

**PREPARATION AND STANDARDIZATION OF AN ANTI-OXIDANT
POLY-HERBAL FORMULATION: PROSPECTIVE ANTI-CANCER
MEDICINE.**

ABSTRACT

Oxidative stress is a causative factor leading to many pathological states. Herbs are one of the natural source of anti-oxidants. Anti-oxidants may safely interact with free radicals to counterbalance or bring to an end the chain reaction before damage may occur at cellular level in any major organ. The objective of the study is to prepare and standardize an anti-oxidant poly-herbal formulation. The active ingredients of the formulation include; *Curcuma longa*, *Nigella sativa*, *Allium sativum*, *Zingiber officinalis* and *Cinnamon zeylanicum*. The formulation may reduce oxidative stress and be of benefit in patients suffering from inflammatory diseases including cancer patients. It may be especially of value in preventing cancer in susceptible population with genetic tendency. Standardization of the formulation was carried out for assessment of its safety, efficacy and quality. The tests performed included; macroscopic evaluation, microscopic analysis, fluorescence analysis, preliminary physical property, phyto-chemical analysis, anti-bacterial, anti-fungal, anti-oxidant and cytotoxicity studies. The formulation was in the conformity to the properties evaluated. The standardization of the preparation was done and the results found are being used for pre-clinical and clinical studies. Furthermore, the results of the study maybe be used as a standard for future reference.

KEY WORDS: Free radicals, pharmacopeial testing, flavonoids, tannins, polyphenols, reactive oxygen species.

INTRODUCTION

Free radicals present in our environment pose a great threat to our health and may serve as a causative factor of diabetes, atherosclerosis, cancer, cardiovascular disease and many other chronic diseases. For the purpose of combating the oxidative stress, external anti-oxidant sources, for example plants are required to strengthen body's defense system. Herbs have both enzymatic and non-enzymatic anti-oxidant defense system against free radicals. Non-enzymatic includes active constituents in plants like flavonoids, carotenoids, tannins, and polyphenols. They have capability of preventing oxidative damage ¹.

Natural anti-oxidants such as flavonoids, tannins, and polyphenols act by donating electrons to intermediate radicals and help in inhibition of lipid peroxidation. Antioxidants are essential to prevent the formation and oppose the actions of reactive oxygen and nitrogen species, which are generated in vivo and cause damage to DNA, lipids, proteins, and other biomolecules. The anti-oxidant system contains exogenous antioxidants (dietary sources) and endogenous antioxidants.

Cancer is one of the principal causes of mortality and morbidity around the globe and the number of cases are constantly increasing estimated to be 21 million by 2030. There is a continuous rise in cancer cases in Pakistan. About 150,000 Pakistani's are diagnosed with cancer every year ².

There are many natural source medicines available in the market but they lack validation of its safety and its efficacy. Our product contain all ingredients with well proved anti-oxidant and anti-cancer effects with no adverse events if given in proper dose. Our product may be of benefit for patients with cancer as well as those individuals who are at high risk of developing cancer. To enhance the immunity in high risk individual to avoid development of cancer and to provide supportive treatment to cancer patient to fight it and get complete cure. The details of the composition of the formulation is as below:

Curcuma longa (turmeric) contains polyphenols, flavonoids, tannins and ascorbic acid. The major anti-oxidant alpha-turmerone (53.4%), beta-turmerone (18.1%) and aromatic-turmerone ³.

Nigella sativa seed are rich in thymoquinone, carvacrol, phenolic compounds and essential fatty acids ⁴.

Allium sativum (garlic) contains potent anti-oxidant compounds including allicin alliin, ajoene, allyl sulphides, diallyl disulphide, diallyl trisulphide, flavonoids, vitamin C and polyphenols ⁵.

Zingiber officinale is abundant in active constituents, such as phenolic and terpene compounds. The phenolic compounds in ginger are mainly gingerols, shogaols, and paradols. Ginger is abundant in active constituents, such as phenolic and terpene compounds ⁶.

Cinnamomum zeylanicum (Cinnamon) possess significant anti-oxidant activity due to its essential oil and other constituents including cinnamaldehyde, eugenol, and linalool ⁷.

METHODOLOGY

Material & Method

Herbal ingredients; *Zingiber officinalis* (rhizome), *Curcuma longa* (root), *Nigella sativa* (seeds), *Allium sativum* (bulb), and *Cinnamon zeylanicum* (barks) were obtained from Karachi, Pakistan. The human cervical carcinoma cell lines, SiHa (HPV-16) and HeLa (HPV-18), were obtained from National Center for Cell Science, Karachi, Pakistan. The cells were grown in DMEM supplemented with 10% FBS. The cells were maintained in a humidified 5% CO₂ incubator at 37°C.

Plant Material and Extraction

The different parts of all the five plants were chopped into small pieces, shade dried at ambient temperature, and stored in airtight container. It was ground into coarse powder in a grinder whenever required. After drugs size was reduced it was passed through sieve of mesh size 18 to obtain desire particle size of our drug.

All the ingredients were reduced to fine powdered form, sieved and then filled in hard gelatin capsules.

The extract of the poly herbal powder drug was obtained by using soxhlet's apparatus using ethanol as a solvent for extraction.

The tests performed for standardization of formulation included; macroscopic evaluation, microscopic analysis, organoleptic evaluation, fluorescence analysis, preliminary physical property, phyto-chemical analysis, thin-layer chromatography, anti-microbial, anti-oxidant activity. Physical property analysis such as bulk density, tapped density, compressibility index and angle of repose for the formulated capsules were also done.

Organoleptic Evaluation

Macroscopic or organoleptic evaluation was performed including assessment of drugs by color, odor, size, shape, taste internal and external markings and texture ⁸.

Microscopic Evaluation

Extract has been visualized under microscope to study powder microscopy. Microscopic evaluation of particles was observed in microscope by using iodine, glycerin, chloral aldehyde at magnification on 4X, 10X and 40X ⁹.

Chemical testing & Phyto-chemical Evaluation

Powder sample was tested phyto-chemically reacting with different chemical agents and triturate with different chemical agents to check the stability, chemical and physical property of the sample with the used chemical agents. Chemical agents; ethyl acetate, HCL acetone, benzene, H₂SO₄, 66%, 5% NaOH 100% H₂SO₄, nitric acid, 5%FeCl₃ were used and triturated with water. Benedicts test for carbohydrate, modified Borntrager's test for Anthrol glycoside, Molish test for carbohydrates, Liebermann's burchard's test for sterols, Froth test for Saponins, Foam test for Saponins, Salkowski test for Sterols, Glycerin test for Tannins, Xanthoprotic test for Protein, Ferric chloride test for Phenols, Alkaline reagent test for Flavonoids, Fehling test A and B, Lead acetate test for Flavonoids, Wagner test, Hager test, has been performed to evaluate the properties of drug use for standardization ¹⁰.

Fluorescence Evaluation

The air dried plant material was subjected to fluorescence analysis under ultra violet light (254nm and 366 nm) and day light after giving treatment with various chemical and organic solvents like 1N Sodium hydroxide in methanol, 1N Sodium hydroxide in distilled water, 50% Nitric acid, 50% Sulphuric acid, 1N Hydrochloric acid ¹¹⁻¹².

Thin-layer Chromatography

Extract of poly-herbal drug in small quantity was dissolved in ethanol (analytical grade) for Thin Layer Chromatography TLC. Ready-made TLC plates (Silica gel 254 fluorescent, Merck, Germany) were used. Sample was applied on TLC plates as described by Stahl¹³. A solvent system that is CHCl₃-MeOH (90:10) used to develop TLC plates and separate UV-active chemical compounds on TLC plates and observed under UV-lights of 254nm and 366nm. After marking, spots were detected by spraying the reagents (Vanillin-Sulphuric acid; 1:100). The R_f value of each spot was determined. Thin chromatography with 100% ethanol, 1:1 chloroform and methanol, 100% methanol and 1:1 benzene and chloroform.

Cytotoxicity Studies

Drug extract sample was dried to perform cytotoxicity activity for standardization. MTT HeLa cell, MTT PC3 cell lines, MTT 3T3 cells lines were treated with sample dried extract and count the number of viable cells¹⁴.

Anti-oxidant activity (DPPH scavenging activity)

3.96 mg of DPPH was dissolved in 20ml of methanol to get stock solution. With 0.5 ml of sample solution was added to 1ml of DPPH solution separately. These solution mixtures were kept at room temperature in dark for incubation period of 30 minutes (incubation period)¹⁵⁻¹⁶. Its absorbance was measured at 517nm. Low absorbance of the reaction mixture indicated higher free radical scavenging activity using the equation.

$$\% \text{ scavenging DPPH free radical} = 100 \times (1 - AE/AD)$$

AE = It is absorbance of the solution, when extract has been added at a particular level

AD = It is the absorbance of the DPPH solution with nothing added (blank without extract)

Anti-bacterial activity

The anti-bacterial activity of different medicinal plants against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi*. All the bacterial isolates were checked and identified on the basis of conventional methods for purity and maintained on nutrient agar at 4°C in the refrigerator for further work. Anti-bacterial activity of crude extract against the test organisms were determined by using agar-well method. Autoclaved Muller Hinton broth was used to keep the bacterial culture in log phase for 2 hours with constant agitation and subsequently wells were dug onto Muller Hinton Agar. Later, 10 microliters of culture were poured into the wells. All plates were incubated at 28 ± 2°C for 24-48 hours and after incubation diameter of zone of inhibition was measured¹⁷⁻¹⁸.

Anti-fungal activity

The test organisms for this study were *Trichophyton rubrum*, *Candida albicans*, *Aspergillus niger*, *Microsporum canis*, *Fusarium lini* and *Candida glabrata*. All the fungal isolates were checked for purity and maintained on Sabour Dextrose agar (SDA) at 4°C in the refrigerator

until required for use. Anti-fungal activities of medicinal plants were tested using agar-well method. Autoclaved distilled water was used for the preparation of fungal spore suspension and transferred aseptically into each SDA plates. All plates were incubated at 28±2 C for 24-48 hours and after incubation diameter of zone of inhibition was measured ¹⁹.

Determination of Bulk Density

Bulk density measurement was carried out by graduated cylinder procedure. The powder sample sufficient to complete the test via a sieve with apertures greater than or equal to 1.0 mm was passed gently. A dry graduated cylinder of 250 ml was taken and powder sample was gently poured without compacting, approximately 100 g of the test sample (m) weighed with 0.1% accuracy. Cautiously the powder was leveled without compacting. Read the unsettled apparent volume (V_0) to the nearest graduated unit. Calculate the bulk density in (g/ml) using the formula m/V_0 . Repeat the procedure thrice to determine the bulk density ²⁰.

Determination of angle of repose

A metal funnel with a circular opening of and a slope of 60° to the horizontal was fabricated. The circular opening was firstly blocked with a finger before of powder was gently charged into it. Removing the finger permitted the powder to flow onto a horizontal surface directly beneath it to form a powder heap ²¹.

RESULTS

Organoleptic Evaluation

Organoleptic observation of the poly herbal powdered drug is as follows:

Color – Brownish-yellow

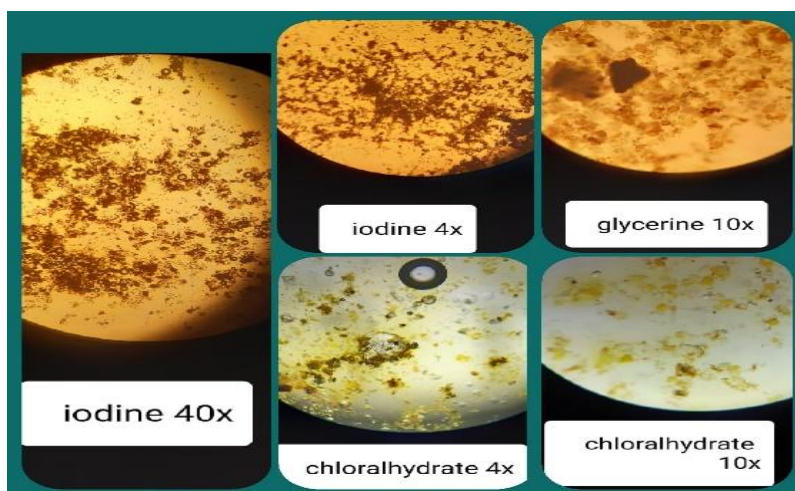
Odor – Spicy, aromatic

Taste – characteristic, slightly astringent

Microscopic Evaluation

Following microscopic features were observed under microscope: cork in surface view, fragments of spirally thickened vessels, covering trichomes, epidermis in surface view, fragments of parenchyma with adherent oleoresins, fibers, sclerids, calcium oxalate crystals, oil cell and starch granules (Figure 1).

Figure 1: Microscopic Examination of poly herbal crude drug



CHEMICAL TESTING & PHYTOCHEMICAL EVALUATION OF CRUDE POLY HERBAL POWDERED DRUG

Phytochemical and chemical testing results are tabulated in tables 1 & 2 respectively. Figure 2 presents the chemical evaluation of poly herbal powdered drug.

TABLE 1: PHYTOCHEMICAL TESTING OF THE POLY HERBAL DRUG

S.N O.	PHYTOCHEMICAL TESTS	OBSERVATION	REMARKS
I	Identification tests for Tannins		
	1. Aqueous extract of sample + 5% Lead acetate	Dense off-white precipitates settled at the bottom	Tannins present
	2. Aqueous extract of sample + 5% Ferric Chloride	Reddish-brown color ring formation	Tannins present
	3. Aqueous extract of sample + dilute KMnO ₄	Dark brown precipitates	
II	Identification tests for Resins		
	1. Aqueous extract of sample + Sulphuric acid (conc.)	Color of solution changes from light yellowish brown to reddish brown	Resins present
	2. Aqueous extract of sample + boric acid	Turbid solution	Resins present
	3. Aqueous extract of sample + Potassium hydroxide	Slight froth formation	Resins present
	4. 2 ml of aqueous extract of sample + 5 ml of dilute acetic acid. Filter. Add ammonium	Not more than slight turbidity produced.	Resins present

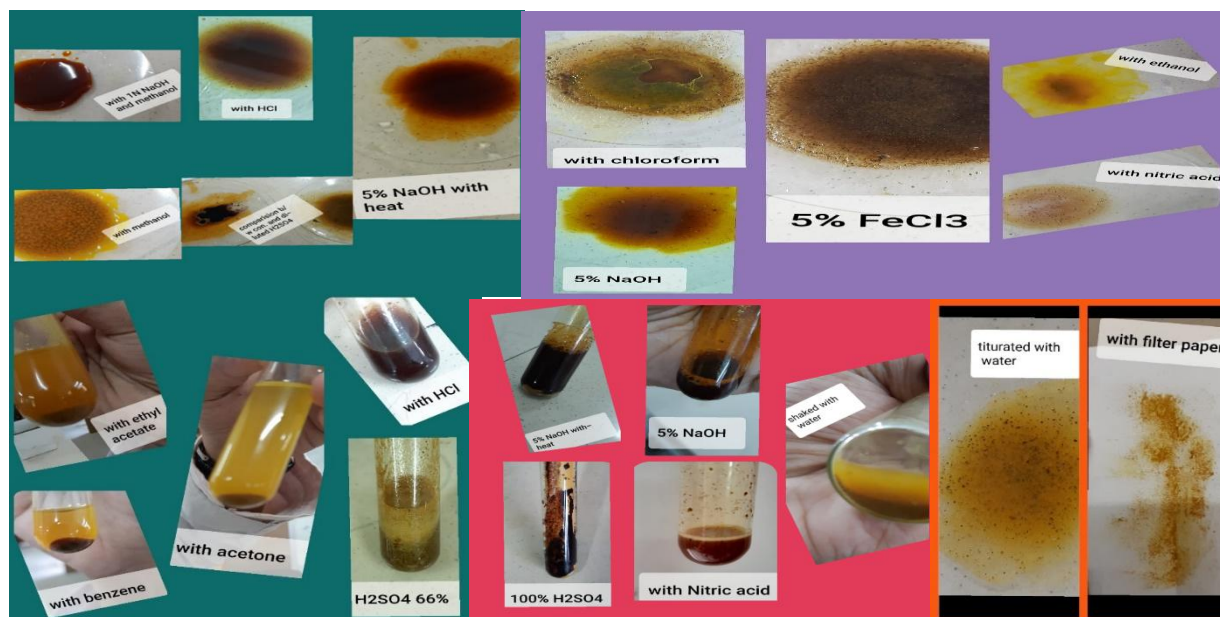
	oxalate solution.		
III	Identification tests for Lipids		
	1. (Iodine absorption test) Aqueous extract of sample + chloroform + iodine	No decolorization	Unsaturated fatty acids absent
	2. (Solubility test) Aqueous extract of sample + ethanol	Clear golden-yellow solution	Fixed oils present
IV	Identification tests for Alkaloids		
	1. Aqueous extract of sample + Wagner's solution	Reddish-brown precipitates	Alkaloids present
	2. Aqueous extract of sample + Hager's solution	Fine yellow precipitates	Alkaloids present
	3. Aqueous extract of sample + Tannic acid solution	Buff precipitates	Alkaloids present
V	Identification tests for Carbohydrates		
	1. 2 ml aqueous extract of sample + 1 ml Fehling solution A + 1 ml Fehling solution B. Heat on water bath for 5 minutes	Brownish-green color	Carbohydrates absent
	2. 2 ml aqueous extract of sample + 1 ml α naphthol + 2 drops of Sulphuric acid	Moove color	Carbohydrates absent
VI	Identification test for Saponin glycosides		
	1. Powdered form of the sample + shaken with distil water	Slight froth formation	Saponin glycosides
VII	Identification tests for Anthraquinone glycosides		
	1. Borntrager's test 1 gm powder sample + 10 ml of Hydrochloric acid 10% + heat on water bath + filter + carbon tetra chloride + ammonia solution	Light /baby pink colored layer	Anthraquinone glycosides present
	2. Modified Borntrager's test 1 gm powder sample + 5 ml of Hydrochloric acid 10% + 5 ml Ferric Chloride + Heat on water bath + filter + carbon tetrachloride + ammonia solution	Dark/magenta pink colored layer	Anthraquinone glycosides present
VIII	Identification tests for Steroids		
	1. Aqueous solution of sample (5 ml) + 2 ml of chloroform + 1 ml of Sulphuric acid	No red color lower layer formation.	Steroids are absent in the sample

	2. Aqueous solution of extract on filter paper	No translucent spot left after solution drying	Fixed oils are absent in the sample
IX	Identification test for Terpenoids		
	1. Aqueous solution of sample (5 ml) + chloroform (2 ml). Heat on water bath and then add Sulphuric acid (3 ml)	Buff colored	Terpenoid present
X	Identification tests for Flavonoids		
	1. Aqueous solution of sample (1 ml) + Lead acetate 10% (4-5 drops).	Dense precipitates settled at bottom, froth formation	Flavonoids present
	2. Aqueous solution of sample + Ferric Chloride 10% (4-5 drops)	Clear golden yellow solution	Flavonoids present

Table 2: REACTION OF CHEMICALS WITH CRUDE POWDER DRUG

REACTION WITH CHEMICALS	OBSERVATION
TITURATE WITH WATER.	Slight clumping Slightly soluble Particles can be seen easily.
SHAKED WITH WATER.	In suspension form No foaming or ppt. seen Particle settle down.
IMMERSED BETWEEN FILTER PAPER.	Smooth texture with no oily layer.
TREATED WITH 5% NaOH SOL.	Color change to reddish brown plus precipitates.
TREATED WITH 5% NaOH SOL. + HEATED.	Darker brownish orange color Thick foam appear.
TREATED WITH 66% H ₂ SO ₄ .	Thick foam Color appear is like iodine solution color.
TREATED WITH 5% FeCl ₃	Brownish black color with thick precipitates.
TREATED WITH CONC. HCl.	Black precipitates Dark color solution (reddish brown).
TREATED WITH CONC. H ₂ SO ₄ .	Purple black precipitates
TREATED WITH CONC. NITRIC ACID	Slight white turbidity with bleach like odor Small white precipitates
TREATED WITH 1N NaOH IN METHANOL.	Reddish brown solution.

FIGURE 2: REACTION OF CHEMICALS WITH CRUDE POWDER DRUG



FLUORESCENCE EVALUATION

See the results of fluorescence analysis of poly herbal powdered drug in table 3.

TABLE 3: FLUORESCENCE ANALYSIS OF POLY HERBAL POWDERED DRUG

Solvent	Visible/ Day light	UV Light (254 nm)	UV Light (366 nm)
Powder + Acetone	Light Orange	Fluorescent green	Brown
Powder + Benzene	Dark Orange	Dark green	Brown
Powder + Chloroform	Yellowish green	Fluorescent green	Dark green
Powder + Ethanol	Yellowish black	Light green	Dark green
Powder + Methanol	Brown	Light green	Dark green
Powder + Ethyl Acetate	Dark yellow	Fluorescent green	Brown
Powder + Distil Water	Light yellow	Fluorescent green	Dark green

THIN-LAYER CHROMATOGRAPHY

See the retention factor of poly herbal crude powdered drug. See table 4.

TABLE 4: THIN LAYER CHROMATOGRAPHY OF POLY HERBAL POWDERED DRUG

Solvent System	Rf value
Ethanol 100%	0.73
Methanol 100%	0.71
Chloroform: Methanol	0.65, 0.72, 0.77

CYTOTOXICITY STUDIES

The powdered drug sample exhibited cyto-toxicity against MTT Hela cell lines and MTT PC3 cell lines. No activity was found against MTT 3T3 cell lines (See tables 5-7).

TABLE 5: CYTO-TOXICITY STUDIES ON MTT HELA CELL LINES

Sample Code	Conc. ($\mu\text{g/ml}$)	% Inhibition/Stimulation	IC ₅₀ \pm SD
APC-31	30	99.4%	15.1 \pm 1.2
Doxorubicin (standard)	30	101.2%	0.9 \pm 1.4

TABLE 6: CYTO-TOXICITY STUDIES ON MTT PC3 CELL LINES

Sample Code	Conc. ($\mu\text{g/ml}$)	% Inhibition/Stimulation	IC ₅₀ \pm SD
APC-31	30	50.5%	29.6 \pm 0.09
Doxorubicin (standard)	30	89.9%	1.9 \pm 0.08

TABLE 7: CYTO-TOXICITY STUDIES ON MTT 3T3 CELL LINES

Sample Code	Conc. ($\mu\text{g/ml}$)	% Inhibition/Stimulation	IC ₅₀ \pm SD
APC-31	30	-25.5%	Inactive

ANTI-OXIDANT STUDIES

The powdered drug sample exhibited potent anti-oxidant activity (See table 8).

TABLE 8: ANTI-OXIDANT ACTIVITY OF THE POLY-HERBAL POWDERED DRUG

Sample Description	Method Used	Results
APC-31	DPPH	90.5% per mg for sample

ANTI-BACTERIAL ACTIVITY

The poly herbal crude drug sample showed moderate inhibition against *Staphylococcus aureus* and *Bacillus subtilis* while insignificant inhibition against all other organisms (See table 9).

TABLE 9: ANTI-BACTERIAL ACTIVITY (MICROPLATE ALAMAR BLUE ASSAY – MABA)

Name of Bacteria	% Inhibition of Compound	% Inhibition of Drug
<i>Escherichia coli</i>	19.11%	84.32%
<i>Bacillus subtilis</i>	47.09%	91.07%
<i>Staphylococcus aureus</i>	44.71%	88.85%
<i>Pseudomonas aeruginosa</i>	No inhibition	84.64%
<i>Salmonella typhi</i>	19.5%	87.27%

ANTI-FUNGAL ACTIVITY

The poly herbal drug sample exhibited insignificant anti-fungal activity (See table 10).

Table 10: IN VITRO ANTI-FUNGAL BIOASSAY

Name of Fungus	Linear growth (mm)		% Inhibition	Standard Drug	MIC (µg/ml)
	Sample	Control			
Trichophyton rubrum	100	100	0%	Miconazole	70
Candida albicans	100	100	0%	Miconazole	110
Aspergillus niger	100	100	0%	Amphotericin B	20
Microsporum canis	100	100	0%	Miconazole	98.4
Fusarium lini	100	100	0%	Miconazole	73.25
Candida glabarata	100	100	0%	Miconazole	110.8

BULK DENSITY DETERMINATION

The observation results of bulk density determination are tabulated in the table 11 below.

TABLE 11: DETERMINATION OF BULK DENSITY

S.NO.	OBSERVATIONS
1	VOLUME BEFORE TAPPING = 40ml
2	VOLUME AFTER TAPPING = 27ml.
3	WEIGHT OF POWDER (Before tapping) = 14.37 gm.
4	WEIGHT OF POWDER (After tapping) = 14.31 gm
5	WEIGHT OF EMPTY CYLINDER = 61.04 gm.
6	WEIGHT OF CYLINDER + POWDER (Before tapping) = 75.41 gm.
7	WEIGHT OF CYLINDER + POWDER (After tapping) =75.35gm.

8	BULK DENSITY (Before tapping):=0.36
9	BULK DENSITY (After tapping):=0.53

ANGLE OF REPOSE DETERMINATION

Angle of repose of the poly herbal powdered sample was 49.2⁰ See the calculations below.

Height: 3.5cm

Diameter: 6cm

Formula: $\tan^{-1} (2h/d)$

$$\tan^{-1} (2*3.5/6) = 49.2^0$$

DISCUSSION

The constituents present in the formulation are rich in flavonoids, polyphenols and tannins. These constituents are already reported to possess anti-oxidant activity and anti-cancer activity²²⁻²⁹. Anti-oxidant defense support provided by the composition of the formulation under study may neutralize these radicals after they have formed by maintaining sufficient anti-oxidant status in the body. The anti-oxidant activity based on the presence of active constituents of the ingredients present in the formulation under study may be of benefit in cancer prevention in population at risk of developing cancer and may be used as an adjuvant therapy along with conventional therapy for cancer in alleviating the side effects accompanied with chemotherapy. It may also reduce the free radicals burden thereby may lead to decrease in chemotherapy doses³⁰. Development of resistance and associated toxic side effects of conventional chemotherapy agents lead to the exploration, development and standardization of known as well as new natural origin active ingredients with anti-oxidant, anti-inflammatory, anodyne and anti-cancer activity. Grigalius & Petrikaite, 2017 studied inter-relation of anti-oxidant effect of trihydroxyflavone with its anti-oxidant effect³¹. Further pre-clinical work is in progress that would lead to clinical studies.

NOTE:

The study highlights the efficacy of "Herbal ingredients" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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