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Development and standardization of Polyherbal formulation for the management of breast cancer

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Abstract
The incidence of breast cancer and related mortality is increasing annually. It is the leading cause of cancer death amongst women worldwide. The current chemotherapeutic agents render toxic effects to the body. Hence, there is a need to find the alternative cure for the management of breast cancer with minimal side effects. The popularity of herbal medicine is rapidly increasing in today's time. The present study deals with development of the polyherbal formulation comprising of the hydro-alcoholic (30:70) extracts of *Curculigo orchioides*, *Curcuma longa, Emblica officinalis*, *Teriminalia bellerica, Teriminalia chebula* and *Withania somnifera*. For the standardization of the raw materials physicochemical studies like ash values, extractive values, phytochemical studies were performed. The Preformulation parameters and parameters for finished product (hard gelatin capsule) include uniformity of weight, disintegration time, moisture content, pH, phytochemical estimation were performed. The cytotoxic activity of the finished product was performed using MTT assay on MCF7 breast carcinoma cell line. The prepared finished product showed significant cytotoxic activity against MCF7 breast carcinoma cell line.

Keywords: breast cancer, polyherbal formulation, mcf7 breast carcinoma cell line, hard gelatin capsule

1. Introduction
Plants are very useful to mankind. Many of them are used exclusively for medicinal purposes. According to the World Health Organization (WHO), "a medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." Such plants are in great demand by pharmaceutical companies for their active ingredients [1, 2].

Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 65-75% of the World's population rely only on medicinal plants as their primary source of medicines. India is one of the few countries in the World which has unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for cure of various diseases [3, 4].

Herbal medicine is the oldest form of health care known to mankind being an integral part in the development of the modern day civilization. In herbal medicine plant based formulation are used to alleviate diseases. But the most important challenges faced by these formulations arise because of their lack of complete evaluation. So, evaluation is necessary to ensure the quality and purity of the herbal product. It is very important to establish a system of evaluation for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous.

Breast cancer causes significant morbidity and mortality amongst women, and metastasis mainly affects outcome of the disease [5]. Lack of effective therapeutic strategies for control and treatment of breast cancers, and the huge financial burden placed on individuals and nations mean urgent action must be taken in the fight against breast cancer. Also, the side effects due to conventional chemotherapy have necessitated the search for newer therapies mostly in the form of natural products. In the recent years, interest in the natural products has grown, and in the light of long-term and safe cancer prevention, current approaches have been focused on the use of food and ethnomedicinal herbs as sources of products that could effectively control cancer [6-8].

In traditional systems of medicine, many plants have been documented to be useful for the treatment of various systemic disorders. Many of the traditional/indigenous systems of medicine are effective but they suffer from lack of complete standardization which is one of the important challenges posed by the traditional systems of medicine. The concept of polyhedral formulation is well documented in the ancient literature. Compared to the single herb, the polyherbal formulation has better and extended therapeutic potential. Hence, the present study was planned to formulate and standardize a polyherbal formulation using plants having known anti-cancer potential.

In poly-herbal preparations it will be very difficult if we want to estimate each and every ingredient in term of their chemical constituent. But if few major constituents having particular therapeutic action indicated in the labelled can be pinpointed then these constituents should be estimated quantitatively along with the other parameters through which presence of all ingredients can be confirmed [9].

2. Materials and methods
2.1 Selection of plant material
All the six plants were selected on the basis of their anti-cancer activity which was previously studied, using MTT assay on MCF 7 Breast cancer cell line.

Plant material used for poly-herbal formulation: following six plants were used for the preparation of polyherbal formulation.

Table 1: Herbal Drugs Used in Poly-herbal Formulation

Name of the plant	Family	Common Name	Parts Used	Uses
Curculigo orchioides	Hypoxidaceae	Kali musli	Rhizomes	Diuretic, anti-cancer, aphrodisiac [10]
Curcuma longa	*Zingiberaceae*	Turmeric	Rhizomes	Anti-inflammatory, antioxidant, anti-cancer [11]
Emblica officinalis	*Euphorbiaceae*	Amla	Fruits	Antioxidant, anti-cancer, anti-inflammatory [12]
Teriminalia bellerica	*Combretaceae*	Baheda	Fruits	Blood purifier, anti-cancer, throat diseases [13]
Teriminalia chebula	*Combretaceae*	Harde	Fruits	Fever, cough, astringent, anti-cancer [14]
Withania somnifera	*Solanaceae*	Ashwagandha	Roots	Anti-inflammatory, anti-tumour, anti-stress, anti-oxidant [15]

2.2 Formula for Poly-herbal formulation

The poly-herbal formulation (capsules) contained the hydro-alcoholic extracts of *Curculigo orchioides* (Rhizomes), *Curcuma longa* (Rhizomes), *Emblica officinalis* (Fruits), *Teriminalia bellerica* (Fruits), *Teriminalia chebula* (Fruits) and *Withania somnifera* (Roots) in the ratio of 1:1:1:1:1:1.

2.3 Preformulation studies

Preformulation parameters such as bulk density, tap density, Compressibility index, Hausner's ratio, and angle of repose were determined for the prepared polyherbal granules and the best trial batch were taken for capsule filling and further studies [16, 17].

Preformulation parameters

2.3.1 Bulk density, tap density and Carr's index [18, 19]

A weighed quantity (15g) of powdered material was taken in a 50ml measuring cylinder and recorded the initial volume (vo). tapped the contents and recorded the powdered volumes after 50 taps(v50).

Fluff density = w/vo g/cc

Tapped density = w/v50 g/cc

Carr's index = Tapped density- Fluff density/ Tapped density * 100 Value for Carr's index below 15 indicate excellent flowing material and value over 20-30 suggested poor flowing material.

2.3.2 Angle of repose [20]

A funnel was fixed at a particular height (1.5, 2.5, 3.5 cm) on a burette stand. A white paper was placed below the funnel on the table. The powdered drug passed slowly through the funnel until it forms a pile. The radius of the pile wasnoted down.

Angle of repose of the powder material was calculated by using the formula:

$\tan\theta = h/r$

$\theta = \tan (h/r)$

Where, h = height of the pile, r = radius.

Values for angle of repose 30^o usually indicate a free flowing material and angle 40^o suggest a poor flowing material.

2.3.3 Hausner's ratio [20]

The basic procedure is to measure the unsettled apparent volume, V0 and the final tap volumeVf of the powder tapping the material until no further volume changes occur. The Hausner's ratio was calculated as follows:

Hausner'sratio = V0 / Vf

Hausner's ratio between 1.00 to 1.11 shows excellent flow and value more than 1.60 shows very, very poor flow.

2.4 Preparation of polyherbal formulation by wet granulation method

The formulation preparation began with trials by adding a different ratio of binders and selecting the quantity of lubricants and preservatives, and finally the procedure was optimized. *Curculigo orchioides* (Rhizomes), *Curcuma longa* (Rhizomes), *Emblica officinalis* (Fruits), *Teriminalia bellerica* (Fruits), *Teriminalia chebula* (Fruits) and *Withania somnifera* (Roots) extracts were powdered (sieve 40), and mixed in the ratio of 1:1:1:1:1:1 and taken for the preparation of capsules by wet granulation technique using 5% starch paste as a binder. The wet mass was passed through sieve number 22 to obtain granules. The granules were dried at 45°C in a tray [21].

2.5 Standardization of polyherbal formulation (hard gelatin capsule)

2.5.1 Capsule evaluation

The poly-herbal capsules were evaluated for their description, average weight, weight variation, moisture content, disintegration time, pH and microbial load and compared with Indian pharmacopoeial standards [22].

- **Average weight:** Twenty capsules were individually weighed and the average weight of the capsule was calculated.
- **Weight variation:** The individual weights of the each capsule should be within the limits of 90% and 110% of the average weight.
- **Moisture content:** Moisture content was determined by using automatic Karl Fischer titration apparatus.
- **Disintegration time:** Disintegration test was performed using the digital microprocessor based disintegration test apparatus. One capsule was introduced into each tube and a disc was added to each tube. The assembly was suspended in water in a 1000 ml beaker. The volume of water at its highest point was at least 25 mm below the surface of the water and at its lowest point was at least 25 mm above the bottom of the beaker. The apparatus was operated and maintained at a temperature of 37 ± 2ºC.
- **pH value:** pH of 1% solution was determined by using a digital pH meter.

2.5.2 Dissolution

Dissolution is considered as a tool for predicting rate of absorption and bioavailability in some cases, replacing clinical studies to determine bioequivalence of drug. We were added six capsules in the basket type dissolution apparatus containing distilled water as a dissolution media. The speed was set on 50 rpm for 1 hour and the sample was drawn at every 10 minutes and the amount of dissolved active ingredient in the solution was calculated as percentage dissolved in 1 hour.

2.5.3 Stability

Pharmaceutical products are generally studied for stability profile at accelerated temperature, humidity and also at different intensities of light. The studies were performed to determine the physical, chemical, and therapeutic changes occurring in the poly-herbal capsule by extrinsic factors [23, 24].

a) **Light**: Sample was stored in different intensities of light i.e. sunrays, fluorescent (tube) light, UV and infrared light for detection of degradation of powder material.
b) **Temperature:** The effect of temperature on the stability of polyherbal capsule was checked by keeping all the capsule at different temperatures i.e. ambient, 35^0C, 50^0C, 55^0C, 65^0C for 30 minutes, 1, 3, and 6 hours.
c) **Humidity**: The effect of humidity on the stability of capsule was checked by keeping the entire capsule at four different humidity percentage i.e. 30%, 50%, 70% and 90%.

Composition of capsule
Each 500 mg capsule contains:
Curculigo orchioides 125mg
Curcuma longa 125mg
Emblica officinalis 125mg
Teriminalia bellerica 125mg
Teriminalia chebula 125mg
Withania somnifera 125mg
Excipients q.s.

2.6 *In-Vitro* Anti-cancer activity of prepared polyherbal formulation

Cytotoxicity assessment: MTT assay [25]

The prepared extracts were tested for its cytotoxicity by MTT-assay. MCF7 cells were seeded in their respective culture medium (200 µl, 1 x 10^4cells/well) in a 96-well plate and incubated at 37 ˚C for 24 h with 5% CO_2 supply. After incubation, the control wells were replenished with fresh medium and the test wells were treated with 100, 250, 500 and 1000 µg/ml of the prepared granules. The cells were further incubated for 48 h maintaining the same conditions. After the treatment incubation period, medium in each well was replenished with 200µl of fresh medium plus 20µl of MTT (0.5 mg/ml). The plate was then incubated for 4 h in the same conditions after which the absorbance was measured at 570 nm using ELISA reader. Percentage cytotoxicity was calculated by the following formula:

$$\% \text{ Cytotoxicity} = [(Ac-At)/Ac)] \text{ X } 100$$

Where,
Ac = mean absorbance of the control wells
At = mean absorbance of the test wells

3. Results

The most important part of any formulation is standardization which ensures the quality, safety and reproducibility. It involves the complete process of bioprospection right from the collection of raw materials to development of finished product. In the present study, standardized polyherbal mixture was formulated in hard gelatin capsule.
Polyherbal formulation composed of six ingredients, belonging to different families, different morphological plant parts and different phyto-constituents.

3.1 Preformulation studies

Preformulation parameters like bulk density, tap density, Carr's index, Hausner's ratio and angle of repose were obtained for the laboratory granules. The granules showed excellent flow property.

Table 2: Preformulation parameters

Sr. No.	Parameters	Results
1	Bulk density	0.8
2	Tap density	0.6
3	Carr's index	19.3
4	Hausner's ratio	1.21
5	Angle of repose	14.16

As per the standards, the flow property of the blend to be filled in the capsule should be in good range and was confirmed by the above parameters. Trail batch IV showed excellent flow characters and batch IV was taken for capsule filling.
The trial IV flow properties were Excellent and all parameters were within the Specified limits. So, fourth trial was chosen for further studies.

Table 3: Evaluation of inprocess Parameters:

Parameter	Trial I	Trial II	Trial III	Trial IV
Flow property	Poor flow	Poor flow	Fair	Good
Uniformity of Filling	-	-	Uniform	Uniform
Uniformity of Weight	-	-	Less weight	Uniform

3.2 Standardization of formulation

3.2.1 Capsule evaluation

Description "light brown" coloured granules packed in "0" size blue capsules. The polyherbal capsules were evaluated for organoleptic characters which include colour, odour, taste and nature.

Table 4: Organoleptic Characters of Capsules

Parameters	Observation
Description	Light brown granule in blue cap and body "0" size capsule
Colour	Light brown granule
Odour	Characteristic odour
Taste	Bitter taste

Table 5: Evaluation of capsules

Parameter	Observation
Average weight	Within limits
Weight variation	Within limits
Moister content(LOD)	3.96%
Disintegration time	9 mins 23secs
pH(1% aqueous solution	6.12± 0.48

Result (n=3) are reported as Mean ± Standard deviation

Table 6: *In Vitro* Dissolution Studies

Time (min)	Abs	Conc. (µg/ml)	Amt (mg/5ml)	Amt (mg/ml)	Amt (mg/900ml)	CDR	%CDR
0	0.057	9.62963	0.04815	0.00963	8.66667	8.6666	3.46664
5	0.361	124.815	0.62407	0.12482	112.333	112.382	44.9526
10	0.446	174.815	0.87407	0.17482	157.333	157.957	63.183
15	0.583	205.185	1.02593	0.20519	184.667	185.541	74.2163
20	0.671	232.222	1.16111	0.23222	209	210.026	84.0104
25	0.737	256.667	1.28333	0.25667	231	232.161	92.8644
30	0.769	272.222	1.36111	0.27222	245	246.283	98.5133

3.2.2 Stability

The stability parameters were analyzed for 30 minutes, 1, 3 and 6 hours of storage at accelerated conditions of temperature, light and humidity were found to be comparable. It was indicating that there gross physical characteristics does not produce any significant change, observation have been tabulated in table 4, 5 and 6 for three Stability parameters

Table 7: Effect of different intensities of lights on polyherbal capsules (500 mg)

Light Source	Sun light				Fluorescence				Tube light				UV Light				Infra-Red (IR)				Lamp Light			
Time of Exposure (hours)	1/2	1	3	6	1/2	1	3	6	½	1	3	6	1/2	1	3	6	1/2	1	3	6	1/2	1	3	6
500mg polyherbal capsule	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

(-) No change, (+) Degradation

Table 8: Stability test of polyherbal Capsule (500mg) at different Temperature

Storage condition	Testing condition	Time Duration (hours)				Result
		1/2	1	3	6	
Ambient	30ºC	-	-	-	-	No change during 6 hours
Warm (30-40 °C)	35ºC	-	-	-	-	No change during 6 hours
Accelerated	50ºC	-	-	-	-	No change during 6 hours
Accelerated	55ºC	-	-	-	+	Degradation start after 4 hours
Accelerated	65ºC	-	-	+	+	Degradation start after 2 hours

(-) No change, (+) Degradation starts

Table 9: Stability of monoherbal Capsule (250 mg) at different Humidity with respect to different Temperature

Temperature	30% Humidity	50% Humidity	70% Humidity	90% Humidity
30%	-	-	-	-
35%	-	-	-	-
55%	-	-	+	++
65%	-	-	++	+++

(+) Degradation (-) No Change

3.3 *In-Vitro* Anti-cancer activity of prepared polyherbal formulation

Polyherbal mixture shows significant cytotoxicity on the MCF7 cell line. The percentage cytotoxicity on MCF7 cell line at different concentration is as shown in Table 10.

Table 10: Zone of inhibition of polyherbal mixture

Sr. No.	Test substance	Concentration			
		100 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml
1	Polyherbal mixture	40.23%	60.57%	76.98%	89.65%
2	Vinblastine (10µg/ml)	91.39%			

4. Discussion

Various types of herbal medicines have been used as curative agents in different parts of the world [26]. Drugs derived from traditional herbs may have possible therapeutic relevance in the treatment of illness [27].

In the present research work *Curculigo orchioides* (Rhizomes), *Curcuma longa* (Rhizomes), *Emblica officinalis* (Fruits), *Teriminalia bellerica* (Fruits), *Teriminalia chebula* (Fruits) and *Withania somnifera* (Roots) were used for the polyherbal 500 mg capsule. First it was formulated and then evaluated for quality herbal product which is very important irrespective of their medicinal content and therapeutic states therefore the pre-formulation and formulation studies of the formulated polyherbal capsule were evaluated.

Preformulation parameters including angle of repose (a traditional characterization method for pharmaceutical powder flow), porosity (packing geometry), Carr's index and Hausner's ratio (a measure of the interparticulate friction) are useful tools in the development of new formulation. A value of <30° indicates 'excellent' flow whereas >56° indicates 'very poor' flow. Based on this, the flow was rated as 'excellent' (Table-2). The CI and HR were found to be 18.4 and 1.19. Lower CI or lower Hausner ratios of a material indicates better flow properties than higher ones. A Carr's index of <10 or HR of

<1.11 is considered 'excellent' flow whereas CI>38 or HR>1.60 is considered 'very very poor' flow [26, 27]. Based on the results obtained (Table-2) flow of selected plant powder was rated as 'good'. Good flow of powder help to avoid the extensive costs and time involved in unloading powders that will not flow out of storage containers. As well as help to achieve the best formulation and improve the quality and consistency of the product.

All the six drugs were approved as quality drug when undergone by phytopharmaceutical evaluation according to the pharmacopoeial standards. 500 mg polyherbal capsules disintegrated in meantime 8.14±15 minutes and *in vitro* condition and we determined the release of a drug from solid dosage format which the substance dissolved in the fluid of gastrointestinal tract. Results indicates that all of six capsules dissolved equal to 90% in 30 minutes and this releasing pattern of drug from their capsule shell *in-vitro* help in predicting the releasing sequence *in-vivo* that developing a tool for bioavailability of drug, as well as in some cases, replacing clinical studies to determine bioequivalence. In light of the phyto pharmaceutical studies of the polyherbal capsule was found almost stable.

Polyherbal mixtures of selected plants were screened for their anti-cancer activity. Poly-herbal mixture of plant shows significant cytotoxicity and thus anti-cancer activity on MCF7 cell line. Further studies using more specific methods are required to explore the constituents responsible for the activity and the mechanism of this activity which might prove important and improved therapies for the treatment and management of breast cancer.

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