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Development and Assessment of Antioxidant, Anticancer Activity of Poly-Herbal Combination

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Research Article

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ABSTRACT

The occurrence of lung cancer and related death is increasing yearly. It is the leading cause of cancer death amongst women worldwide. Hence, there is a need to find the alternative cure for the treatment and management of lung cancer with minimal side effects. The present research work objective was development of the poly-herbal capsule formulation comprising of the hydro-alcoholic (20:80) extracts using *Ocimum sanctum, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula, Piper longum, Piper nigrum* and *Zingiber officinale* for the management of lung cancer disease. This poly-herbal capsule formulation is having potential antioxidant and anticancer bioactive molecule. The poly-herbal capsule formulation was formulated and standardized as per WHO guidelines of quality standardization. The optimized poly-herbal capsule formulation was evaluated by using standard evaluation parameters of capsule formulation. The antioxidant and anticancer activity was determined by using DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) and Ferric Ion Reducing Power Assay (FRAC) and (MTT) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide on A549 lung cancer cell line. The evaluation results of pharmaceutical parameters i.e. weight variation; and moisture analysis was found to be within official limit. The dissolution studies represented that drug release of maximum up to 98.67% at 30 minutes. The DPPH assay and FRAC assay activity of the poly-herbal extract is found to potent as similar standard drug. The optimized finished poly-herbal formulation showed significant cytotoxic activity against A549 lung cancer cell line. The results of all emulation parameters revealed that it could be used for the treatment and management of lung cancer disease.

INTRODUCTION

Herbal plants are most important for mankind for treatment and management of diseases. According to the World Health Organization (WHO), "natural plants are a plant having more bioactive molecules that have been reported their therapeutic benefits, or which are mother sources of chemo-pharmaceutical semi-synthesis." Such medicinal plants are in highly demand by the pharmaceutical companies for their bioactive ingredients.

Medicinal plants have been reported worldwide in traditional medicines for the treatment of various chronic diseases. According to various reports estimated that even modern time approximately 65-75% of the World's populations depend on medicinal plants for treatment and management of diseases [1].

The various therapeutic systems that herbal plants and their parts are used for the treatment of diseases like homeopathic, Chinese, Unani and Ayurvedic systems. In herbal medicine plant based formulation are very economic and less side effects that used to alleviate diseases. The quality of herbal products assurance is main issue for maintain the standard at worldwide. Therefore need to standardization and evaluation. It is necessary to ensure the quality and purity of the herbal product. These standardized herbal products increase quality and enhance the more acceptability and export quality of plant medicine in world market.

The most common cancer has been reported in are lung cancer disease. This lung cancer creates significant morbidity and mortality amongst humans, and metastasis mainly affects outcome of the disease. Lack of effective therapeutic drugs for control and treatment of lung cancers, and the huge financial burden placed on individuals and nations mean urgent action must be taken in the fight and treatment and management of lung cancer disease. The side effects produce by conventional chemotherapy for long time treatment. These side effects can be removed by using herbal bioactive medicines at economic level. In the recent years, peoples interest highly increases towards natural products for safe cancer prevention, current approaches have been focused on the use of food and ethno medicinal plants as sources of natural products that could used for effectively control cancer diseases [2,3].

In poly-herbal formulation is an excellent concept and suitable and right for the treatment and management of chronic diseases such as lung cancer. Many researchers have been reported and found good results but need to more research work for development of potential natural products. Our objective of present research work for the development of poly-herbal capsule formulation for the treatment and management of lung cancer disease where used potential sources of herbal drugs [4].

MATERIALS AND METHODS

Selected plants materials were collected from local market like as *Ocimum sanctum, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula, Piper longum, Piper nigrum* and *Zingiber officinale*. The plants materials are verified by the Central Ayurvedic Research Institute, Jhansi, Uttar Pradesh with accession number CARI/H/13212021, CARI/H/13222021, CARI/H/13232021, CARI/H/13242021, CARI/H/13252021, CARI/H/13262021, CARI/H/13272021, CARI/H/13282021, CARI/H/132292021, by Pharmacognosist, Dr. Sandeep Kumar Singh. Plant materials were used for poly-herbal formulation: Following eight plants were used for the preparation of poly-herbal formulation. All the required chemicals and reagents are analytical grade were used. The cell lines were obtained from the National Center for Cell Sciences pune (Table 1).

Table 1. Herbal drugs used in poly-herbal capsule formulation.

Name of the plant	Common name	Parts used	Uses
Ocimum sanctum	Tulsi	Leaves	anti-cancer, aphrodisiac etc.
Curcuma longa	Turmeric	Rhizomes	antioxidant, anti-cancer etc.
Emblica officinalis	Amla	Fruits	Antioxidant, anti-cancer etc.
Teriminalia bellerica	Baheda	Fruits	anti-cancer, throat diseases etc.
Teriminalia chebula	Harde	Fruits	cough, astringent, anti-cancer etc.
Piper longum	Long pepper	Fruit	Anti-inflammatory, anti-tumour etc.
Piper nigrum	Kali mirch	Fruit	antioxidant, antitumor, analgesic etc.
Zingiber officinale	Ginger powder	Rhizome	antioxidant, anticancer etc.

Preparation of ethanolic extract of polyherbal formulation

Preparation of ethanolic extract of *Ocimum sanctum, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula, Piper longum, Piper nigrum* and *Zingiber officinale* were powdered and extracted with 80% absolute ethanol using Soxhlet apparatus for 6 h. The extracts were evaporated to dryness under reduced pressure using rotator evaporator at 60°C and stored at 4°C until use [5].

Phytochemical analysis

One gram of each ethanolic plants extracts of *Ocimum sanctum, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula, Piper longum, Piper nigrum* and *Zingiber officinale* were dissolved in 100 ml of its own mother solvent to obtain a stock of concentration 1% w/v and were used for the phytochemical analysis of carbohydrates, proteins, sterols, alkaloids, tan-nins, glycosides, flavonoids, phenolic compounds, and saponins [6].

Total tannin content

The Folin-ciocalteu method was used to determine the total tannin content. Tannic acid solutions (20 to 100 µg/mL) were prepared as a standard set. Using a UV-visible spectrophotometer, the absorbance of standard and test solutions was

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measured with a blank at 725 nm wavelength. The total tannin content of the polyherbo-ceutical formulation was calculated as mg of tannic acid equivalent (TAE)/g.

Total bitter content

A gravimetric process was followed to determine the total bitter content.

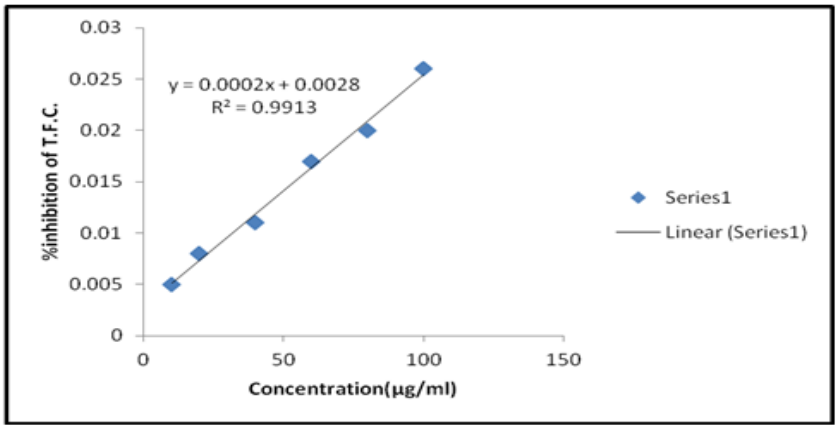
Total alkaloid content

A gravimetric extraction process followed to determine the total alkaloid content.

Total flavonoid content or quercetin equivalent

The total flavonoid content was determined by using *in-vitro* an aluminum chloride complex-forming assay. Poly-herbal formulation and standard quercetin were dissolved in ethanol solvent and processed to calculate as quercetin equivalents (%QE). Aluminum chloride of 10% solution was added to each prepared dilution and allowed to stand for 5 minutes [7,8]. Then after 1M sodium hydroxide solution was added consecutively. A UV-visible spectrophotometer was used to calculate the absorbance of solution at 510 nm. The total flavonoid content in poly-herbal formulations was estimated using a standard quercetin curve and regression equation. Standard curve of rutin is shown in Figure 1.

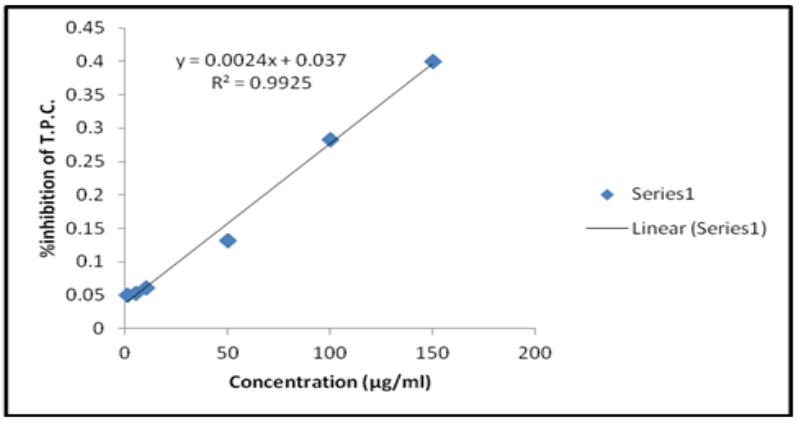
Figure 1. Standard curve of rutin for total flavonoid content.



Total phenolic content or gallic acid equivalent

The total phenolic content of poly-herbal formulation was determined by using *in-vitro* folin-ciocalteu method. The absorbance was measured at 765 nm using UV-visible spectrophotometer [9,10]. The standard gallic acid curve and regression equation were used to calculate total poly-phenol content in poly-herbal formulation. Standard curve of galic acid is shown in Figure 2.

Figure 2. Standard curve for gallic acid.



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Microbial contamination

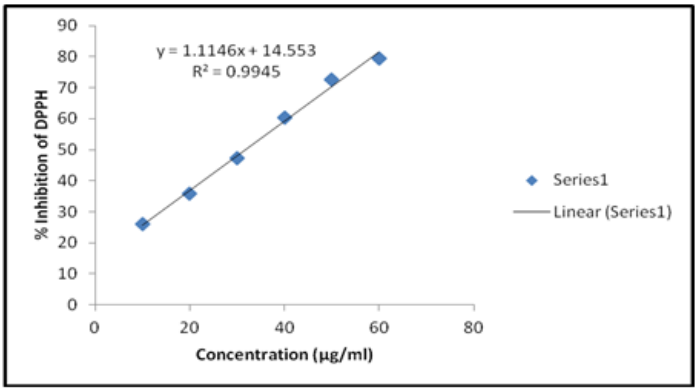
Total bacterial count, fungal count, and pathogen analysis were performed to ensure the viable count of microorganisms and limits followed as prescribed in Ayurvedic Pharmacopoeia of India [11-13].

In-vitro anti-oxidant activity (DPPH radical scavenging activity)

In-vitro anti-oxidant activity was determined by using free radical scavenging activity (DPPH radical scavenging activity) of poly-herbal formulation. It was performed as per the method of "Blois" with a little changes. In brief, a 0.2 mM DPPH radical solution in etthanol was prepared, and then 3 mL of this solution was mixed with 3 mL of the sample solution in different concentrations (1-15 mg/mL) of the formulation. This mixture was kept in the dark for 30 minutes; the absorbance was measured at 517 nm. The DPPH solution absorbance is inversely proportional to the DPPH radical scavenging activity. The DPPH solution without sample solution was used as a control. Gallic acid was used as a standard in the concentration of 0.005–0.5 mg/Ml. Inhibition concentration at 50% (IC50) was calculated by plotting a calibration curve with concentration and percentage inhibition at each point. Percentage inhibition was calculated for standard and polyherboceutical formulation by using the given formula (Figure 3).

% inhibition=[(Absorbance of blank–Absorbance of formulation) × 100]/Absorbance of blank.

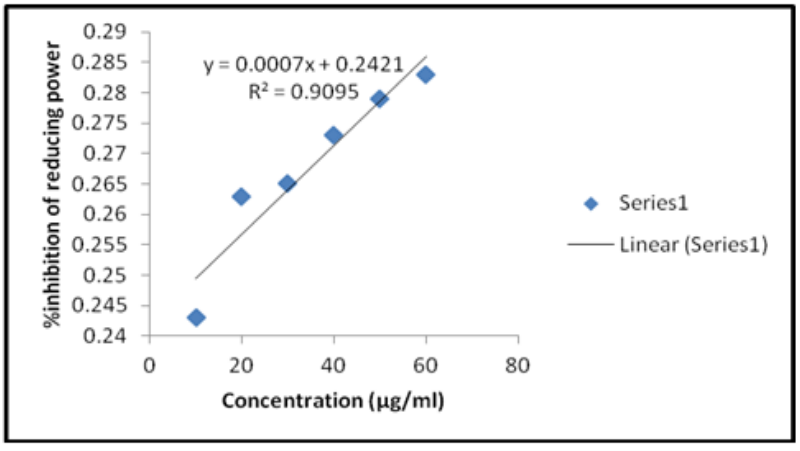
Figure 3. Graphical representation of DPPH radical scavenging activity of polyherbal extract.



In vitro anti-oxidant activity (Reducing power assay)

The reducing power of poly-herbal formulation was determined by the method of "Oyaizu" with some modifications. The reduction of ferric to ferrous ion is directly proportional to the absorbance indicating the sample (3-20 mg/mL) in double-distilled water was mixed 2.5 mL of phosphate buffer incubated at 50°C for 20 minutes after which 1.5 mL of trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes from all the tubes, 0.5 mL of ferric chloride was added [11-13]. The absorbance was measured at 700 nm on a spectrophotometer. Gallic acid was used as a standard for comparison at a concentration of 0.12-0.36 mg/mL. The water was used as a blank instead of additives. Comparative evaluation of concentration value calculated at 0.5 of absorbance for standard and poly-herbal formulation (Figure 4).

Figure 4: Graphical representation of determination of reducing power of the polyherbal extract.



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Preparation of polyherbal formulation

The polyherbal formulation (capsules) contained the ethanolic extracts of herbal plants material were taken in the ratio of as Group A: *Ocimum sanctum, Curcuma longa, Emblica officinalis*; Group B: *Teriminalia bellerica, Teriminalia chebula, Piper longum* and Group C: *Piper nigrum and Zingiber officinale)* for their similar phytoconstituents pattern, and further we have choosen the proper ration of drug as Group A (2): Group B (2): Group C (1), i.e. (2:2:1). The quality control tests of the poly-herbal formulation were performed as per the WHO guidelines. The quality control tests are ash content, extractable matter, foaming index, loss on drying, tannin content, foreign matters, and specific powder characteristic tests such as angle of repose and bulk density were undertaken and significant results were recorded [14].

Preparation of formulation by wet granulation method

The poly-herbal formulation preparation start with hit and trials by adding a different ratio of binders and selecting the quantity of lubricants and preservatives, and finally the procedure and was optimized. *Ocimum sanctum, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula, Piper longum, Piper nigrum and Zingiber officinale* extracts were finely powdered (sieve 40), and mixed in the ratio of 2:2:1 and taken for the preparation of capsules by wet granulation technique using 20% lactose solution as a binder. The wet mass was passed through sieve 20 to obtain granules. The granules were dried at 45°C in a tray dryer. The granules were lubricated with 1% magnesium stearate. Diluents and preservatives were added. The optimized formulation was showed very good flow properties. After this, the granules from the optimized batch (20% lactose) were filled in capsules colored yellow of size "0" in a capsule filling machine. The capsules were then deducted and transferred into poly bags, labeled, and the samples were evaluated as per the testing requirements. Each 500 mg of herbal capsule contained the extracts of *Ocimum sanctum* (50mg), *Curcuma longa* (50 mg), *Emblica officinalis* (50 mg), *Teriminalia bellerica* (50 mg), *Teriminalia chebula* (50mg), *Piper longum* (50mg), *Piper nigrum* (25 mg),and *Zingiber officinale* (25 mg), and lactose and excipients quantity sufficient (q.s.).

Capsule filling and packing

The capsules were filled in a manual capsule filling machine (300 capsules in a single operation) under a controlled environment (25 ± 5°C and less than 60% relative humidity). The filled capsules were de-dusted, sealed, and stored in bottles and containing silica gel packets for moisture-free storage.

Preformulation studies

Preformulation parameters were used for the evaluation such as bulk density, tap density, Compressibility index, Hausner's ratio, and angle of repose were determined for the prepared poly-herbal granules and the best trial batch were taken for capsule filling and further studies [15-17].

Bulk density, tap density and carr's index

A weighed quantity (15g) of poly herbal powdered materials were taken in a 50 ml measuring cylinder and recorded the initial volume (vo). Tapped volume the contents and recorded the powdered volumes after 50 taps (v50). This process was repeated in three times and average were calculated and noted.

Fluff density (B.D.) = weight of powder/volume of powder g/ml.

Tapped density (T.D.) = weight of powder/volume occupied by powder g/ml g/ml

Carr's index (C.I.) = Tapped density- Fluff density/ Tapped density x 100

Value for Carr's index below 15 indicate excellent flowing material and value over 20-30 suggested poor flowing material.

Angle of repose

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 was noted 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 30o usually indicate a free flowing material and angle 40o suggest a poor flowing mate

Hausner's ratio

The basic procedure is to measure the unsettled apparent volume, V_0 and the final tap volume V_f of the powder tapping the material until no further volume changes occur. The Hausner's ratio was calculated as follows:

Hausner'sratio=T.D. / B.D.

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

E-ISSN: 2347-226X
P-ISSN: 2319-9857

Standardization of formulation

Physicochemical parameters of raw materials were determined as per the guidelines of the WHO, which includes moisture content, total ash value, water soluble ash, acid insoluble ash, heavy metals, water soluble extractive, alcohol soluble extractive, and acidity (pH).

pH value

pH of 1% solution was determined by using a digital pH meter.

Ash value and extractive value

Total ash, water soluble ash, acid soluble ash and extractive values were determined as the procedure described elsewhere.

Limit test for heavy metals

Qualitative estimation of heavy metals was done for the detection of arsenic and lead as per the Ayurvedic pharmacopoeial procedures.

Capsule evaluation

The poly-herbal capsules were evaluated for their description, microbial load, uniformity of dosage units, weight variation, disintegration time, and moisture content, and compared with Indian pharmacopoeial standards.

Microbial load analysis

For the safe use of the poly-herbal capsules, microbial count was done and it was checked whether the total aerobic viable count, yeasts and molds were within the prescribed limits and the microorganisms, *Escherichia coli, Clos-tridia, Salmonellae, Shigella, Pseudomonas, and Staphylococcus*, were absent in the final formulation.

Weight variation

Twenty capsules were individually weighed and the average weight of the capsule was calculated.
Weight variation=Average weight- Individual weight/ Average weight x 100

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 ± 2°C.

Dissolution

Dissolution of preparation of poly-herbal formulation was determined. The dissolution is key a tool for predicting rate of absorption and bioavailability in some cases, replacing clinical studies to determine bioequivalence of drug. We were used 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.

Stability

Developed poly-herbal capsule formulation was studied stability profile at accelerated temperature, humidity and also at different intensities of light. The stability studies were performed and results determine by using following parameters such as physical, chemical, and therapeutic changes occurring in the poly-herbal capsule by extrinsic factors.

a) Light: Developed poly-herbal capsule formulation 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 developed poly-herbal capsule formulation and was checked by keeping all the capsule at different temperatures i.e. ambient, 35°C, 50°C, 55°C, 65°C for 30 minutes, 1,3,and 6 hours.

c) Humidity: The effect of humidity on the stability of capsule was investigated by using the entire capsule at four different humidity

percentage i.e. 30%,50%,70% and 90%.

Composition of capsule

Each 550 mg capsule contains:

Ocimum sanctum 50 mg, *Curcuma longa* 50 mg, *Emblica officinalis* 50 mg, *Teriminalia bellerica* 50 mg, *Teriminalia chebula* 50 mg, *Piper longum* 50 mg, *Piper nigrum* 25 mg and *Zingiber officinale* 25 mg Excipients q.s.

***In-Vitro* anti-cancer activity of prepared poly-herbal formulation**

Cytotoxicity assessment using MTT assay

Maintenance of cell lines: The A549 (Human alveolar lung adeno-carcinoma cell line) were purchased from NCCS, Pune, India. The cells were maintained in DMEM high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^{0}C temperature in the CO_2 incubator and sub-cultured for every 2days [18,19].

Background of the study: MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm.

Materials:

1. Cell lines:
a) A549-Human alveolar lung adenocarcinoma cell line (From NCCS, Pune)
2. Cell culture medium: DMEM-High glucose media-(Cat No: 2120785, Gibco)
3. Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
4. Fetal Bovine Serum (#RM10432, Himedia)
5. MTT Reagent (5 mg/ml) (# 4060 Himedia)
6. DMSO (#PHR1309, Sigma)
7. D-PBS (#TL1006, Himedia)
8. 96-well plate for culturing the cells (From Corning,USA)
9. T25 flask (# 12556009, Biolite - Thermo)
10. 50 ml centrifuge tubes (# 546043 Torson)
11. 1.5 ml centrifuge tubes (Torson)
12. 10 ml serological pipettes (Torson)
13. 10 to 1000 ul tips (Torson)

Equipments:

1. Centrifuge (Remi: R-8^{o}C).
2. Pipettes: 2-10 µl, 10-100 µl, and 100-1000 µl.
3. Inverted microscope (Biolink)
4. 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China)

Assay controls:

(i) Medium control (medium without cells)

(ii) Negative control (medium with cells but without the experimental drug/compound)

E-ISSN: 2347-226X
P-ISSN: 2319-9857

(iii) Positive control-(Medium withy cells treated with 20 ug/ml of sample/drug)

Steps followed:

1. Seed 200 µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 24 hours.

2. Add appropriate concentrations of the test agent (Mentioned in the results - Excel sheet).

3. Incubate the plate for 24 hrs at 37°C in a 5% CO_2 atmosphere.

4. After the incubation period, takeout the plates from incubator, and remove spent media and add MTT reagent to a final concentration of 0.5 mg/mL of total volume.

5. Wrap the plate with aluminium foil to avoid exposure to light.

6. Return the plates to the incubator and incubate for 3 hours. (Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.)

7. Remove the MTT reagent and then add 100 µl of solubilisation solution (DMSO).

8. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.

9. Read the absorbance on a spectrophotometer or an ELISA reader at 570 nm wavelength.

10. The IC50 value was determined by using linear regression equation i.e. Y=Mx+C.

Here, Y=50, M and C values were derived from the viability graph.

11% Cell viability is calculated using the below formula:

% Cell viability=[Abs of treated cells/Abs of Untreated cells] x 100

RESULTS

The most significant part of developed formulation is standardization that ensures the quality, safety and reproducibility. It involves the entire process right from the plant collection and other raw materials to development of finished product. In the present research work, standardized development of poly-herbal mixture was formulated using hard gelatin capsules. Poly-herbal capsules formulation composed of six ingredients, belonging to diverse families, different morphological plant parts and different phyto-constituents.

Phytochemical investigation of all eight plant drugs

Proper and complete identification is one of the most important parameter incase of herbal medicine because the formulation cannot produce desired effects, if the herbs are not properly identified.

Chemical composition of drugs

Before the blends filled in to the capsule dosage form it was necessary to evaluate the presences of different chemical composition of the herbs. In case of herbal medicine the pharmacological efficiency can only be estimated by evaluating the chemical composition of herbs (Table 2). All these extracts were tested and found to be sterols, alkaloids, tannins, glycosides, flavonoids, phenolic compounds, and saponins present that revealed that this combination is suitable for therapeutic activity.

Table 2. Chemical composition of eight herbal plants used in the preparation of polyherbal capsule.

Chemical composition	Polyherbal formulation
Proteins	-
Lipids	-
Alkaloids	+
Glycosides	+
Sterols	+
Triterpenes	+
Tannins/Flavonoids	++

E-ISSN: 2347-226X
P-ISSN: 2319-9857

Steroids	++

Total flavonoid content

The absorbance on concentration curve for to linear with the rutin standard. The concentration is expressed as mg/mL on X-axis and the corresponding observed absorbance on Y-axis. The flavonoid concentration of sample determined by extrapolation was calculated as 1.83% w/w given in Table 4 and rutin linear curve are shown in Figure 1. Total flavonoid content or rutin equivalent was determined of developed poly-herbal capsule formulation. Total flavonoid content or rutin equivalent was found to be 478 µg/ml (Table 3).

Table 3. Total flavonoids in the ethanolic extracts of poly-herbal formulation.

S. No.	Conc	abs.	Rutin eq.
1	1 µg/ml	0.098	478 µg/ml

Total poly-phenols content

The absorbance on concentration curve for to linear with the gallic acid standard. The concentration is expressed as mg/mL on X-axis, and the corresponding observed absorbance on Y-axis. The poly-phenol concentration of the sample determined by extrapolation was calculated as 0.11% w/w given in Table 4, and gallic acid linear curve is shown in Figure 2. Total phenolic content or gallic acid equivalent was determined of developed poly-herbal capsule formulation. Total phenolic content or gallic acid equivalent was found to be 286.66 µg/ml extract.

Table4. Total phenol in the ethanolic extracts of poly-herbal formulation.

S.No.	Conc	abs.	Gallic acid Phenolic content
1	1 mg/ml	0.725	286.66 µg/ml extract

Antioxidant activity

Anti-oxidant activities were determined using two different methods: DPPH free radical scavenging activity and Ferric Ion Reducing Power Assay (FRAC) with gallic acid serving as a standard natural anti-oxidant compound. The reducing power of different extracts of polyherbal stable with increasing amount of sample.

The anti-oxidant power of the sample was evaluated with a comparison of inhibition concentration at 50% and 0.5 of absorbance value with standard gallic acid (Figure 3). Poly-herbal formulation had an IC50 value of 28.07 µg/mL in scavenges DPPH free radical (Figure 4). Ferric ion reduction property was also much effective and found 0.5 of absorbance at 7.10 µg/mL. Good anti-oxidant property may be high amounts of total flavonoids, poly-phenols, and tannins present in a sample. The results are given in Table 5. It is evident that reduction of oxidative stress is directly correlated with anti-hypertensive activities of Poly-herbal formulation.

Table 5. IC50 values of Standard and Poly-herbal formulation.

Antioxidant assay	Sample	IC50 (µg/ml)
DPPH	Quercetin	6.258
	Poly herbal formulation	28.07
ABTS	Quercetin	0.1995
	Poly herbal	7.1

E-ISSN: 2347-226X
P-ISSN: 2319-9857

	formulation	

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 outstanding flow property.

Flow property of powdered extract

The powdered material of poly-herbal capsule formulation was filled in size 1 capsules. Prior to filling of powder in capsules, its flow property was properly checked. The flow property of polyherbal formulation was found to be best and under acceptable limit as per Indian Pharmacopoeia. The results are shown below in Table 5. 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. The optimized poly-herbal capsule formulation showed excellent flow characters and was taken for capsule filling. All parameters were within the specified limits.

Table 6. Preformulation parameters.

Parameters	**Poly-herbal formulation**
Bulk density	0.823g/ml
Tapped density	0.702 g/ml
Carrs index	14.7023
Hausners ratio	1.14 ± 0.04
Angle of repose	19.04

Standardization of formulation

Capsule evaluation

Description "light brown" coloured granules packed in "0" size blue capsules. The poly-herbal capsules were evaluated for their description, microbial load, uniformity of dosage units, weight variation, disintegration time, and moisture content, and compared with Indian pharmacopoeial standards.

Organoleptic characters like brown colour, characteristic odour and taste was found. The Physiochemical parameters results were found such as pH 7.7, moisture content 1.01%, average weight 550 mg and weight variation 2.83%. The pharmacognostic powder characters results are loss on drying 2.55%, total ash 5.66%, acid-insoluble ash 1.28%, and water-soluble ash 3.51%, water-soluble extractive value 16.72%, ethanol-soluble extractive value 13.38%, arsenic not more than 5 ppm, microbial load analysis, presence of *E. coli* (should be absent), presence of salmonella (should be absent), presence of streptococcus (should be absent), presence of pseudomonas (should be absent) and total microbial count of yeast and molds are under limit (Table 7).

Tablet 7. Standardization of poly-herbal capsule.

Name of the test	Observations
Organoleptic characters	
Description	Brown colour powder
Colour	Brown
Odour	Characteristic
Taste	Bitter
Physiochemical parameters	

E-ISSN: 2347-226X
P-ISSN: 2319-9857

pH	7.7
Moisture content	0.0101
Average weight	563 mg
Weight variation	0.0283
Disintegration time (Mean ± SEM)	3 min 25 seconds ± 0.21
Loss on drying	0.0255
Total ash	0.0566
Acid-insoluble ash	0.0128
Water-soluble ash	0.0351
Water-soluble extractive value	0.1672
Ethanol-soluble extractive value	0.1338
Limits for heavy metals	
Arsenic not more than 5ppm	Complies
Lead not more than 10ppm	Complies
Microbial load analysis	
Total microbial count NMT 1000 cfu/g	113 cfu/g
Yeast and molds	Nil
Presence of *E. coli* (should be absent)	Absent
Presence of *Salmonella* (should be absent)	Absent
Presence of *Streptococcus* (should be absent)	Absent
Presence of *Pseudomonas* (should be absent)	Absent

Disintegration

Disintegration test was performed (550mg) by using six capsules. The minimum time of disintegration was 6 minutes and the maximum time observed was 13 minutes. All the six capsules fulfill the criteria of dissolution according to the Indian Pharmacopoeia (Table 8).

Table 8. Disintegration and dissolution pattern of poly-herbal capsule.

No of capsules	Disintegration time (Min)
1st Capsule	6 Min
2nd Capsule	8 Min
3rd Capsule	9 Min
4th Capsule	11 Min
5th Capsule	12 Min
6th Capsule	13 Min
Mean X	9.83
± S.D.	2.64
%CV	26.856

Dissolution

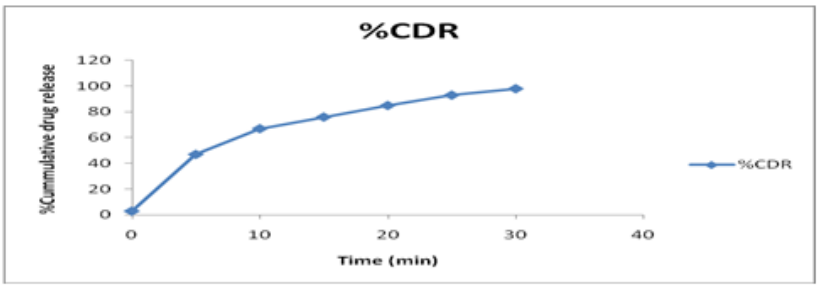
The optimized poly-herbal formulation of dissolution was studied by using *in-vitro* method. The % of cumulative drug release of bioactive drug molecule is maximum 98.6734 % up to 30 minutes (Table 9 and Figure 5).

E-ISSN: 2347-226X
P-ISSN: 2319-9857

Table 9. *In-Vitro* Dissolution Studies.

Time (min)	Abs	%CDR
0	0.0571	3.6864
5	0.3612	47.852
10	0.446	67.1841
15	0.5833	76.2171
20	0.6712	85.0103
25	0.7371	93.8643
30	0.769	98.6734

Figure 5. *In-vitro* % cumulative drug release of poly-herbal capsule.



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 10,11 and 12 for three stability parameters.

Table 10. Effect of different intensities of lights on poly-herbal capsules.

Light source	Sun light				Fluorescence				Tube light				UV Light				Infra-Red (IR)				Lamp light			
Time of exposure (hours)	01-Feb	1	3	6	01-Feb	1	3	6	½	1	3	6	01-Feb	1	3	6	01-Feb	1	3	6	01-Feb	1	3	6
600 mg polyherbal capsule	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 11. Stability test of poly-herbal capsule at different temperature.

Storage condition	Testing condition	Time duration (hours)				Result
		44593	1	3	6	
Ambient	30°C	-	-	-	-	No change during 6 hours after
Warm (30-40 °C)	35°C	-	-	-	-	No change during 6 hours after
Accelerated	50°C	-	-	-	-	No change during 6 hours after
Accelerated	55°C	-	-	-	+	Degradation start after 4 hours

E-ISSN: 2347-226X
P-ISSN: 2319-9857

Accelerated	65°C	-	-	+	+	Degradation start after 2 hours

Table 12. Stability of polyherbal capsule at different humidity and temperature.

Temperature	30% Humidity	50% Humidity	70% Humidity	90% Humidity
0.3	-	-	-	-
0.35	-	-	-	-
0.55	-	-	+	++
0.65	-	-	++	+++

In-Vitro anti-cancer activity of prepared polyherbal formulation

Polyherbal mixture shows significant cytotoxicity on the A549 cell line. The percentage cytotoxicity on A549 cell line at different concentration is as shown in Table 13 (Figure 6-8).

Table 13. Table showing the IC50 concentrations of the test compounds, S1 against A549 cell lines after the incubation period of 24 hrs.

S. NO	Sample code	IC_{50} (µG/ml) A549 Cell
1	S1	17.68

Figure 6. Overlaid Bar graph showing the IC50 values of Test compounds against A549cell lines by MTT study.

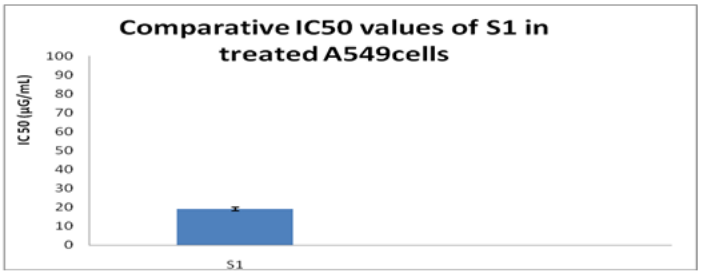


Figure 7. Graph showing the % cell viability of Test compounds against A549cell lines by MTT study.

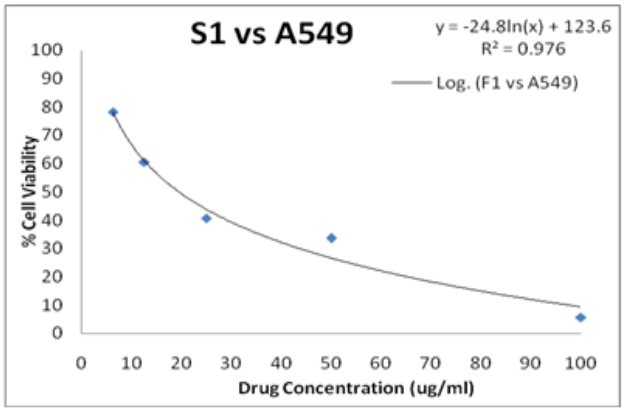


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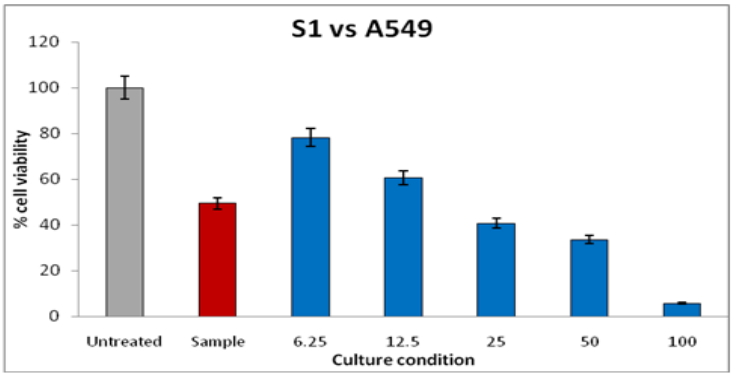
Figure 8. Bar graph showing the % cell viability of Test compounds against A549cell lines by MTT study.



The Observations in Statistical data of MTT cytotoxicity Study suggesting us that against A549cell lines test compounds namely S1 showing significant cytotoxic potential properties with the IC50 concentrations at 19.12 uG/ml used in the study. The compound, S1 showing effective cytotoxicity on A549 cells and may be considered as potent anti-lung cancer agent due to their low IC50 values on A549 cells. Overall, S1 compound showing effective anti-cancer potency on Human lung cancer cells. Further studies like cell cycle study by PI staining, Apoptosis study by Annexin V/PI staining, Apoptotic Protein expressions like Caspase 3,7,9, Bcl2,p53 and ROS study to evaluate the mechanism of action of test compounds *viz.*, S1 behind the anticancer potential in *in-vitro* conditions.

DISCUSSION

Different types of poly-herbal dosage forms have been used as therapeutic agents for the treatment of different disease. These bioactive derived molecules are obtained from traditional herbs. It may have potential therapeutic relevance in the treatment of cancer or many diseases. Therefore need do more research work for development of best combination of herbal formulation. In the present research work *Ocimum sanctum* 50 mg, *Curcuma longa* 50 mg, *Emblica officinalis* 50 mg, *Teriminalia bellerica* 50 mg, *Teriminalia chebula* 50 mg, *Piper longum* 50 mg, *Piper nigrum* 25 mg and *Zingiber officinale*25 mg were used for the poly-herbal 550 mg capsule. Poly-herbal combination of capsule formulation was developed with wet granulation method and then evaluated for quality poly-herbal product. It is very significant irrespective of their medicinal content and therapeutic states therefore the pre-formulation and formulation studies of the formulated polyherbal capsule solid dosage form were evaluated. Preformulation parameters are with 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 inter-particulate friction). These are useful tools in the development of new poly-herbal formulation. A value of <30° indicates 'excellent' flow whereas >56° indicates 'very poor' flow. Based on this, the flow was rated as 'excellent'. The CI and HR were found to be 14.7023and 1.14 ± 0.04. Lower CI or lower Hausner ratios of a material indicates better flow properties than higher ones. 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 eight herbal drugs were approved as quality drug when undergone by phytopharmaceutical evaluation according to the pharmacopoeial standards. Each poly-herbal capsule 550 mg disintegrated in meantime 13.14 ± 15 minutes and in vitro condition. The dissolution of drug determined where the release of a drug from solid dosage format which the substance dissolved in the fluid of gastrointestinal tract. Dissolution results represented that all of six capsules dissolved equal to 90% in 30 minutes. Drug releasing pattern of drug from capsule shell during *in-vitro* study is predicting the releasing sequence. The correlations of *in-vitro* and *in-vivo* results are developing a tool for bioavailability of drug, and to determine bioequivalence. In light of the phyto pharmaceutical studies and stability studies of the poly-herbal capsule was found almost stable.

Poly-herbal mixtures of selected plants were screened for their anti-cancer activity. Poly-herbal dosage form of plant shows significant cytotoxicity and thus anti-cancer activity on A549 cell line. Further studies using further precise methods are necessary to explore the constituents responsible for the activity and the mechanism of this activity which might confirm important and better therapies for the treatment and management of lung cancer [20,21].

CONCLUSION

Thus our study findings demonstrate that the poly-herbal capsule formulation evaluation of oral dosage form (550 mg capsule according to the Indian Pharmacopeia was successfully done. In the present research work, poly-herbal formulation was evaluated for its ability to reduce oxidative stress well. The oxidative stress is a vital factor for the pathogenesis of cancer disease, and decreases it; antioxidant may have been seen shown to be beneficial in the management of lung cancer disease.

Therefore, it can be concluded that the formulated poly-herbal solid dosage form, containing eight good anticancer bioactive molecule. It can be used for the treatment and management of cancer diseases.

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