the above mixture. Washed the residue with boiling water and transferred the residue from the cloth to a beaker.

Then added 50 ml of 2.5 % v/v sodium hydroxide solution and heated to boil (maintained at boiling point for 30 s and stirred constantly). Strained and washed with hot water as mentioned earlier. Transferred the residue in a clean dried silica crucible and weighed. The percentage of crude fiber was calculated.

Analysis of Heavy Metals

For Lead, Cadmium and Arsenic

About 2.5 g of the well homogenized sample was weighed accurately in the microwave digester vessel. About 5 ml of suprapure concentrated nitric acid and 2 ml of 30 % hydrogen peroxide were added. The tubes were closed with the cap. The microwave digester was operated. After completion of digestion process, the vessel is cooled thoroughly. The solution was transferred into a 25 ml standard flask and diluted to the mark with distilled water. This is then taken for analysis by Atomic Absorption Spectrometry (AAS) [8].

For Mercury

About 5 g of the well homogenized sample was weighed accurately in the digestion flask. About 10 mg of Vanadium pentoxide and 20 ml of sulphuric acid- nitric acid (1:1) v/v were added. The flask was kept initially at low boil for 6 min and at strong boil for 10 min. After completion of digestion process, the vessel is cooled and washed with water. The solution was transferred into a 50 ml standard flask and diluted to the mark with distilled water. This is then taken for analysis by CVAAS (Cold Vapor Atomic Absorption Spectrometry) [9].

Individual working standards were prepared for the calibration of mercury (0.01, 0.02, 0.05, 0.075, 0.10, and 0.15 mg/ l) and for arsenic / Cadmium (0.02, 0.05, 0.10, 0.20, 0.50, and 1.00) by diluting a suitable aliquot to a desired volume with 3 % nitric acid.

Microbiological Analysis

Detection of *Enterobacteriaceae*

Enumeration method

Pretreated material (violet red bile agar) in lactose broth was incubated for 2 h at 30 °C for revivification of *bacteria*. The pretreated homogenized material which is in lactose broth was inoculated by transferring 1 ml, 0.1 ml, and 0.01 ml of sample in to *Enterobacteriaceae* enrichment broth mossel (100 ml) individually and then incubated (35 °C for 24 h). After incubation, a subculture was prepared by using violet red bile agar with lactose on a plate and again incubated (35 °C for 24 h). Absence of red or reddish color colonies showed that the sample was passed the test for *Enterobacteriaceae*.

Detection of *Escherichia coli*

Identification test

About 1 g of pretreated sample in lactose broth was taken and inoculated it into a 100 ml Macconkey broth and incubated (45 °C for 24 h). After incubation, a subculture was prepared with Macconkey agar on a plate and again incubated (45 °C for 18-24 h). The presence of *Escherichia coli* is detected by the growth of red non-mucoid colonies of gram-negative rods surrounded by a reddish zone of precipitation. The sample passed the test as no such colonies were detected. Confirmatory test was performed by taking one of the perfect suspected colonies, inoculated into the tryptophan broth and incubated at 43.5 °C for 24 h. After incubation, 2 drops of Indole reagent was added. Red color ring was not appeared; the test confirmed the absence of *Escherichia coli*.

Detection of Total fungal count

Enumeration method

About 10 ml of the sample was inoculated into 100 ml of sodium chloride peptone solution and 1 ml of this broth was again inoculated into yeast extract agar medium. The plates were incubated at 25 °C for 5 d. Presence of aerial growth as *Yeast fungus* and mucoid-like colonies as *Moulds* are regarded as positive for the detection of total fungal count. The colonies were not detected and confirmed the absence of total fungal^[10].

Pesticidal analysis

Standard

Stock solution (1 mg/ml): About 10 mg of each pesticide standard was weighed and transferred in to individual 10 ml volumetric flask and made up the volume with ethyl acetate.

Intermediate solution (10 µg/ml): About 1 ml of each stock solution was transferred in to individual 100 ml volumetric flask and made up the volume with ethyl acetate.

Working solution (0.1 µg/ml): About 1 ml of each intermediate solution was transferred in to individual 100 ml volumetric flask and made up the volume with ethyl acetate.

Sample

Extraction was done by taking approximately 5 g of the crushed sample and homogenized it for 1 min at 10,000 revolutions per minute (RPM). About 1 gm of the homogenized sample was taken and 50 ml of ethyl acetate and 25 g of sodium sulfate were added. The sample was again homogenized for one min at 10,000 RPM. After the each homogenization, the turrax in the sequence-water-ethyl acetate-water was cleaned to remove the solid matrix clinging to the turrax. The extract was centrifuged at 1200 RPM in a high volume centrifuge for one min. About 1 ml of the supernatant extract from high volume centrifuge was transferred into an eppendorf tube (capacity-2 ml) and 25 mg of Primary secondary amine was added. Then the tube was centrifuged at 10,000 RPM for 2-3 min. About 1-2 µl of extract was taken from the eppendorf tube and injected into GC-MS^[11].

RESULTS AND DISCUSSION

Physical Evaluation

Ash values are useful for the evaluation of purity of a crude drug by detecting excess of earthy matters. The presence of ash was determined as total, acid-insoluble and water-soluble ash and the values were calculated as 8.65 ± 0.114 , 2.01 ± 0.070 , and 4.25 ± 0.035 respectively. Extractive value represents the nature of the constituents of the crude plant and it was detected by specific solvents (alcohol and water) and the values were calculated as 18.96 ± 0.081 , 14.72 ± 0.061 respectively. Moisture content and excessive woody material were determined by the method of loss on drying (LOD) and crude fiber content and were found to be 1.5 ± 0.026 , 43.5 ± 0.529 respectively. The results are shown in Table 1.

Heavy Metal Analyses

Trace level of metal contents is essential for nutritional consideration in the crude plant. The detection limit of Lead, Cadmium, Arsenic and Mercury were found to be 0.05 ppm. The results were found to be complied with the WHO specification. It means that the total concentration of metal content were negligible. (Table 2) (fig.1).

Pesticide Analyses

Pesticide residues were separated from the matrix by using extraction method. After purification, the residues in concentrated eluates were identified by comparing the retention time of the sample with the standard and quantified its residue level. The results were found to be complied with the WHO specifications (Table 3) (fig. 4, 5).

Microbiological Analyses

Microbiological testing is important in order to ensure that the sample is safe for use. Qualitative and quantitative estimations of specific viable microorganisms were found with satisfactory results. It showed that *Enterobacteriaceae* and total fungal count were found to be significantly lower levels and also *Escherichia coli* was not present in the sample. The results are given in the (Table 4) (fig. 2,3).

Table 1: Physical Evaluation

Parameters	Percentage Yield (% W/W)
Total ash value	8.65 ± 0.114
Acid - Insoluble ash value	2.01 ± 0.070
Water - Soluble ash value	4.25 ± 0.035
Alcohol – Soluble extractive value	18.96 ± 0.081
Water – Soluble extractive value	14.72 ± 0.061
Moisture content	1.5 ± 0.026
Crude fiber content	43.5 ± 0.529

% W/W – Percentage weight by weight, NMT- Not more than, NLT- Not less than

Table 2: Heavy Metals

Test	Observed result	Specification as per WHO
Lead	BDL(D.L-0.05 ppm)	10 ppm
Cadmium	BDL(D.L-0.05 ppm)	0.3 ppm
Arsenic	BDL(D.L-0.05 ppm)	3.0 ppm
Mercury	BDL(D.L-0.01 ppm)	1 ppm

ppm – Parts per Million, BDL - Below Detection Level, DL - Detection Limit

Table 3: Pesticidal Analysis

Compound	Standard		Sample	
	RT	Conc. (ppb)	RT	Conc. (ppb)
HCH	14.84			Not Detected
Dieldrin	27.33	100	-	(D.L: 0.005
Aldrin	28.67			mg/kg)

RT – Retention time, C- Concentration, ppb- Parts per Billion

Table 4: Microbial Analysis

Test	Observed result	Specification as per WHO
Total Fungal count	<10 CFU/ml	NMT 10 ³ CFU/ml
<i>E.coli</i>	Absent/ml	Absent/ml
Enterobacteriaceae	<10 CFU/ml	NMT 10 ³ CFU/ml

CFU – Colony-Forming Unit



Figure 1: *Enterobacteriaceae*



Figure 2: Total *funga* count



Figure 3: *E.coli*

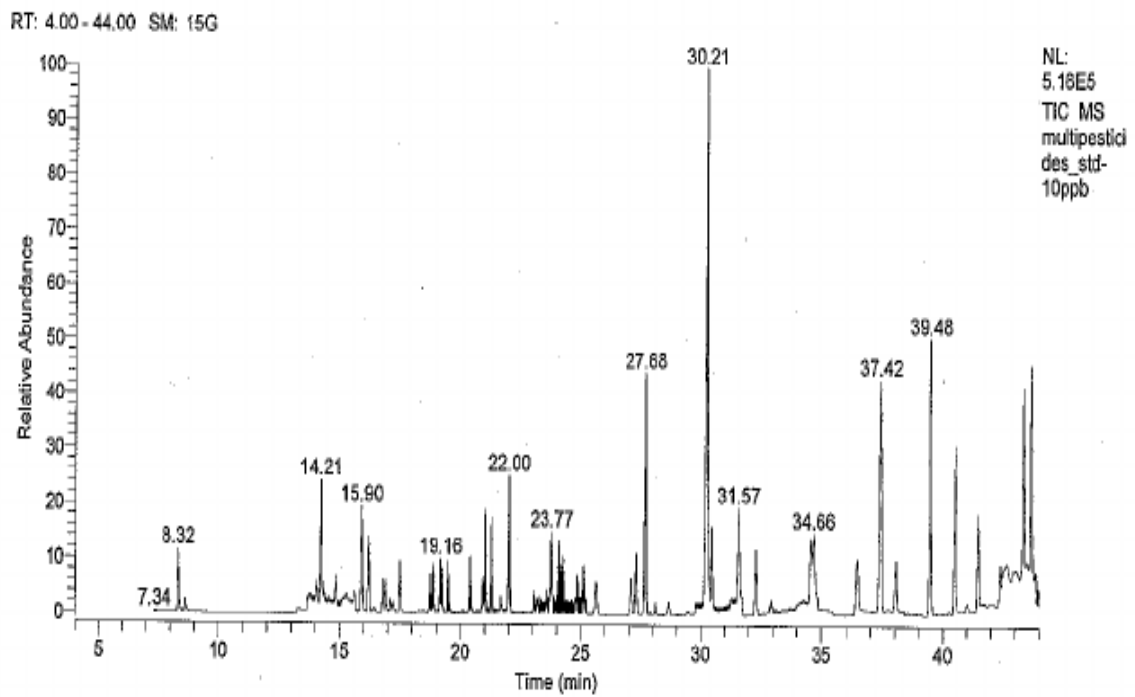


Figure 4: Pesticide standard

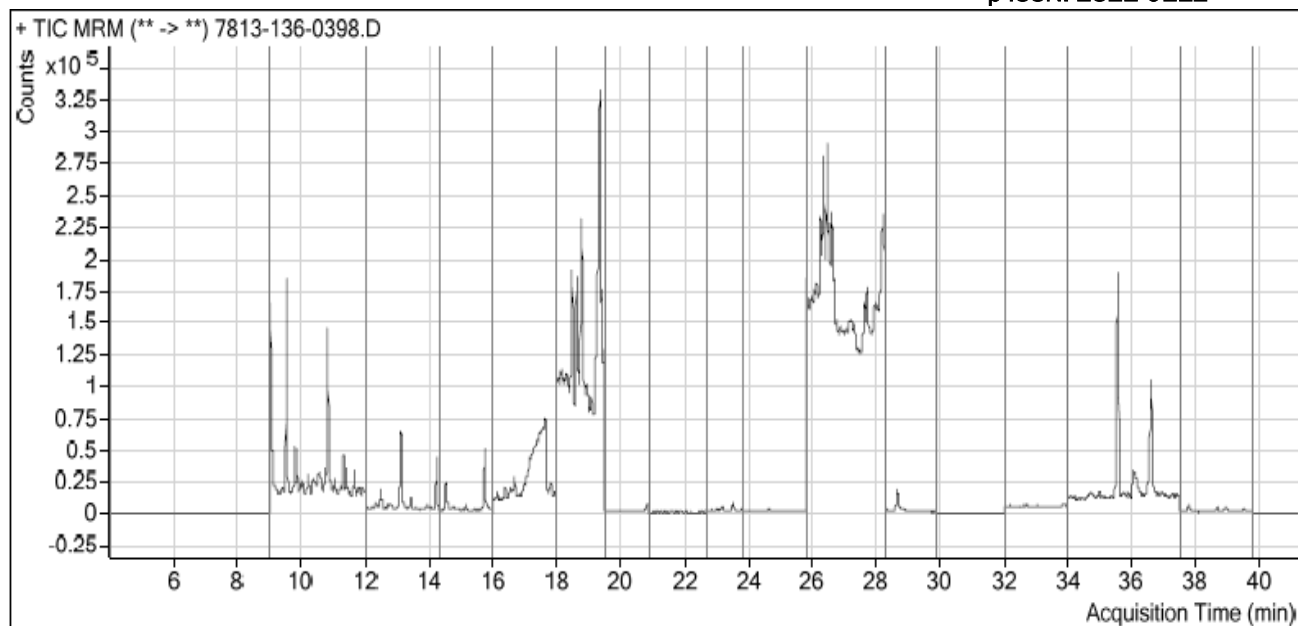


Figure 5: Pesticide sample

CONCLUSION

Modern approach of standardization of selected medicinal plant was performed. The quality and purity has been achieved by analyzing various parameters and compared with standard specifications mentioned in the official guidelines. Thus in conclusion, the herbal extract of *Calanthe triplicata* is free from heavy metals, pesticides and microbial contaminants and it can be used for further analysis.

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REFERENCES

1. KR Khandelwal. Text book of Practical Pharmacognosy (Techniques and Experiments), 17th ed. Nirali Prakashan Publishers; 2007.
2. Pulok K. Mukherjee. Text book of Quality control of herbal drugs (An approach to evaluation of Botanicals), 1st ed. Business Horizons Publishers; 2002.
3. Sreenivasulu Munna, KN Jayaveera, C Madhusudhana Chetty, K Gnanaprakash, K Adinarayana. Heavy metal analysis of various parts of *Ficus mollis* (Vahl) by HPTLC. Int J Chem Tech Res. 2010; 2 (2): 807-812.
4. Klaus-J Appenroth. Definition of Heavy metals and their role in biological systems. 2010; 19-29.
5. Vandana garg, Vir Ji Dhar, Anupam Sharma, Rohit Dutt. Facts about standardization of herbal medicine: a review. J Chinese Integr Med. 2012; 10 (10): 1077-1083.
6. Identification & taxonomic classification of microorganism(s) represented for use as supplements under the Fertilizers Act. Canadian Food Inspection Agency (CFIA) 2013.
7. Method validation and quality control procedures for pesticide residues analysis in food and feed, National Food administration, Sweden. Document number SANCO / 10684/ 2009: 3-40.
8. United States Department of Agriculture Food Safety & Inspection Service - GLG - TM 3.01 REC.01, 2012.
9. Official Methods of Analysis of AOAC International, 18th ed. Chapter 9, Gaithersburg, MD, USA, official method 993.14, AOAC, 2005.
10. Quality control methods for herbal materials. Updated edition of Quality control methods for medicinal plant materials, World Health Organization 1998. ISBN 978 92 4 1500739: 1-135.

11. Pesticide analytical manual (multi residue methods) 304 method for fatty foods: Food and Drug administration, Washington 3rd ed. (1), 1999.
12. K Mythili, C Umamaheswara Reddy, D Chamundeeswari, PK Manna. GC-MS analysis of phytocomponents and in-vitro inhibitory effects of *Calanthe triplicate*. J Natural Prod. 2013; 6:141-146
13. K Mythili, C Umamaheswara reddy, D Chamundeeswari, PK Manna. Phytochemical screening and in-vitro anti-oxidant activity of *Calanthe triplicate*. World Acad Sci Eng Technol. 2012;69.