Original Research Article

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 purpose of the study was 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 exhibited positive results against 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. Enzymatic and non-enzymatic anti-oxidant defense system against free radicals is displayed by the herbs. 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 giving electrons to intermediate radicals and help in inhibition of lipid peroxidation. Anti-oxidants are vital to avert the formation and oppose the actions of reactive oxygen and nitrogen species that are produced *in vivo* and cause damage to DNA, lipids, proteins, and other biomolecules. The anti-oxidant system contains exogenous anti-oxidants which includes, dietary sources and endogenous anti-oxidants.

Cancer is one of the chief causes of mortality and morbidity world-wide and the number of cases are continuously growing; assessed 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 α-turmerone (53.4%), β-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 (ginger) is abundantly contains phenols and terpene compounds as their active constituents. The phenolic compounds in ginger includes: gingerols, shogaols, and paradols. Ginger is rich 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. They were authenticated and formulated in to dosage form by Dr Farah Saeed.

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% CO_2 incubator at 37°C.

Plant Material & Extraction

The different parts of all the five plants were cut 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, weighed the amount of powder (500 mg) and then filled in hard gelatin size 0 capsules (https://www.capsulesizes.com/capsule-size-chart/) [8] in specific ratio.

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 tests conducted encompassed: bulk density, tapped density, compressibility index and angle of repose for the poly-herbal capsules.

Organoleptic Evaluation

Macroscopic or organoleptic evaluation was performed including assessment of drugs by color, odor, size, shape, taste internal and external markings and texture [9].

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 [10].

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, H_2SO_4, 66%, 5% NaOH 100% H_2SO_4, nitric acid, 5% $FeCl_3$ were used and triturated with water. Benedicts test for carbohydrate; modified Borntrager's test for Anthraquinone 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 and Lead acetate test for Flavonoids were performed to evaluate the properties of drug use for standardization [11].

Fluorescence Evaluation

The air dried plant material was exposed to fluorescence analysis under ultra violet light 254nm and 366 nm respectively as well as 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 [12-13].

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 defined by Stahl [14]. A solvent system that is $CHCl_3$-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 Rf 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 and anti-cancer activity for standardization on breast, prostate and cervical cancers in 96-well micro-plates by standardized MTT colorimetric

Assay[15]. MTT Hela cell, MTT PC3 cell lines, MTT 3T3 cells lines were treated with sample dried extract and count the number of viable cells [16].

Anti-oxidant activity (DPPH scavanging 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) [17-18]. Its absorbance was measured at 517nm. Low absorbance of the reaction mixture indicated higher free radical scavenging activity using the equation.

% scavenging DPPH free radical = 100 × (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 the poly-herbal extract was carried out against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi*. All the bacterial isolates were retained on nutrient agar at 4°C in the refrigerator for further work. Anti-bacterial activity of poly-herbal 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 measured [19-20].

Anti-fungal activity

Anti-fungal activities of the poly-herbal extract was tested using agar-well method. The test organisms for this study were *Trichophyton rubrum*, *Candida albicans*, *Aspergillus niger*, *Microsporum canis*, *Fusarium lini* and *Candida glabarata*. All the fungal isolates were checked for purity and maintained on Sabourd Dextrose agar (SDA) at 4°C in the refrigerator until required for use. 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 [21].

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 gm of the test sample (m) weighed with 0.1% accuracy. Cautiously the powder was leveled without compacting. The un-settled apparent volume (V_0) was read 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 [22].

Determination of angle of repose

A metal funnel with a circular opening of and a slope of 60° to the horizontal was used to carry out the experiment. The circular opening was firstly blocked with a finger before poly-herbal powder was gently poured into it. Removing the finger permitted the powder to flow onto a horizontal surface directly beneath it to form a powder heap [23].

RESULTS

Organoleptic Evaluation

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

Color – Brownish-yellow

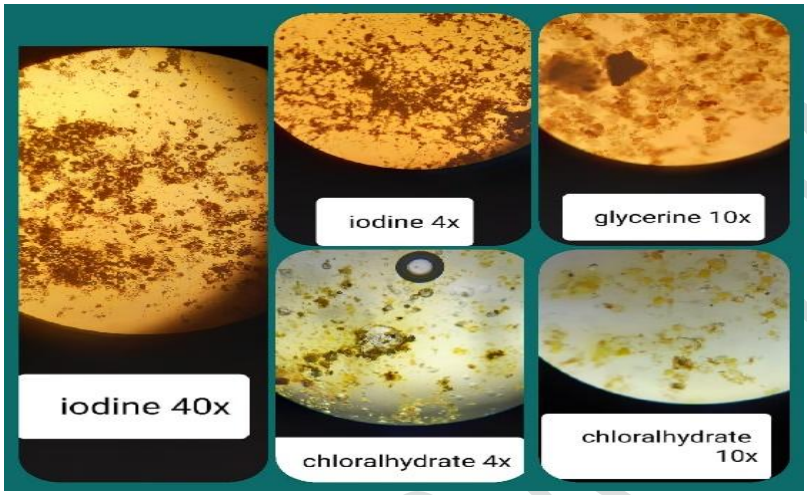
Odor – Spicy, aromatic

Taste – characteristic, slightly astringent

Microscopic Evaluation

Following microscopic features were observed under microscope of the poly-herbal powdered drug: cork in surface view, fragments of spirally thickened vessels, covering trichomes, epidermis in surface view, fragments of parenchyma with adherent oleoresins, fibers, scelerids, 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.NO.	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 KMnO4	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 oxalate solution.	Not more than slight turbidity produced.	Resins present
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 napthol + 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	Dark/magenta pink colored layer	Anthraquinone glycosides present

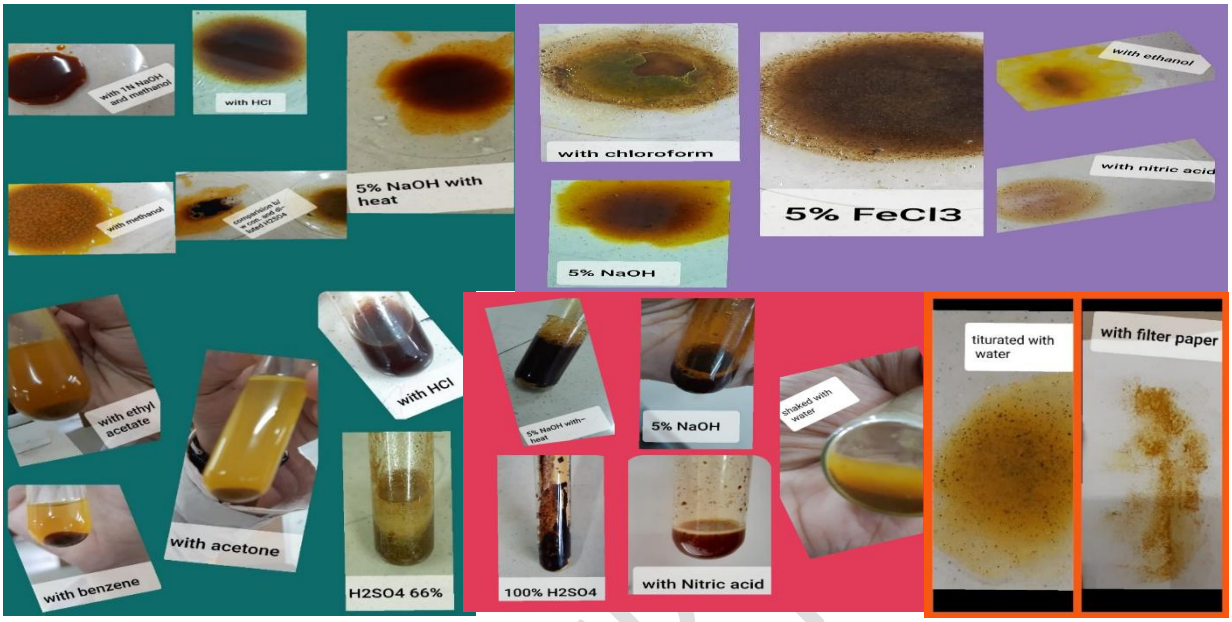
	solution		
VIII	**Identification tests for Steroids**		
	1. Aqueous solution of sample (5 ml) + 2 ml of chloroform + 1 ml of Sulphuric acid	No red color lower layer formation.	Steroids are absent in the sample
	2. Aqueous solution of extract on filter paper	No translucent spot left after solution drying	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_2SO_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_2SO_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

Solvent			Visible/ Day light	UV Light (254 nm)	UV Light (366 nm)

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

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

TABLE 3: FLUORESCENCE ANALYSIS OF POLY HERBAL POWDERED DRUG THIN-LAYER CHROMATOGRAPHY

See the retention factor of poly herbal crude powdered drug. The Rf value validates the thymoquinone, phenolic and flavonoids present in the poly-herbal drug powder. 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 powered 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. (µg/ml)	% Inhibition/Stimulation	IC_{50} ± SD
APC-31	30	99.4%	15.1 ± 1.2
Doxorubicin (standard)	30	101.2%	0.9 ± 1.4

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

Sample Code	Conc. (µg/ml)	% Inhibition/Stimulation	IC_{50} ± SD
APC-31	30	50.5%	29.6 ± 0.09
Doxorubicin (standard)	30	89.9%	1.9 ± 0.08

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

Sample Code	Conc. (µg/ml)	% Inhibition/Stimulation	IC_{50} ± SD
APC-31	30	-25.5%	Inactive

Bacteria	Inhibition of Compound (%)	Inhibition of Drug (%)
Escherichia coli	19.11%	84.32%
Bacillus subtilis	47.09%	91.07%
Staphylococcus aureus	44.71%	88.85%
Pseudomonas aeruginosa	No inhibition	84.64%
Salmonella typhi	19.5%	87.27%

ANTI-OXIDANT STUDIES

The powered drug sample exhibited potent anti-oxidant activity (See table 8). The results exhibit outstanding anti-oxidant activity.

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)

ANTI-FUNGAL ACTIVITY

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

Table 10: IN VITRO ANTI-FUNGAL BIOASSAY

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

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**

Angle of repose of the poly herbal powdered sample was 49.2^0 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 activity [24-31]. 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 effect (90.5%) 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 doses [32].

The poly-herbal powdered extract exhibited prominent anti-proliferative activity against MTT Hela (99.4%) and PC3 (50.5%) cell lines and insignificant effect against 3T3. The extract exhibited moderate anti-bacterial activity and insignificant anti-fungal activity. Our anti-oxidant and cytotoxicity and anti-microbial results are supported by the work of Nair *et al*. 2000; Ruan *et al*. 2021; Anjum *et al*. 2020; Toninet *et.al*. 2019 and Rao *et al*. 2019) [33-37].

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 effect [38]. Further pre-clinical work is in progress that would lead to clinical studies.

CONCLUSION

The study highlights the efficacy of "Herbal ingredients" which is an ancient tradition, used in some parts of India. In the current study the herbal ingredients are mixed in specific ratio and their pharmacognostic, phytochemical, biological studies results showed promising therapeutic efficacy. The toxicity studies are in progress and clinical studies are in consequent future plan. This combination drug may be used after the successful completion of rigorous studies.

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