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Preliminary Phytochemical Studies, Evolution of antimicrobial and *in vitro* cytotoxic studies of selected polyherbal mixture of dietary importance

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Abstract

Background: In the present study we were evaluated pharmacognostical; antimicrobial and cytotoxic studies of the aqueous and Ethanolic extracts of the dietary polyherbal powders because they are great importance in the treatment of various metabolic disorder. Materials and methods: the following powder mixtures of *Malus pumiplus* (fruit), *Momordica chirata* (fruit) powder, *Citrus limon* (fruit), *Pleurotus ostreatus* (Mushroom), and *Triticum aestivum (wheat grass)* were extracted with distilled water and with ethanol. Results and conclusion: The result indicates that the above solvents having better extractive values with good bioactive components like Polyphenols, Glycosides, and Saponins. These are responsible bioactive phytochemical used in the herbal therapy. The aqueous and the Ethanolic were evaluated for The anti-microbial activity by cup plate method and the diameter of zone of inhibition is nearly equal to the standard drug and both the extracts showed potential cytotoxic activity through the in-vitro cytotoxic studies by MTT assay.

Keywords: Dietary powders, preliminary phytochemicals, antimicrobial, *in-vitro* MTT assay.

Introduction

Many countries plants are used as a source of medicines to treat infections and other disorders and some of the most powerful and potent drugs used nowadays were derived from plants. Various parts of plant (root, fruit, stem, flower, modified plant organs and twigs exudates) having different therapeutic properties are used as herbal medicines[1]. They are collected on a minute scale to utilize by folk healers and local communities, while several others are collected as raw material in larger amounts to trade them in the market for numerous herbal industries[2]. Natural bioactive compounds in plants play a key role in plants defense system and are also well known for their unambiguous physiological action on human body. Amino acids, proteins common sugars and chlorophyll are plants primary metabolites whereas secondary metabolites comprise of flavonoids, alkaloids, tannins, saponins, and terpenoids.

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In view of the tremendous importance of secondary metabolites as therapeutic agents; they are becoming parts of the integrative health care system as alternative or supportive medicines [3].

Material and methods

Collection and authentication

We are purchased the following material from local licensed herbal supplier in Vijayawada *Malus pumiplus* (Apple) powder. *Momordica chirata* (Karela) powder, *Citrus limon* (Lemon) powder, *Pleurotus ostreatus* (Mushroom) powder, and *Triticum aestivum (wheat grass)* powder. The above materials were preserved in our Pharmacognosy lab for the further experimental purpose.

Chemicals and requirements

Human Brest cancer cell lines(MCF7)(Sigma-Aldrich, USA),Culture media (RPMI-1640): Contains 20 mM HEPES, L-glutamine and phenol red with pH >7.2 (Sigma-Aldrich, USA) ,MTT powder (Sigma, USA), DMSO, Microliter plate reader (ELISA reader) ,96-well microliter plate (flat-bottomed) etc.,

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[4].

Evaluation of Preliminary phytochemical and physicochemical properties of crude extracts

The above aqueous and Ethanolic extract were used for preliminary phytochemical studies according to standard guidelines [5-8].

Evaluation of antimicrobial activity of crude extracts

The term microbiological assay is a biological assay performed with micro-organisms like Bacteria, Yeast, Moulds, etc. This involves the measurement of the relative potency or activity of compounds by determining the amount of test material required for producing stipulated effect on suitable organism under standard conditions.

The procedures employed in microbial assay. (Cup Plate Method)

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

In cup plate method, the antimicrobial substance diffuses from the cup through a solidified agar layer in a Petri dish or a plate to an extent so that the growth of added micro-organism is inhibited entirely in a circular area or zone around the cavity containing the solution of a known quantity of antimicrobial substance. The antimicrobial activity is expressed as the zone of inhibition in millimeters, which is measured with a zone reader [9].

Were screened for antimicrobial activity against a wide spectrum of micro-organisms and the activity was compared with appropriate reference standards (Penicillin for both gram-positive and gram-negative organisms and fluconazole for fungal strains).

Cell viability- MTT Assay

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, the working solution (5 mg/mL) will be prepared on the day of experiment by dilution(10).

Experimental Procedure for *In vitro* Cytotoxic Activity by MTT assay

The cell lines were prepared and cryopreserved using reagents such as DMSO which preserve the cell during freezing. DMSO is toxic at room temperature. The frozen ampoule is brought to room temperature by slow agitation (thawing). The frozen cry vials plunged into the water bath and is rapidly thawed until it gets liquefied. Solution, centrifuged with saline for 10 min to remove the DMSO. The saline is discarded and aliquot is taken for cell counting, cell viability and for sub-culturing. MTT assay is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of living cells. The assay was carried out using (3-(4, 5dimethyl thiazol-2yl) - 2, 5-diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity.

Preparation of Herbal extract for the assay

i. 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.
ii. 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.
iii. 500 µl of 48h culture of MCF 7 cell lines at a concentration of 105 cells/ ml was added to each well.
iv. 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.
v. The plate was microscopically examined for confluent monolayer of cells, turbidity, and toxicity.

Assay Process

i. 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.
ii. 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.

iii. 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.
iv. The suspension was transferred to a spectrophotometer cuvette and the optical density (OD) was measured at 540nm using DMSO as blank.
v. The % cell viability was calculated with the following formula:
 a. Cell viability % = Mean OD of wells receiving each plant extract dilution / Mean OD of control wells x 100.

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:

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

Where, At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells) % cell inhibition = 100 − % cell survival

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

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.

Physical nature of crude extract and Extractive values of powder mixture

The physical and extractive values are tabulated respectively in Table 1 and 2

Physic chemical properties of extracts

The physic-chemical values like loss on drying and ash values are tabulated in Table 3 and 4

Preliminary phytochemical studies

The Above extracts are subjected to test for identification various phytochemicals according to standard procedures and result showed in Table .5

In -vitro Antimicrobial activity of polyherbal extract.

Various concentrations 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 showed in Table.6and Figure 1 & 2.

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 7.

Discussions

In the past decade, there has been renewed attention and interest in the use of traditional medicine globally[11]. 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[12] In India, the coverage of rural population by the modern health system varies between different regions from three to thirty percent[13-15].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 antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs [16-18].

Nowadays, multiple drug-resistant strains have been developed due to the indiscriminate use of commercial antimicrobial 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 antibacterial activity[19-21].

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents[22]. The first step towards this goal is the *in vitro* antibacterial activity assay[23,24].Due to the increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents have Leading compound to the screening of several medicinal plants for their potential anti-microbial activity(25, 26).Therefore the present study was conducted to evaluate the antibacterial and anti-fungal activity for extract of polyherbal powder mixture containing *Malus pumiplus* (Apple) powder. *Momordica chirata* (Karela) powder, *Citrus limon* (Lemon) powder, *Pleurotus ostreatus* (Mushroom) powder, and *Triticum aestivum (wheat grass).*[27,28]

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 (Table.5) which are responsible for the pharmacological activities[29-31].

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 it's *in vitro*-cytotoxic activity on MCF 7using MTT assay both extracts are having high *in vitro* cytotoxic activity when compared with standard drug Tamoxifen (Table.7).

Table: 1 Physical Properties of polyherbal powder mixture extracts

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

Table: 2 Extractive values of polyherbal extract

S.No	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 Powdermixture extract	1.028	0.2084	0.7196

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 miture extract	1.8

Table: 4 Determination of ash values

S.No.	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

Table: 5 Preliminary phytochemical investigation

S.No	Name of the chemical test	AQPHE	EPHE
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

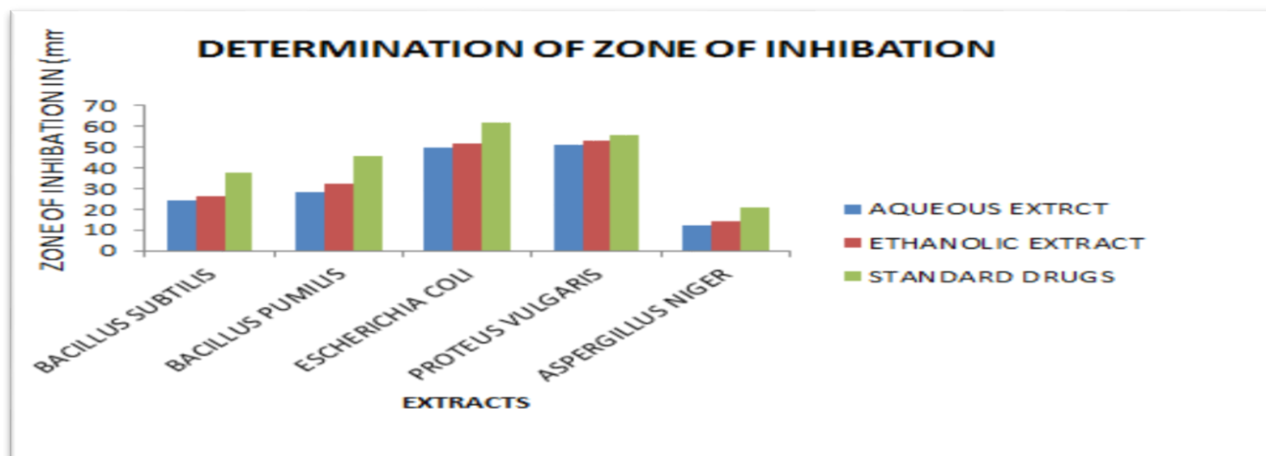
Table:6 Average Zone of Inhibition (mm) Against Various Microorganisms with Poly Herbal Extracts

S.No	Name of the organism tested	Average zone of inhibition (mm) against various microorganisms with poly herbal extracts			
		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	*Aspergillusniger*	12±1.24	14±1.52	21±1.19	0

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	Dilutions	*in vitro* cytotoxic activity		
			Aq.extract	**Eth.extract**	**Tamoxifen**
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

Figure 1: Average Zone of Inhibition (mm) Against Various Microorganisms with Poly Herbal Extracts



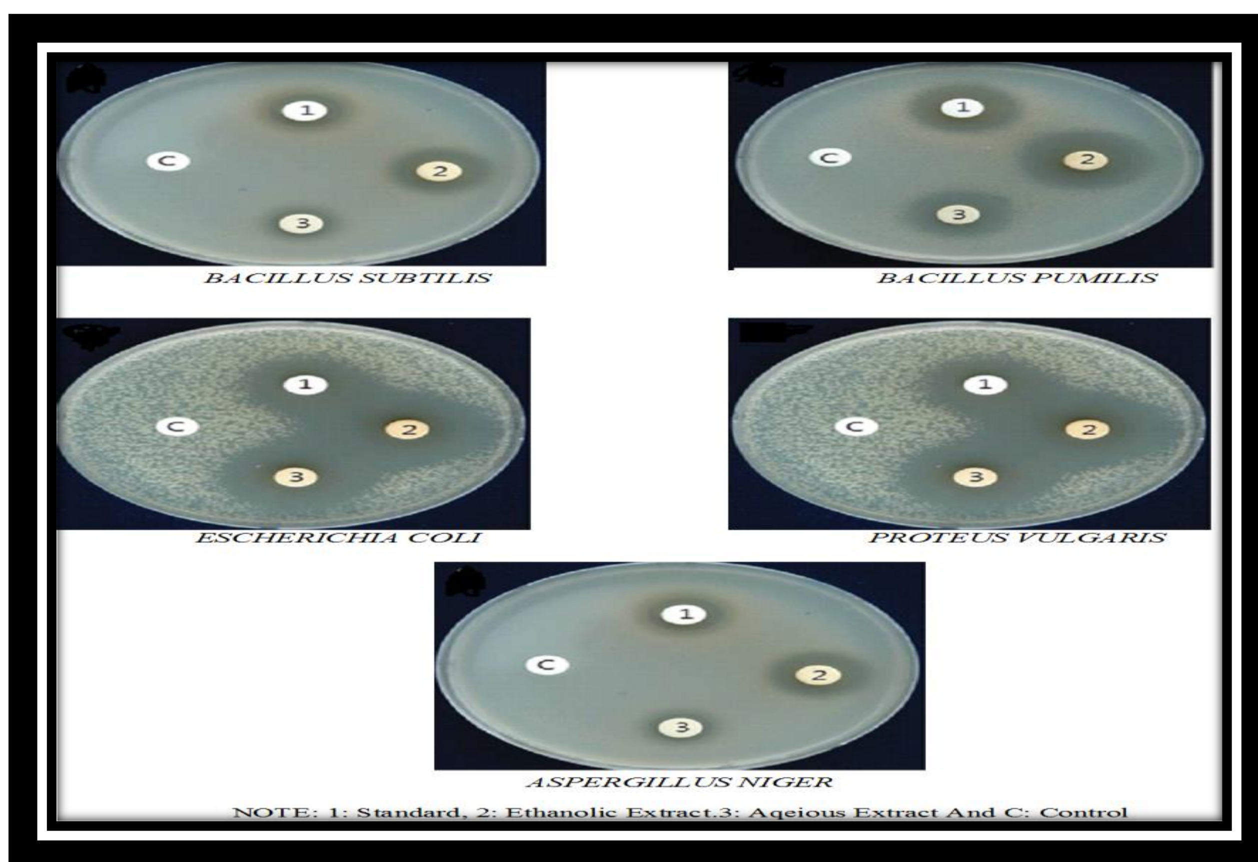


Figure.2: Zone of Inhibition

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 antibacterial and antifungal activity of *Maluspumiplus* (Apple) powder. *Momordica chirata (Karela)* powde*r, Citrus limon* (Lemon) powder, *Pleurotusostreatus*(Mushroom)powder,and*Triticumaes tivum*(wheat grass) polyherbal powder mixture

Extracts of both Aqueous and Ethanolic has been demonstrated for the first time against phytopathogenic bacteria. Thus these herbal powders and its combinations can be utilized as an alternative source of useful drugs. Further research is required with this polyherbal mixture to isolate potentate bioactive molecules, characterize and elucidate the structure of the bioactive compounds of this plant for industrial drug formulation.

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