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"PHYSICOCHEMICAL, ANTI-MICROBIAL ACTIVITY AND CYTOTOXIC ACTIVITY OF POLYHERBAL POWDER"

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ABSTRACT

The present study was carried out to evaluate the Physicochemical, Anti microbial activity and Cytotoxic activity of polyherbal powder mixture of aqueous extract and ethanolic extract was done by standard procedures. Evaluation of Antimicrobial activity by using standard procedures for cup plate method and zone of inhibition was determined. Cytotoxicity was carried against for both aqueous and ethanolic extract. The cell viability of MCF7 Cancer cell line for their extract was determined.

KEYWORDS: Cytotoxic activity, Standard procedures, Zone of Inhibition, MCF7 Cell line, Antimicrobial activity.

INTRODUCTION

Herbal wealth of India Now-a-days natural products are becoming an integral part of human health care system, because of popular concern over toxicity and resistance of modern drugs.[1] The WHO estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern Pharmacopoeias still contain at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. Demand for medicinal plant is increasing in both developing and developed countries due to increasing recognition of natural products, being non narcotic, having less side-effect, easily available at affordable prices and sometime the only source of health care available to

the poor. Medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of India.[2] Demand for medicinal plant is increasing in both developing and developed countries, and the bulk of the material trade is still from wild harvested sources on forest land and only a very small number of species are cultivated.[3] The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Plants are known to produce a variety of compounds to protect themselves against a variety of their pathogens and therefore considered as potential source for different classes of antimicrobial substances. Plants used in traditional medicine contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases. The substances that can either inhibit the growth of micro-organisms or kill them are considered as candidates for developing new drugs for treatment of various infectious diseases. The plants used in traditional system of medicine are well known as remedies for diseases in the rural areas of developing countries. Herbal medicines have been used in developing countries as an alternative to Allopathic medicines. Our extensive review of literature has revealed a variety of medicinal plants possessing anti-inflammatory and hepatoprotective activity which also exhibited good antimicrobial properties. On the basis of these reports the plant extracts were subjected for antimicrobial activity against various bacteria and fungal strains.[4]

MATERIAL AND METHODS

Collection and authentication

We are purchased the following material from local licensed herbal supplier inVijawada *Maluspumiplus* (Apple) powder. *Momardicachirata* (Karela) powder, *Citrus lemon*(Lemon) powder, *Pleurotusostreatus*(Mushroom)powder, and *Triticumaestivum (wheat grass)* powder. The above materials were preserved in our Phramacognosy lab for the further experimental purpose.[1-3]

Extraction of polyherbal powder mixture

All the above five powders are mixed in the equal proportions to get 200 gm. and then the powder herbal mixture used for extraction using soxhilation with distilled water and ethanol for six hours. Then the extraction was dried under reduced pressure using Rotary evaporator. Then the both dried extracts are preserved in the desiccators and used for further purpose.[17]

Determination of water soluble extractive

The same procedure was followed as directed for the determination of the water soluble extractive by using chloroform water instead of alcohol. The extractive values of drugs were calculated and recorded.[13]

Loss on drying

2 gm. of powdered drug was taken in tarred china dish. Dried in the oven at 100 or 105℃, cooled in a desiccator and watch. After that the loss was recorded as moisture. The procedure was continued for at least two common readings.[13-15]

The procedures employed in microbial assay were,

Cup Plate Method

In the present study, antimicrobial screening was carried out by cup plate method.[18]

Test Organisms

The following strains procured from Division of Biotechnology, Hindu college of pharmacy, Guntur were employed for the present study.

Gram-positive organisms: Bacillus pumilis, Bacillus subtilis.

Gram-negative organisms: Proteus vulgaris, Escherichia coli.

Fungal strains: Aspergillusniger, Penicilliumchrysogenum.

Fungi

Aspergillusniger: Aspergillusniger causes asprigillosis in man which is a frequent infection of the external ear called Ostomycosis.[9]

Standardization of micro-organisms

One loop-full of micro-organisms were inoculated into 100 ml of sterile medium and incubated for 24 hrs at 37°C for bacterial culture and for 48 hrs at 27°C for fungal culture.

After 24 h/48 h of incubation, 1 ml of broth containing the micro-organisms was added to 9 ml of peptone water.

10 fold serial dilutions were made in the range of 10-1 to 10-10.

100 µl of the dilutions ranging from 10-5 to 10-8 were spread over the sterile nutrient agar (SDA) plates and kept at 37 and 27°C for 24 / 48 hours respectively.

The number of colony forming units (CFU) was counted and number of micro-organisms per 1 ml of stock culture was calculated.[15-18]

Preparation of test and standard solutions

The stock solution of test compounds was prepared by dissolving the dried extracts of both ethanolic and aqueous polyherbal mixture at a concentration of 100, 200 and 300mg/ml in dimethylsulphoxide (DMSO) respectively. The stock solution of reference standards (Penicillin and Fluconazole) was prepared at a concentration of 10 mg/ml in sterile water. Antimicrobial activity was screened by adding 0.05 ml stock solution to each cup by micropipette.[4,13]

Culture medium

Nutrient broth for bacteria: Beef extract - 0.35%, Sodium chloride - 0.5%, Peptone - 0.5%.

Nutrient agar for bacteria: Beef extract - 0.3%, Sodium chloride - 0.5%, Peptone - 0.5%, Agar - 2.0%, pH - 7.2-7.4.

The sterilized medium was cooled to 40°C and poured into the Petri dishes to contain 6 mm thickness. The media was allowed to solidify at room temperature.

Evaluation of antibacterial and antifungal activity

Determination of zone of inhibition by cup plate method

The cup plate assay of drug potency is based on measurement of the diameter of zone of inhibition of microbial growth surrounding cylinders (cups), containing various dilutions of test compounds. A sterile borer was used to prepare four cups of 6 mm diameter in the agar medium spread with the micro-organisms and 0.1 ml of inoculums was spread on the agar plate by spread plate technique. Accurately measured (0.05 ml) solution of each concentration and reference standards were added to the cups with a micropipette.

All the plates were kept in a refrigerator at 2 to 8°C for a period of2 hours for effective diffusion of test compounds and standards. Later, they were incubated at 37°C for 24 hours. The presence of definite zone of inhibition of any size around the cup indicated antibacterial activity. The solvent control was run simultaneously to assess the activity of dimethyl sulphoxide and water which were used as a vehicle. The experiments were performed three times. The diameter of the zone of inhibition was measured and recorded.[4-7]

Cell viability- MTT Assay

MATERIALS AND EQUIPMENTS

1. A549 Lung adenocarcinoma cell line (Sigma-Aldrich, USA); 2. Culture media (RPMI-1640): Contains 20 ml HEPES, L-glutamine and phenol red with pH >7.2 (Sigma-Aldrich, USA); 3. MTT powder (Sigma, USA); 4. Dimethyl sulfoxide; 5. Microtiter plate reader (ELISA reader); 6. 96-well microtiter plate (flat-bottomed); 7. Inverted microscope; 8. Sterile falcon tubes (15 mL); 9. Multi-channel pipettes; 10. Serological pipettes; 11. CO2 incubator; 12. Sterile pipette tips; 13. Laminar flow hood; 14. Hemocytometer; 15. Benchtop centrifuge; 16. Centrifuge tubes.

PREPARATION OF REAGENTS MTT stock solution: Dissolve 500 mg MTT powder in 10 mL phosphate buffer solution. Stir the solution with a magnetic stirrer for about 1 hour in the dark. Filter the sterilized solution with a 0.22 mm filter (Millipore, Ireland) and then store it in 10-mL aliquots (50 mg/mL) at -20°C (van Meerloo et al., 2011). The working solution (5 mg/mL) will be prepared on the day of experiment by dilution.[2,5,8,9]

Procedure

In vitro Cytotoxic Activity by MTT assay

Preparation of Herbal extract for the assay

- 0.5 ml of stock (100 mg/ ml) herbal extract was dissolved in 4.5 ml of DMSO for a concentration of 10 mg/ ml.
- The fresh working suspension was filtered through 0.45 µm membrane filter prior to the assay. Using the 1 mg/ ml concentration herbal extract, nine serial doubling dilutions of the extract of 500µl each was prepared in DMSO to get the concentration of the extract as indicated and the diluted extracts will be transferred to 10 wells of a 12 well culture plate.
- 500 µl of 48h culture of MCF 7 cell lines at a concentration of 105 cells/ ml was added to each well.
- Two control wells received only cell suspensions without plant extract. The plate was incubated in a humidified CO2 incubator at 37°C for 4 - 6 h.
- The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.[2-9]

Assay Process

- After incubation, the medium from the well was aspirated carefully and then discarded. Each well was washed with Eagle's Minimum Essential Medium (EMEM) without Fetal

Calf Serum (FCS). 200 µl of MTT solution (5mg MTT/ ml of PBS, pH 7.2) will be added to each well.

- The plate was incubated for 6-7 h at 37°C in a CO2 incubator with 5% CO2. After incubation 1 ml of DMSO was added to each well and mixed with pipette and left for 45s at room temperature.
- Purple formazan was formed in the wells. Cell control and solvent controls were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments.
- The suspension was transferred to a spectrophotometer cuvette and the optical density (OD) was measured at 540nm using DMSO as blank.
- The % cell viability was calculated with the following formula.

  Cell viability % = Mean OD of wells receiving each plant extract dilution / Mean OD of control wells x 100.[2-9]

Determination of IC50

IC50, the concentration of compound required to inhibit 50% cell growth, was determined by plotting a graph of Log (concentration of Extract) vs % cell inhibition. A line drawn from the 50% value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of the compound). The antilog of that value gives the IC50 value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula: $\text{(At} - \text{Ab)}$ % cell survival = ---------- × 100 $\text{(Ac} - \text{Ab)}$

Where, At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells) % cell inhibition = 100 − % cell survival.[2-9]

Data interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

% cell survival= {(At-Ab) / (Ac-Ab)} x100

Where, At= Absorbance value of test compound Ab= Absorbance value of blank Ac=Absorbance value of control

% cell inhibition= 100-cell survival[2-5]

PHYTOCHEMICAL ANALYSIS

For ethanolic and aqueous polyherbal powder mixture

*The phytochemicals found in ethanolic extract are: Phenolic Compounds And Tannins; Phytosterols And Triterpenoids; Saponins; Glycosides; Proteins And Amino Acids.

*The phytochemicals found in aqueous extract are: Carbohydrates; Proteins And Amino Acids; Alkaloids; Glycosides; Phenolic Compounds And Tannins; Phytosterols And Triterpenoids; Saponins.[3]

The above performed tests results are mentioned in Table: 5 Preliminary phytochemical investigation in Results and Discussion.

PHYSICO-CHEMICAL PARAMETERS

Ash Values calculated are: Total ash value, Acid insoluble ash, Sulphated ash value.[15]

The ash values are mentioned in Table: 4 Determination of ash values in Results and discussion.

RESULTS AND DISCUSSION

The polyherbal powder mixture was extracted with Distilled water and Ethanol using the soxhilation and the residue obtained from both solvents are dried. The crude extract used to conduct the various evaluation tests and value as follows.

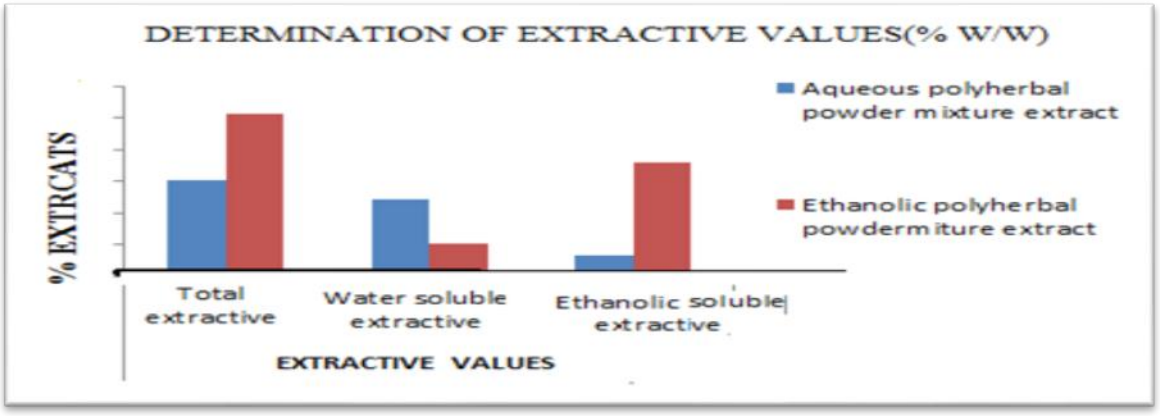
Table 1: Physical Properties of polyherbal powder mixture extracts.

S.No	Name of the extract	Physical status	color
1	Aqueous polyherbal powder mixtureextract	solid	Blackish brown
2	Ethanolic polyherbal powder mixture extract	Semi solid	Greenish black

Determination of Physico-chemical values

Table: 2 Extractive values of polyherbal extract

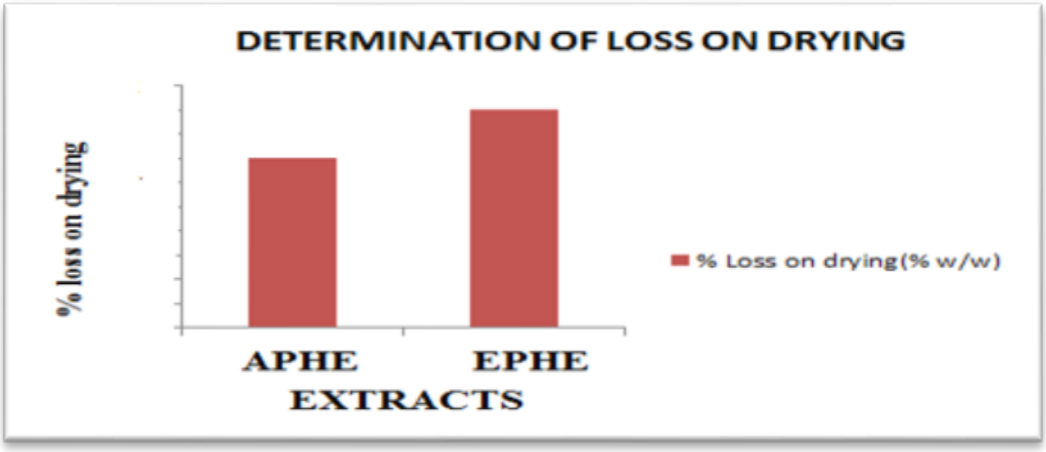
sno	Name of the extract	Total extractive value(% w/w)	Water soluble extractive value(% w/w)	alcohol soluble extractive value (% w/w)
1	Aqueous polyherbal powder mixture extract	0.608	0.4864	0.1314
2	Ethanolic polyherbal powder mixture extract	1.028	0.2084	0.7196



Graph 1: Extractive values of polyherbal extract.

Table 3: Determination of loss on drying.

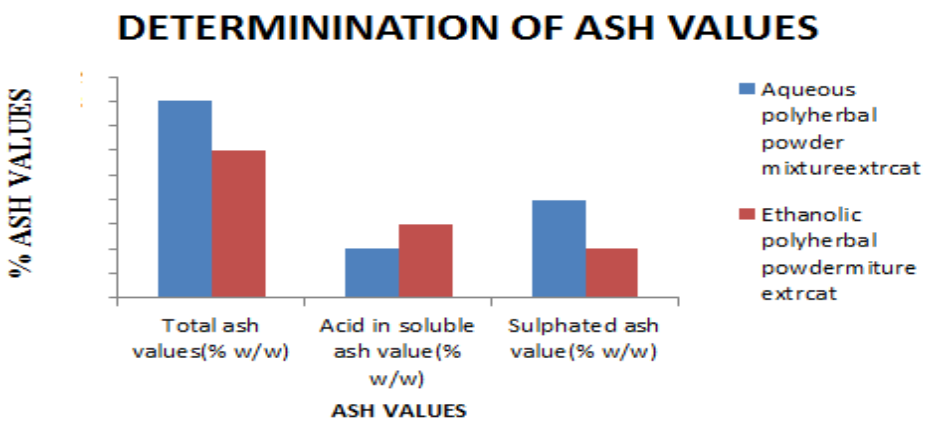
S. NO	Name of the extract	%Loss on drying (% w/w)
1	Aqueous polyherbal powder mixture extract	1.4
2	Ethanolic polyherbal powder mixture extract	1.8



Graph:2 Determination of loss on drying.

Table: 4 Determination of ash values.

sno	Name of extractive	Total ash values (% w/w)	Acid in soluble ash value(% w/w)	Sulphated ash value(% w/w)
1	Aqueous polyherbal powder mixture extract	08	02	04
2	Ethanolic polyherbal powder mixture extract	06	03	02



Graph: 3 Determination of ash values.

Table: 5 Preliminary phytochemical investigation.

s.no	Name of the chemical test	Aqueous polyherbal powder mixture extract	Ethanolic polyherbal powder mixture extract
1	CARBOHYDRATES		
	Molisch's Test:	+	-
	Fehling's Test	+	-
	Barfoed's Test	+	-
	Benedict's Test:	+	-
2	PROTEINS AND AMINO ACIDS		
	Millon's Test:	+	+
	Biurette Test:	+	+
	Ninhydrin Test	+	+
3	ALKALOIDS		
	Mayer's Test:	-	-
	Wagner's Test:	+	-
	Hager's Test:	-	-
	Dragendroff's Test	-	-
4	GLYCOSIDES		
	Borntrager's Test:	+	+
	Legal's Test:	+	+
5	SAPONINS		
	Foam or Froth Test	+	+
6	PHYTOSTEROLS AND TRITERPENOIDS		
	Liebermann – Burchard's test	+	+
7	PHENOLIC COMPOUNDS AND TANNINS		
	Ferric chloride test:	+	+
	Gelatin test:	-	-
	Lead acetate test:	+	+
	Alkaline reagents:	-	-
	Shinoda test or Magnesium – Hydrochloric acid reduction	-	-
8	Oils and fats	+	+

+Positive for the given test :- Negative for given test

Determination of antimicrobial activity using cup plate method

Various concentration of Aqueous and Ethanolic extracts are used to determine the antimicrobial activity using cup plate method and zone of inhibition was determined. the experiment is repeated and average diameter of zone inhibition values are calculated and the value as follows.

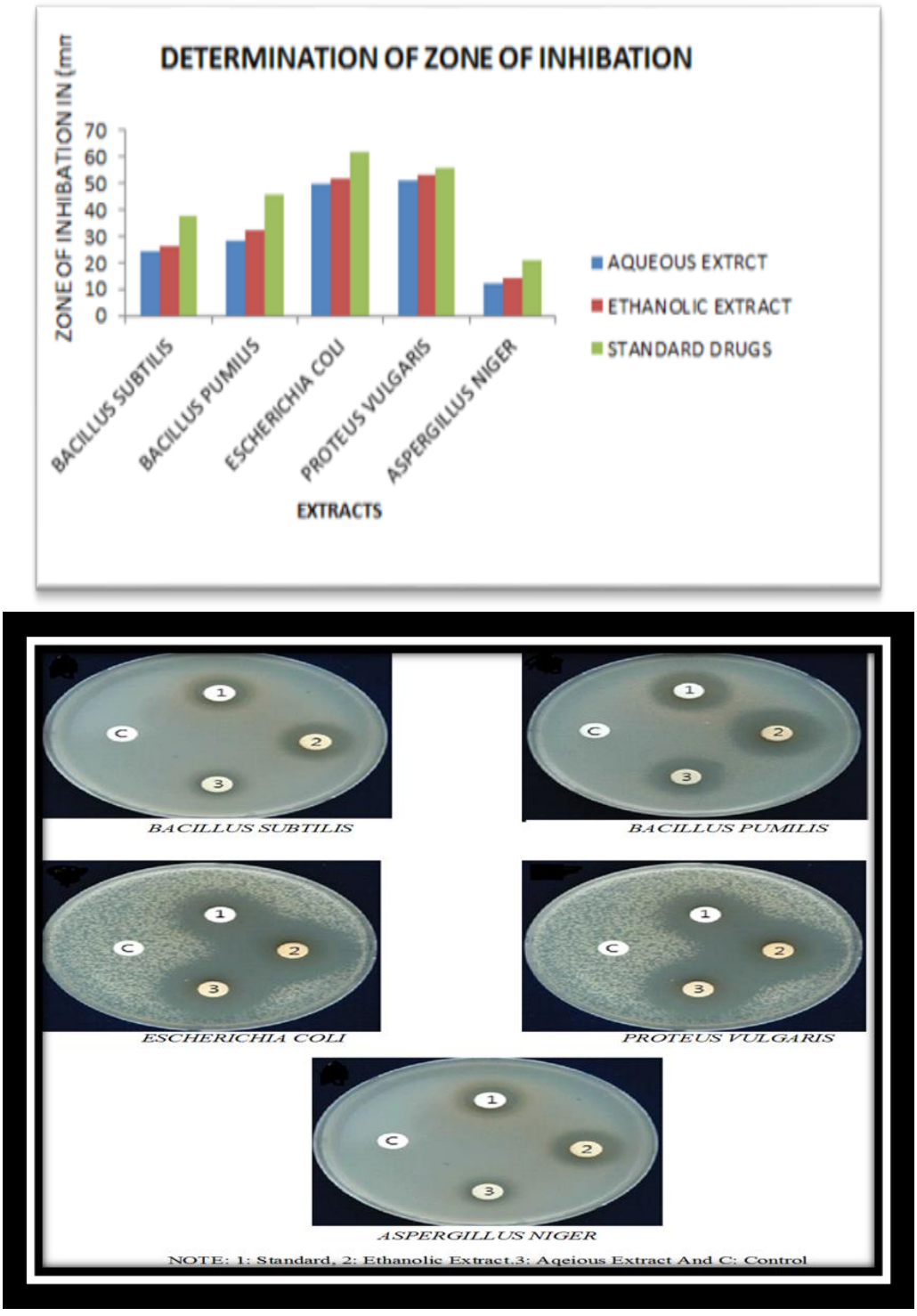


Figure.7 Zone of inhibition.

Table 6: Average Zone of Inhibition (mm) Against Various Microorganisms with Poly Herbal Extracts.

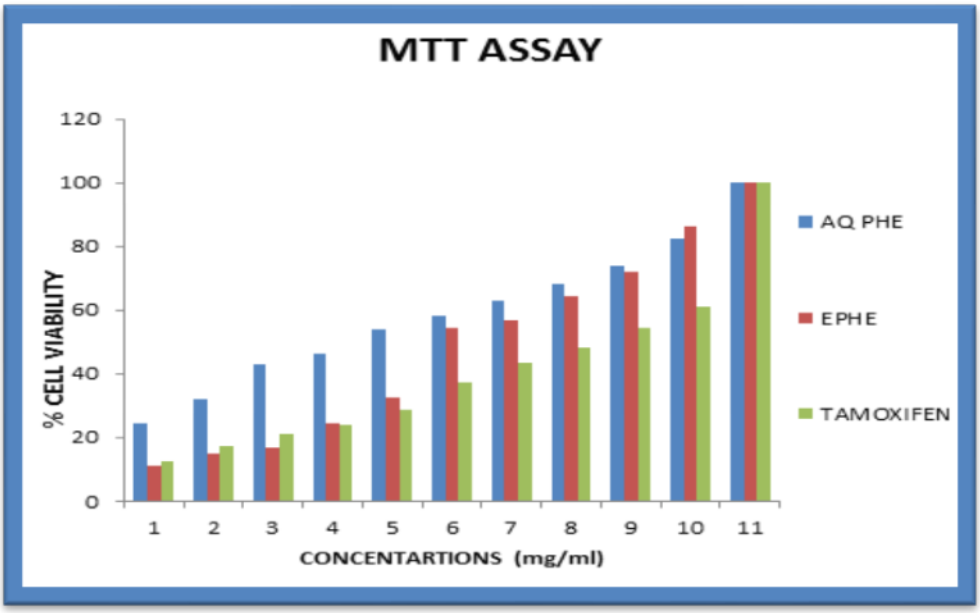
S.No	Name Of The Organisam Tested	Name Of The Polyherbal Extract			
		Aqueous	Ethanolic	Standard	Control
1	*Bacillus Subtilis*	24±1.1	26±0.59	38±1.24	0
2	*Bacillus Pumilis*	28±1.5	32±1.43	46±1.21	0
3	*Escherichia Coli*	50±1.56	52±1.41	62±1.42	0
4	*Proteus Vulgaris*	51±1.34	53±1.59	56±1.61	0
5	*Aspergillus Niger*	12±1.24	14±1.52	21±1.19	0

Determination In -vitro cytotoxic activity using MTT assay

This in vitro cytotoxic activity carried against for both aqueous and Ethanolic extracts. The cell viability of mcf7 cancer cell line for these extracts was determined and the values given in the below table.

Table: 7 *In vitro* Cytotoxic Activity of Aqueous and Ethanolic extract of Poly herbal powder mixture On Breast Cancer Cell Lines (MCF 7 CELLLINE).

S. NO	CONCENTRATION (MG/ML)	DILUTIONS	IN VITRO CYTOTOXIC ACTIVITY		TAMOXIFEN
			AQ PHE	EPHE	
1	1000	NEAT	24.56 ±0.59	11.32 ±0.25	12.33 ± 0.51
2	500	1:01	32.23 ±0.32	14.96 ±0.89	17.18 ±1.58
3	250	1:02	42.92 ±0.57	16.96 ±1.23	20.98 ±1.47
4	125	1:04	46.35 ±0.44	24.36 ±0.89	24.13 ±0.55
5	62.5	1:08	53.87 ±0.14	32.59 ±1.05	28.78 ±0.87
6	31.25	1:16	58.23 ±0.57	54.55 ±1.47	37.17 ±0.69
7	15.625	1:32	62.77 ±0.22	56.79 ±1.69	43.46 ±0.54
8	10	1:64	68.02 ±1.06	64.32 ±0.58	48.19 ±1.25
9	7.8125	1:128	74.12 ±1.08	72.19 ±0.47	54.33 ±1.06
10	3.125	1:256	82.64 ±1.89	86.23 ±0.55	61.22 ±0.88
11	CELL CONTROL	-	100	100	100



Graph: 4 *In- vitro* Cytotoxic Activity of Aqueous and Ethanolic extract of Poly herbal powder mixture On Breast Cancer Cell Lines (MCF 7 CELLLINE).

DISCUSSION

In the past decade there has been renewed attention and interest in the use of traditional medicine globally. Medical plants continue to provide health security to millions of rural people all over the world. According to WHO estimated over 80% of people in developing countries depend on traditional medicines for their primary health needs. In India the coverage of rural population by the modern health system varies between different regions from three to thirty percent. Millions of rural households in India use medicinal plants in a self-help mode. Thus, for some 4-5 million people, traditional medicine is the only alternative source of health in the absence of the ailing government run healthcare systems. They are supported by over one million traditional, village based carriers of the herbal medical traditions. Medicinal plants represent a rich source of anti-microbial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs.

Nowadays, multiple drug resistant strains have been developed due to the indiscriminate use of commercial anti-microbial drugs commonly used for infectious diseases treatment. Unfortunately, bacteria have the genetic ability to transmit and acquire resistance to drugs and chemicals. Beyond the increasing prevalence of antibiotic resistance among pathogenic bacteria, undesirable side effects of some synthetic antibiotics add urgency to the search for new infection-fighting strategies, as well. Scientists and pharmaceutical industries consider medicinal plants as a good choice, because these natural resources have ordinary fewer side

effects, are costless and effective against broad spectrum of antibiotic resistant bacteria. In many parts of the world, the extracts of medicinal plants are used for their antibacterial, anti-fungal and anti-viral properties. Plant species used in folk medicine are potential for discovering extracts with active biological compounds that have anti-bacterial activity.

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vitro anti-bacterial activity assay. Due to the increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents have lead to the screening of several medicinal plants for their potential anti-microbial activity therefore the present study was conducted to evaluate the anti-bacterial and anti-fungal activity for extract of poly herbal powder mixture containing *Maluspumiplus* (Apple) powder. *Momardicachirata*(Karela) powder, *Citrus limon*(Lemon) powder, *Pleurotusostreatus*(Mushroom)powder, and *Triticumaestivum (wheat grass).*

The above polyherbal powder was extracted with distilled water and ethanol as solvents and the physical, physicochemical parameters like extractive values (Table: 1&2), Loss on drying (Table: 3), Ash values (Table: 4). This Physicochemical Values will be Considered as The Primary Parameters for standardization of herbal formulation according to the WHO guidelines. The preliminary phytochemical investigation gives evidence the extract having various bioactive phytochemical which are responsible for the pharmacological activities.

The aqueous and Ethanolic extracts are evaluated for its antimicrobial activity using the cup plate method and the results tabulated in the Table: 6 and diameter of the zone of inhibition was calculated. The results showed that both extracts having good anti-microbial activity by comparing with the standard drug as Chloramphenicol. In other experimental procedure both extracts are evaluated for its in vitro-cytotoxic activity on MCF 7using MTT assay both extracts are having high in vitro cytotoxic activity when compared with standard drug Tamoxifen.

CONCLUSION

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their anti-microbial activity may provide new antimicrobial substances, hence in the present investigation the anti-bacterial and antifungal activity of Maluspumiplus (Apple) powder. Momardicachirata(Karela) powder, Citruslimon(Lemon) powder,

Pleurotusostrea-tus(Mushroom) powder, and Triticumaestivum (wheat grass) polyherbal powder mixture extracts of both aqueous and ethaonolic extract has been demonstrated for the first time against phytopathogenic bacteria. Thus these plants can be utilized as an alternative source of useful drugs. Further studies are needed with this plant to isolate, characterize and elucidate the structure of the bioactive compounds of this plant for industrial drug formulation.

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