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Research Article

STANDARDIZATION AND PHYTOCHEMICAL INVESTIGATION OF CALENDULA OFFICINALIS, CASSIA TORA AND MOMORDICA CHARANTIA SEED EXTRACT.

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Abstract:

Purpose: "Standardization" the process of developing and implementing technical standards and to maximize compatibility, safety, repeatability and quality of the drugs is one of the important measures in view of the various practical problems encountered from time to time especially in the field of herbal drugs and Ayurveda.Scientific data pertaining to the standardization of the herbal drugs could be of immense value to substantiate efficacy, safety or toxicity of an herb. Hence present study was intended to standardize Calendula officinalis flowers, Cassia tora and Momordicacharantia seed extracts, since these are widely used in large number of herbal, ayurvedic and homeopathic formulations.

Methodology: Systematic investigations were carried out to assess various quality parameters as per WHO guidelines such as morphological, microscopical and proximate analysis including ash values, moisture contents and extractive values and TLC for Calendula officinalis flowers, Cassia tora and Momordicacharantia seed extracts.

Results: Results indicated the authenticity of the herbal drugs used in the study. Organoleptic and proximate analyseswere well within the acceptable limits. TLC system was developed for identification of the extracts. Quantitative estimation of total saponins, phenols and flavonoid contents were determined. Herbs tested indicated absence of heavy metals and arsenic. Qualitative phyto chemical analysis of extracts obtained using different solvents revealed presence of glycosides, saponins, triterpenes, phenols, tannins and carbohydrates in different extracts.

Conclusion: Study was successful in establishing quality standards for the flowers of Calendula officinalis, seed of Momordicacharantia and Cassia tora. These preliminary studies may offer great help in initial procurement and assessment of quality of the crude drugs when these are being used as raw materials for preparations of herbal formulations.

Key words: *Standardization, Calendula officinalis, Cassia tora, Momordicacharantia, proximate analysis, quantitative study.*

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INTRODUCTION

There is much interest created by natural resources especially"herbs"as source of therapy. Medicinal herbs have been known for centuries and are highly valued all over the world as a rich source of therapeutic agents for prevention of diseases and ailments. Use of plants as a source of medicine has been inherited and is an important component of the health care system in many countries in the world.[1]

Cassia tora (F:Cesalpinaceae), Momordicacharantia, (F:Cucurbitaceae) and Calendula officinalis (Asteraceae)are some of the herbs which have been used since ages for their wide range of medicinal properties. They are used as one of the ingredients in wide range of herbal, ayurvedic and homeopathic formulations for internal as well as external preparations[2,3,4] WHO encourages, recommends and promotes traditional/herbal remedies in natural

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health care programs because these drugs are easily available at low cost, safe and people have faith in them. The WHO assembly in number of resolutions has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards.[5] Standardization is necessary to make sure the uniform quality product throughout the world. Herbal formulations manufactured using "standardized extracts" can claim status of standard preparations much easily rather than trying to standardize the formulations prepared using non "standardized" extracts without prior after manufacturing using extracts which are not standardized.

Hence in the present study, an attempt was made to assesthe pharmacognostic parameters of seeds of Cassia tora, Momordicacharantia, flowers of Calendula officinalis. Standardization of aqueous extract of these herbs using physical, phytochemical, analytical and microbiological methods were also evaluated.

MATERIALS AND METHOD

Plant Material

Samples of Momordicacharnatia and Cassia tora seeds were obtained from Natural remedies, Bangalore. Dried flowers of Calendula officinalis was procured from Himalaya herb stores, Saharanpur, India. The crude drugs were identified at Regional Research Institute, Bangalore, by Botanist Mr.P.Santanam. Voucher specimens (Calendula officinalis:CA-tsr/2005, Cassia tora: CT-tsr/2005, Momordicacharantia:Mc-tsr/2005)of all the herbs were deposited in the laboratory as reference samples.

Extraction of the herbs.

50grams each of dried seeds of Momordicacharnatia, Cassia tora and Dried flowers of Calendula officinalis were powdered and extracted in soxhlet apparatus using petroleum ether, benzene, chloroform, methanol, ethanol and water successively. Each time before extraction with the next solvent the powder was air dried to remove the adhering solvent. Extracts were concentrated by evaporating the solvent in water bath. Extracts were labeled and stored in airtight containers at room temperaturefor further studies.

Phytochemical analysis of the different extracts was carried out as per the standard procedures[5,6,7,8]

Standardization: The procedures followed for the standardization of the aqueous extracts Calendula officinalis, Momordicacharantia, and Cassia tora are:

a) Description: Description of the extract includes physical appearance of the extract by visual examination under diffused light.
b) Solubility: Solubility of the extract was observed with water, 95% and 50% ethanol.
c) Identification: By Thin Layer Chromatography.[9]

i) Cassia tora: Adsorbent : Silica gel 60 F254, Solvent system: Ethyl acetate:
Methanol: Water 80:19:1, Sample preparation: 1 gm of the extract is dissolved in 5 ml of Dimethyl formamide (DMF)and volume was made upto 50ml with methanol, filtered and used for TLC, Standard Preparation: Emodin 1mg/ml in methanolic DMF, Detection : UV - 366nm (Fig. 5).

ii) Calendula officinalis: Adsorbent :Silica gel 60 F_{254} Solvent system: Ethyl acetate:Butanone: Formic acid:water 5 : 1 : 1, Sample preparation: 1 gm of the extract is dissolved in 5 ml of DMF andvolume was made upto 50ml with methanol, filtered and used for TLC. Standard Preparation: Rutin 1mg/ml in methanolic DMF. Detection: By spraying Vanillin Sulphuric Acid reagent (Fig. 3).

iii)Momordicacharantia: Adsorbent : Silica gel 60 F254, Solvent system: Toulene : Acetone : Water 5 :15:1, Sample preparation: 1 gm of the extract is dissolved in 5 ml of DMF and volume was made upto 50ml with methanol, filtered and used for TLC.Standard Preparation: beta sitosterol-d-glucose 1mg/ml in methanolic DMF, Detection : By spraying 10% alcoholic Sulphuric Acid (Fig. 4).

d) Moisture Content: Moisture content of the extracts of all the three extracts was determined by IR moisture balance.
e) Ash Values:The total ash, acid insoluble ash and water-soluble ash values were determined for the extracts using the standard procedure.[5]
e) Test for Pb,Ni,Cd:[5]

Preparation of the extracts: The extracts were accurately weighed, finely powdered and taken up for the heavy metal analysis.

Preparation of digestion mixture: A ternary digestion solution of three acids was prepared by mixing 100 ml of concentration HNO_3, 10 ml of

concentration H_2S0_4 and 40 ml of 60% $HClO_4$ and allowed to cool and stored in a reagent bottle.

Wet oxidation of Plant samples : The extracts were treated with the ternary acid mixture in a silica crucible without exerting pressure for the oxidation of the extracts. Blank digestions (in duplicate) were run on the reagents added in the same amounts as employed in the determinations.

Cd/Pb/Ni contents in the extracts : Pretreated sample with HNO_3 was placed in digestion flask and then mixed with appropriate amount of the ternary acid mixture, consisting of 5 ml for 1-2 gms of powdered extracts. Digestion was carried out at 180^0C to 200^0C until dense white fumes of H_2SO_4 : $HClO_4$ were evolved. The digestion was continued at 180^0C to 200^0C until the acid was largely volatilized and the residues in the flask were clear white and only slightly moist with H_2SO_4. The residue was diluted with glass distilled water and made up to definite volume in a volumetric flask. Then the solution was ready for the estimation of different toxic heavy metals like Cd, Pb and Ni with the help of Atomic Absorption Spectrophotometer (AAS) Perkin Elmer model AAnalyst 100.

Calculation : mg/kg of heavy metals (Cd, Pb and Ni) in plants = Sample dilution X AAS reading in mg/kg.

f) Limit test for Arsenic:

Preparation of the sample by acid digestion: Accurately weighed aqueous herbal extract (5 gms) was powdered and treated with water (2.5ml) followed by nitric acid (5 ml) and sulphuric acid (2 ml) carefully. This was kept in a fuming cupboard with repeated addition of nitric acid until no further darkening takes place, until a clear solution with copious vapors of sulphur dioxide was obtained indicating the complete removal of organic matter. The mixture was cooled and treated with a mixture of water (7.5) and 25g/l ammonium oxalate (2.5ml). This was further heated until fumes of sulfur trioxide developed. This was cooled and transferred to 25ml volumetric flask, volume was adjusted with water and used for the limit test.

Preparation of the standard stain: Standard stain was prepared by mixing 10 ml of stannated hydrochloric acid and 1 ml of dilute arsenic to 50ml of water and treated as described in the general test, as per Indian Pharmacopoeia , which yields a stain on mercuric bromide paper As R referred to as the standard stain. The intensity of the stain produced by the sample (extract) was compared to that of the standard.

g) Microbial test : Total viable aerobic count- Plate count method[8]

Sample : One gram of the extracts was dissolved in 100 ml of buffered sodium chloride- peptone solution pH 7.0.

For bacteria : 1 ml of the pretreated extracts solution was mixed with 15 ml of sterilized liquefied casein-soybean digest agar at a temperature not exceeding 45^0C, and transfer aseptically into a sterilized petriplate. Petriplate were in an incubated at 30 ± 50^0C for 5 days and the number of colonies formed observed.

For Fungi : 1 ml of the pretreated extracts solution was mixed with 15 ml of sterilized liquefied Sabouraud glucose agar at a temperature not exceeding 45^0C, and transfer this aseptically into a sterilized petriplate. Incubate the petriplate in an incubator at 20 ± 50^0C for 5 days and observed for the colonies formed.

h) Assay of the constituents : Gravimetry[9]

Determination of saponins [9]: 5gms of the extract was extracted with 25ml of 90%v/v of methanol by refluxing for half an hour, filtered and the residue was extracted again twice by taking 25 ml of methanol. The solvent was evaporated and the resulting soft extract was refluxed with 25ml pet ether for half an hour, cooled and solvent was removed by decantation. The residue left was refluxed with 25ml of ethyl acetate, cooled and decanted. The remaining residue was extracted with 25 ml of n-butanol successively for 3 times. The combined n-butanol extract was evaporated to remove the solvent and the residue was dissolved in 5 ml of 90%methanol. This was filtered and concentrated. This concentrated extract was added drop by drop with constant stirring to 25 ml of solvent ether in a tared beaker, which precipitates the saponins. The precipitate was allowed to settle in the beaker and solvent ether was allowed evaporate, the residue was dried to a constant weight at low temperature.

The percentage of saponins was calculated by the formula

$$\% \text{ of Total saponins} = B\text{-}C/A \times 100$$

Where as A= Weight of the extract taken,

B = Weight of the tarred dish + saponins,

C = Weight of the tarred dish

Determination of total bitters: 3 gms of the extract was refluxed with 50 ml of alcohol on a water bath for half an hour and filtered. This was repeated until bitterness is observed in the residue. The combined filtrate was evaporated and the residue was washed repeatedly with water. The aqueous solution was shaken repeatedly with 25, 20 and 15 ml of pet ether in a separator. The pet ether layer was discarded and the aqueous layer was repeatedly shaken with 25, 20,15 and 15ml of ethyl acetate. The ethyl acetate layer was collected in a tarred beaker and evaporated to dryness and weighed.[9]

The percentage of Total bitters was calculated by the formula

$$\% \text{ of Total Bitters} = B\text{-}C/A \times 100$$

Whereas A= Weight of the extract taken,
B = Weight of the tarred dish + Total bitters
C = Weight of the tarred dish

All the experiments were done in triplicated and values were expressed in terms of mean±SEM.

RESULTS AND DISCUSSION

In an attempt to standardize, aqueous extracts of Calendula officinalis, Momordicacharantia and Cassia tora, were subjected to various analysis by following standard procedures as described in pharmacopoeia and official books.[10] Extracts were standardized with respect to their appearance, solubility, chromatographic identification, moisture content, ash values, heavy metals, arsenic , microbial contamination (total bacteria, fungal count) and assay of active substances (total saponins and total bitters).

Identification of the crude drugs is the most important aspect to begin any study with regard to the herbal drugs. In this aspect morphological, organoleptic and microscopical characters of the crude drugs were evaluated and are presented (Table. No.1, Plate No. 1 and 2).

Plate. No.1. Photographs of dried
1) seeds of Cassia tora,
2) Dried flowers of Calendula officinalis and
3) seeds of Momordicacharantia.

Table 1: Morphological features of seeds of Cassia tora, Momordicacharantia and for the dried flowers of Calendula officinalis.

Sample	Colour	Odour	Taste	Form	Size-Length Diameter
Cassia tora	Light brown	Mild andcharacteristic	Mucilaginous, Slightly bitter	Rombohedral	3-4 mmlong
Momordica-charantia	Greenish yellow	characteristic	Bitter	Laterally compressed, Corrugate margin and sculptured on both surfaces	8-13mmlong 7-9 mmlong 2-3 mmlong
Calendula officinalis.	Orange yellow	Faint and characteristic	Bitter and salty	Whole or partially broken up flower heads with shiny, yellowish red, female lingulate florets with tridentate top without papus and few short tubular florets	

Plate. No.2. Photographs of Microscopic charaters of dried seeds of
1)Cassiatora,flowers of
2) Calendula officinalis and seeds of
3) Momordicacharantia

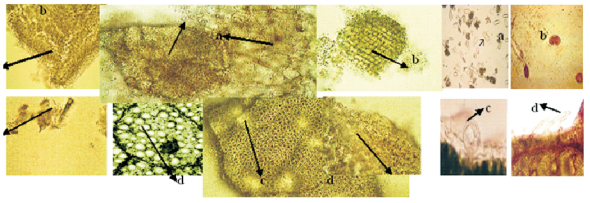

Plate. No.3. Thin layer chromatography of
a) Calendula officinalis,
b) Cassia tora and
c) Momordicacharantia

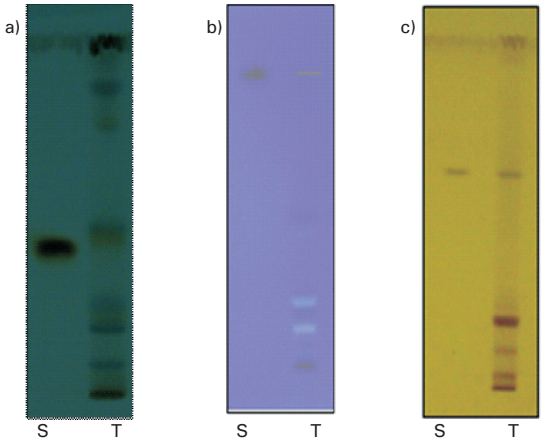

Fig. 3. a – S: Rutin , T: Calendula officinalis extract
Fig. 3. b – S:Emodin , T:Cassia toraextract
Fig. 3. c – S: Beta-sitosterol-d-glucose, T –Momordicacharantia officinalis extract

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of the drugs is not too high thus it could discourage bacterial, fungal or yeast growth[11]. The experimental

results of moisture content and ash values indicated that the foreign organic matter, metallic salts or silica are well within the permissible limits.

Moisture content , total ash, acid insoluble ash and water soluble ash contents of Calendula officinalis was found to be 1.34%, 0.54%, 0.01% and 0.12% respectively, in Momordicacharantia extract it was 0.65%, 0.56%, 0.01% and 0.11% respectively, in case of Cassia tora it was found to be 1.39%, 0.46%, 0.01% and 0.09% respectively. All the tested extracts indicated absence of lead, nickel, cadmium or arsenic as evident by the results of the heavy metal analysis and limit test for arsenic.

Quantification of the phyto-constituents can also be an indicative of the fact that the process of extraction has not affected structural integrity of the constituents. Hence quantitative analysis for the presence of total saponin contents of Calendula officinalis extract was found to be 16.3±1.50% and in case of Cassia tora it was found to be 23.4 ±1.25*. Total bitters in Momordicacharantia extract was found to be 12.1±1.62%. (Table 2).

Table 2 : Standardization data of vacuum dried aqueous extract of seeds of Cassia toraCalendula officinalis and Momordi-cacharantia

Sl. No	Parameters		Parameters Cassia tora officinalis	Calendula	Momordicacharantia
1	Color		Blackish brown	Dry dark brown,	Cream
2	odour		Agreeable, characteristic	Agreeable and charateristic	Agreeable and very faint
3	Nature		Crystalline solid	crystalline	Dry powder
4	Taste		Bland	charateristic	Bitter
5	Solubility	Water Alcohol 50%v/v Alcohol95%v/v	Soluble Insoluble Partially soluble	Soluble Insoluble Partially soluble	Soluble Insoluble Partially soluble
6	Moisture content (%)		1.39±0.56*	1.34	0.65
7	Total ash(%)		0.46±0.02*	0.54	0.56
8	Acid insoluble ash(%)		0.01±0.01*	0.01	0.01
9	Water soluble ash(%)		0.09±0.02*	0.12	0.11
10	Total Saponins(%)	23.4 ±1.25*	16.3±1.50*	---	
11	Total bitters		----	---	12.1±1.62*
12	Limit test for Arsenic		Passes	Passes	Passes
13	Heavy metals	Nickel Cadmium Lead	Nil Nil Nil	Nil Nil Nil	Nil Nil Nil
14	Microbiological test	Bacteria Fungi	Absent Absent	Absent Absent	Absent Absent

*Values are mean ± SEM of values done in triplicate

Standardization being one of the one of the biggest challenges in the field of natural products, different people have different meanings to the word standardization. As per the latest edition of European Pharmacopoeia, standardization means "adjusting the herbal drug preparation to a defined content of a constituent or a group of substances (total saponins, total bitters, total alkaloids, total glycosides, total flavanoids, phenols etc). These compounds exert therapeutic effect and account for medicinal property of the medicinal herb. Most of the herbal extracts are made from crude herbs and can vary in percentage of active constituents, which further influences the therapeutic activity of herbs. Depending upon the source, the variation in the chemical composition of the medicinal plants is enormous, a possible cause of inconsistent therapeutic effects of herbal products. Thus, standardization involves creating clear links between a plant's chemistry to its biology by using physical, phytochemical, analytical and microbiological methods.[9]

Equally important in the evaluation of the crude drugs and the extracts is the phytochemical analysis. Pharmacological efficacy of any of the herbal drug is due to the present of type of phyto-constituents in them.[12,13] Phyto-constituents present in the were identified in the extracts by preliminary qualitative analysis. Presence of tannis, saponins and other phenolic compounds could explain the vaious pharmacological properties of Cassia tora, calendula officianalisand Momordica charantia[14-18] (Table.no3)

Table 3: Phytoconstituents present in different extracts of Calendula officinalis, Cassia tora and Momordicacharantia

Sl. No	Phyto constituents	Calendula officinalis				Cassia tora				Momordicacharantia			
		P.E	Met	Eth	Aq	P.E	Met	Eth	Aq	P.E	Met	Eth	Aq
1	Alkaloids	-	-	-	-	-	-	-	-	+	+	+	+
2	Carbohydrates	-	+	-	+	+	-	+	+	+	+	+	+
3	Glycosides	-	+	+	+	+	+	+	+	-	+	+	-
4	Saponins	-	+	+	+	+	-	+	+	+	-	-	+
5	Triterpenes	+	-	+	+	+	-	+	+	-	+	-	-
6	Fats & Oil	+	-	-	-	+	-	+	-	-	-	-	-
7	Resins	-	-	-	-	-	-	-	-	-	-	-	-
8	Phenols	-	-	-	-	-	-	-	-	-	-	-	-
9	Tannins	-	-	-	-	-	-	-	-	+	+	+	+
10	Flavanoids	-	+	+	+	-	-	-	+	+	-	-	-
11	Proteins	-	-	-	-	-	-	-	-	+	+	+	+
12	Diterpenes	-	+	+	+	-	-	-	-	-	-	-	-

P.E =Pet ether extract, Met = Methanolic extract,
Eth = Ethanolicextract, Aq = Aqueous extract, + = Present , - = Absent

CONCLUSION

Study was successful in establishing quality standards for the flowers of Calendula officinalis, seed of Momordicacharantia and Cassia tora. These preliminary studies may offer great help in initial procurement and assessment of quality of the crude

drugs when these are being used as raw materials for preparations of herbal formulations.

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