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STANDARDIZATION AND CYTOTOXIC POTENTIAL OF A POLY HERBAL FORMULATION- CHATHURMUKA CHOORANAM

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ABSTRACT

Siddha system of medicine is an ancient traditional system intertwined with the mythology and culture of ancient Tamil civilization. The present study investigates the Standardization, Phytochemicals and Cytotoxic potential of a poly herbal siddha formulation Chathurmuka chooranam. Various parameters performed which include organoleptic, physiochemical and flow properties. Its cytotoxicity was determined by MTT assay in Hep G2 cell line. Phytochemicals investigation was also analyzed which revealed the presence of alkaloids, phenols, and flavanoids. The cytotoxicity of the crude extract was found to be very potent against cancer cell line .The results obtained may pave the way for the quality assurance and control organization mostly depends upon the plant drugs as the primary health care needs.

KEYWORDS: Chathurmuka chooranam, Standardization, Phytochemicals and Poly herbal formulation, MTT assay.

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INTRODUCTION

Plants are the superior combinational contribution still providing concealed secrets of their therapeutic properties in relieving several diseases in the human man kind. Natural products have inspired many developments in organic chemistry leading to advances in synthetic methodologies in developing several analogues of lead compound with therapeutic potential [1].Poly herbal formulations are the selection of systematic and regularized approach in the traditional system of medicine and it will pave the way for the development of new drugs from plants source. Various marketed formulation shows dose variation, content variation and lack of standardization which affect its therapeutic activity, therefore it is imperative to establish of quality control parameters for herbal of siddha formulations which will be in alignment with modern technology [2].Standardization is a system to ensure that every packet of medicine that is being sold has the correct amount and will induce its therapeutic effect [3] Standardization and quality control depends upon the nature of crude drug and compound drugs, it's source i.e. factors associated with raw materials which are beyond of human control like seasonal, geographical, age of the plant, time of collection, type of drying etc [4].It is a now accepted that qualitative and quantitative analysis of major bioactive chemical components of a crude drug constituents an important and reliable part of quality control protocol, since any change in quality of the drug directly affects the constituents. Such analysis need to be developed for every aspect of poly herbal extracts and also single herbs [5]. This chooranam currently used for several ailments such as relieving toxicity, haemorrhage, and anticonvulsant properties etc. The formulation containing ingredients such as *Plumbago zeylanica, Curculigo orchioides, Tinospora cordifolia, Semecarpus anacardium* and *Asparagus racemosus*. However, literature revealing standardization and cytotoxicity about this chooranam is totally lacking, hence the present investigation was undertaken. The objective of the present study is to evaluate physio-chemical properties such as ash value, extractive value, flow properties, fluorescence analysis, phytochemicals and its toxicity.

MATERIALS AND METHODS

Preparation of herbal formulation and solvent extraction

Each one gm of a formulation contains equal amount of *Plumbago zeylanica* (root), *Curculigo orchioides*(rhizome), *Tinospora cordifolia*(whole plant) *Semecarpus anacardium*(nut)and *Asparagus racemosus*(rhizome).The plants were authentified in Botanical survey of India,Coimbatore and ABS botanical garden, Salem. 100 g of the dried powder was taken separately and cold macerated with pet ether, chloroform and (1:1) ratio of hydro-ethanolic solvent with occasional stirring for 3 days. After 3 days, the suspensions was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature ($<40^0$ C) under reduced pressure in a rotary evaporator. The yield of crude extract of each solvent extraction was found to be petroleum ether-2.35%, chloroform extract-3.57% and hydro-ethanolic extract -9.64% which were stored in an air-tight desiccator's and used for further analysis.

Organoleptic properties [6]

Organoleptic evaluation refers to evaluation of formulation by color, odor, taste, texture etc. The organoleptic characters of the sample were carried out based on the method described by Siddique *et al.*

Physiochemical properties[7]

Determination of total ash

Total ash determination constitutes detecting the physiological ash (ash derived from Plant (tissue) and non physiological ash (ash from extra generous matter, especially sand and soil adhering to the surface of the drug). For its detection, 2g of powdered material of the formulation was placed in a suitable tared crucible of silica previously ignited and weighed. The powdered drug was spread into an even layer and weighed accurately. The material was incinerated by gradually

increasing the heat, not exceeding 450°C until free from carbon, cooled in a desiccator, weighed and percentage ash was calculated by taking in account the difference of empty weight of crucible & that of crucible with total ash.

Acid insoluble ash

The ash obtained as above was boiled for 5min with 25ml of dilute hydrochloric acid;The insoluble matter was washed with hot water and the percentage of acid-insoluble ash was calculated.

Water soluble ash

The ash was boiled for 5 minutes with 25 ml of water; collected insoluble matter in an ash less filter paper, washed with hot water, and ignited for 15 minutes at a temperature not exceeding 450 ° C. Subtract the weight of the insoluble matter from the weight of the ash; the difference in weight represents the water-soluble ash. The percentage of water-soluble ash with reference to the air-dried drug was calculated

Alcohol soluble extractive value

5g of coarsely powdered air-dried drug was macerated with 100ml of alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowed to stand for eighteen hours. It was then filtered rapidly taking precautions against loss of solvent. 25ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish at 105°C to constant weight and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air dried drug and is represented as% value.

Water Soluble Extractive Value

5g of coarsely powdered air-dried drug was macerated with 100ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowed to stand for eighteen hours. then the contents were filtered and the filtrate was evaporated to dryness.The water soluble extractive percentage was calculated.

Loss on drying

Loss on drying is the loss of mass expressed as percent w/w. About 10g of drug sample of the formulation was accurately weighed in a dried and tared in flat weighing bottle and dried at 105 °C for 5hrs. Percentage was calculated with reference to initial weight

Determination of pH

The pH of the formulation in 1% w/v and 10% w/v of water soluble portions was determined using standard glass electrode at 240 according to the prescribed standard method in Indian Pharmacopoeia.

Fluorescence analysis [8]:

One mg of powdered drug of formulation were exposed to ultraviolet light at wavelength of 366nm and in daylight while wet after being treated with different reagents

Flow properties[8]

Bulk density and Tap density

The term bulk density refers to a measure used to describe a packing of particles or granules. The equation for determining bulk density (D), Db=M/Vb Where M is the mass of the particles and V is the total volume of the packing. The volume of the packing can be determined in an apparatus consisting of a graduated cylinder mounted on a mechanical tapping device. 100gm of weighed formulation powder was taken and carefully added to the cylinder with the aid of a funnel. Typically the initial volume was noted and the sample was then tapped until no further reduction in volume was noted. The initial volume gave the Bulk density value and after tapping the volume reduced, giving the value of tapped density

Angle of repose

The fixed funnel and the free standing cone method employs a funnel that is secured with its tip at a given height, which was taken 2.5 cm (H), above the graph paper that is place on flat horizontal surface. Powder or granulation was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. Tan _ = H/ R or _ = arc tan H/R Where _ is the angle of repose, R being the radius of the conical pile

Hausner ratio

The equation for measuring the Hausner ratio is: Df / Do, where D_f = Tapped density and Do = Bulk density.

Phytochemical investigation [9]

For the preliminary phytochemical analysis Pet ether, Chloroform and Hydroethanolic extract were investigated by the presence or absence of different phytoconstituents such as alkaloids,phenols,terpenoids, steroids, sugar, tannin, glycosides and flavonoids etc. were detected by usual prescribed methods .

Cytotoxicity of the formulation tested against Hep G 2 cell line [10]

Cell culture and medium

Hep G 2(Liver cancer cell line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of Hep G 2 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in an humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).

Cytotoxicity assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 μl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 μl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 μl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \text{ X } 100 \right)$$

RESULTS

1. Organoleptic Properties

The result obtained for the formulation was a coarsely powdered material with fragrant odour, dark brown colour and sweetish taste.

2. Physiochemical Properties

The physiochemical properties of the formulation were tabulated in the Table-1.

Table 1

Physiochemical properties

Parameters	% yield(w/w)
Total ash	11.86
Acid-insoluble ash	3.05
Water soluble ash	2.40
Pet ether soluble extractive value	9.82
Chloroform soluble extractive value	13.42
Alcohol extractive value	20.4
Water soluble extractive value	18.4
P^H	4.5 to 5

3. Flow properties

The physical properties of the formulation were tabulated in the Table-2

Table 2
Physical Properties of the Formulation

Parameters	Formulation
Bulk density	0.485 ±0.24 g/ml
Tap density	0.7206± 0.52g/ml
Angle of repose	47.23 ±0.24
Hausner's ratio	1.485±0.44
Loss on drying	4.42±0.25 w/w

4. The fluorescent analysis

The fluorescent analysis was examined under the UV and Day light were tabulated in the Table: 3

Table 3
Fluorescent Analysis of the Formulation

Reagents	Day-light	UV light
Powder + Conc.Sulphuric acid	Reddish brown	Reddish brown
Powder + Aluminum ferric chloride	Blackish green	Blackish green
Powder +Picric acid	Yellowish	Yellowish blue
Powder + Aqueous mercuric chloride	Light brown	Dark brown

5. Phytochemical investigation

The preliminary phytochemical investigation shows the presence or absence of various phytochemicals in different solvent extraction which are helpful in predicting their therapeutic properties which tabulated in Table: 4.

Table 4
Phytochemical Investigation of the Formulation

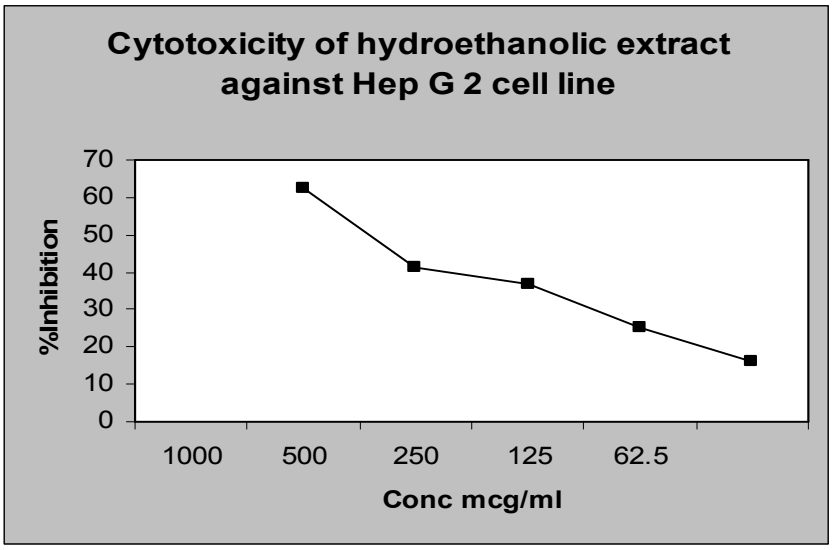
Plant constituents	Pet ether extract	Chloroform extract	Hydro ethanolic extract
Alkaloids	-	-	+
Phenols	-	+	+
Terpenoids	+	+	+
Steroids	+	+	+
Sugar	-	+	+
Tannin	-	-	+
Glycosides	+	-	+
Flavonoids	+	+	+
Fixed oils	+	-	-

+=presence and - = absence

6. Cytotoxicity of the hydro-ethanolic extract against Hep G 2 cell line

The maximum inhibition at 1000 µg/ml was found to be 63% of inhibition against liver cell line. Graphical representation of the hydroethanolic extract concentration against percentage inhibition was showed in the Fig: 1.

Figure 1
Cytotoxicity against Hep G 2 Cell Line



DISCUSSION

In recent years, there has been a great demand for plant derived products in developing countries. These products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics [11]. Due to lack of infrastructures, skilled manpower reliable methods and stringent regulatory laws most of these manufacturers produce their product on very tentative basis [12]. The organoleptic evaluation provides the simplest and quickest means to establish the identity and thereby ensure quality of a particular sample and these features are useful in judging the material in its entirety and in powder form [13]. In this study, it was revealed that the crude drug used for preparation of formulation lie within the significant limit with good quality. The amount of minerals and earthy materials in the plant material was indicated by total ash content. The water-soluble extractive value indicated the presence of sugar, acids and inorganic compounds. Less or more extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying, or storage or formulating [14,15]. Loss on drying at 105°C was determined since the presence of excess moisture is conclusive to the promotion of mould and bacterial growth, and subsequently to deterioration and spoilage of the drug [16].If the rate is too slow the drying rate also changes, here the rate of formulation dried was proper so the drying limit was also proper. The P^H of 1% w/v and 10%w/v solution revealed that the formulation was suitable for human use.

Flow properties of the granules can be judged from the angle of repose and Hausner's ratio. Angle of repose has been used as an indirect method of quantifying powder flowability because of its relationship with interparticle cohesion. The angle of repose <30° indicates free flowing material and >40° with poor flow properties. Hausner's ratio was related to interparticulate friction and could be used to predict powder flow properties. It showed that powder with low interparticulate friction, such as coarse sphere, had ratio of approximately 1.2 whereas less free flowing powder such as flakes have Hausner's ratio greater than 1.6 [17, 18]. A value for angle of repose was found to be in the range of 47.23 to 49.85 with poor flow properties but the Hausner's ratio of the present formulation has moderate interparticulate friction Bulk characterization is necessary to avoid misleading predictions of stability or solubility which depends on a particulate flow ability of granules or powder. Bulk density and tapped density is useful for

determination of packing of powder materials [19]. The fluorescent analysis of powder indicates the nature of phyto constiutents present or adulterant. In this study, there was no such fluorescent material found in the formulation. The preliminary phytochemical analysis indicates the nature of phytoconstiutents. Hydro-ethanolic extract showed maximum presence of phytoconstiuents than the pet ether and chloroform extract. So, the hydro-ethanolic extract was tested against the liver cancer cell line. The cytotoxic activity of crude drug was found to significantly inhibit the cancer cell line. A substance is cytotoxic if it inhibits vital metabolic processes or it causes disorder in living organisms resulting in perversion of behavior or death [21].The cytotoxicity may be mainly due to the active ingredients and secondary metabolites in the hydroethanolic extract of the formulation.

CONCLUSION

The present investigation of chathurmuka churnam was evaluated by various standardization parameters such as physicochemical standards, organoleptic parameters and safety evaluation showed that the contents of formulation presents within the permissible limits as per WHO. The therapeutic potential of the formulation may be due to the presence of phytochemicals present in the hydro-ethanolic extract. Further studies is too carried out by apoptotic process and also by in vivo methods.

CONFLICT OF INTEREST

Conflict of interest declared none.

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