Indian Journal of Drugs, 2018, 6(2), 112-127 ISSN: 2348-1684

Research Article

EVALUATION OF ANTHELMINTIC POTENTIALS OF NEWLY DEVELOPED STANDARDIZED ORAL POLYHERBAL FORMULATION

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Received: 08.12.2017
Accepted: 22.06.2018

Access this article online

Website:
www.drugresearch.in

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ABSTRACT

Objective: The present study was designed to develop a safe, effective formulation comprising extracts of *Aegle marmelos* fruits, leaves of *Psidium guajava* and seeds of *Manilkara zapota* as anthelmintic agent in the form of convenient, stable dosage form, standardized to establish scientific evidence for anthelmintic activity. **Methods:** Pharmacognostic evaluation, successive solvent extraction, phytochemical screening of extract was done. Antioxidant activity by DPPH assay and *in-vitro* anthelmintic activity of extracts and its mixtures, was carried out. Herbal formulation comprising mixture of extracts was developed, evaluated for physicochemical properties and anthelmintic activity. **Results:** Ethanolic extracts of *Aegle marmelos*, *Psidium guajava* and *Manilkara zapota* exhibited highest percentage of inhibition by DPPH assay. *Psidium guajava* exhibited highest percentage of inhibition (93%) followed by *Manilkara zapota* (54%) and *Aegle marmelos* (37%). *Manilkara zapota* showed better anthelmintic results with less paralysis time (8-9mins) and death time (12-15mins), followed by *Psidium guajava* (20 -55mins) and *Aegle marmelos* (32 and 68mins). Antioxidant and anthelmintic potentials of combined herbal mixture was also effective than that of individual extracts. 1% of herbal mixture comprising extracts *Aegle marmelos*, *Psidium guajava and Manilkara zapota* developed as oral polyherbal formulation. **Conclusion:** The studies revealed that the herbal extract mixture selected for formulation was effective with *in-vitro* anthelmintic activity. Developed polyherbal oral formulation consisting of extracts mixture of three plants was also found to be effective as anthelmintic agent by *in-vitro.*

KEY WORDS: Antioxidant, Anthelmintic, central composite design DPPH, polyherbal.

INTRODUCTION

Helminthiasis is a macroparasitic worm disease of humans and animals infested with worms like pinworm, roundworm or a tapeworm. Helminths are the most common infectious agents of humans in developing countries like India, causing a global burden of disease and contribute to prevalence of malnutrition, anemia, eosinophilia and pneumonia. Patel J et al. (2012) Diseases caused by helminthes are of chronic and debilitating in nature. Weakness, loss of appetite and decreased feed efficiency along with infection of intestinal worms leads to parasitic gastroenteritis and there are number of pathways by which parasite burden may affect congnition Arvind K et al. (2010). Helminths are divided into three groups: Cestodes (tapeworms); Nematodes (roundworms); and Trematodes (flukes). The helminths differ from other infectious organisms in that they have a complex body structure. They are multicellular and have partial or complete organ systems like muscular, nervous, digestive, and reproductive. Anthelmintics are the drugs that acts against infections caused by parasitic worms, which are generally administered orally. Anthelmintics acts by mechanism such as: a)

Affecting the nervous system followed by muscle paralysis. b) Affecting glucose uptake and thus disturbing with energy mechanism. (Lippincott, 2000). Even though many allopathic drugs are effectively used as anthelmintics, many are associated with toxic effects to the host. It is also reported that there is increased resistance by helminthes parasites for the synthetic drugs. Side effects caused by synthetic drugs include vomiting, headache, abdominal pain and diarrhoea. The Indian medicinal system viz., Ayurveda, Siddha and Unani system predominantly use plant based raw materials in most of their preparations and formulations. (Irfan AK, 2007). As per WHO survey around two billion people are reported to be suffering from parasitic worms. Developing nations which suffer most, from this tropical disease have a little to invest in anthelmintic drug discovery and hence the therapy. Plants are known to provide a rich source of botanical anthelmintics. We here in propose to explore scientifically the anthelmintic potential of three traditionally used medicinal plants of India and an effort to substantiate the folklore claims. (Neha S, 2010). In this regard, after thorough review, three medicinal plants are selected for the present study, which are *Aegle marmelos* (whole fruit), *Psidium guajava* (stem and leaves) and *Manilkara zapota* (seeds). *Aegle marmelos* commonly called Bael, belongs to family Rutaceae. Fruits are used in the treatment of diarrhea, dysentery and also reported to have the anthelmintic activity. Gangadhara A et al. (2013). *Psidium guajava L*. belonging to family Myrtaceae is also known as Amrut or Peyara in common. Guava leaves decoction is found to have antibacterial, antioxidant and also anthelmintic activities. Mohamed I et al. (2012). *Manilkara zapota* known as Sapodilla and belongs to family Sapotaceae, common names are Sapota or Chikoo. Medicinal uses of Sapodilla extracts are analgesic, antioxidant and anthelmintic. Pankaj K et al. (2011). Even though, the selected plants are reported as anthelmintic agents traditionally, not much scientific data is available with regard to anthelmintic potentials of the selected parts of the plant especially in combination; hence the present study is designed: -To evaluate *in-vitro* anthelmintic activities of individual extracts and compare the activity with their combination. - To develop and standardize liquid oral formulation comprising of combined extracts and its evaluation in *in-vitro* anthelmintic activity.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Collection and authentication of plant material

Dried fruits of *Aegle marmelos* was obtained from local market Amruth kesari, Bangalore. Fresh leaves and stem parts of *Psidium guajava* and seeds of *Manilkara zapota* were collected in Bangalore. Identification and authentication of all three plants and their parts was done at Ayurveda Dietetics Research Institute, Jayanagar, Bangalore. Photographs of selected medicinal plants are shown in **Fig no. 1 to 3.**

2.2. Evaluation of crude drugs

2.2.1. Organoleptic and Macroscopic evaluation

The aerial parts of samples were subjected to macroscopical identification based on Colour, odour, taste and shape of the drug. Morphological observations are recorded in **Table no 2.**

2.2.2. Microscopic evaluation

Powders of the different parts were examined for microscopic features using chloral hydrate, phloroglucinol - HCl and iodine mounts.

2.2.3. Proximate analysis:

Physicochemical constituents:

Organic and inorganic constituents present in a drug or plants play a significant role in identification of crude drugs. Physical constants like ash value, moisture content and extractive values help in establishing the pharmacopoeial standards of drug. The organic analysis helps to understand the organic constituents of drug, while chromatographic profile may be compared with authentic samples

which in turn help in identification of drug. Analysis was carried out in triplicate and mean ± SEM is given in **Table no. 3 and Graph no. 1.**

a) **Determination of moisture content by Loss on drying method:** About 3g of the drug was weighed accurately in a watch glass, kept in hot air oven at 105◦c and dried for a period until constant weight was obtained. Weight loss on drying was noted and difference in weight gives the moisture content of powdered drug. Total moisture content of crude drug was noted.
b) **Determination of ash value**: About 3 g of powdered drug was weighed accurately and placed in tarred silica crucible and incinerated at 450^0C in muffle furnace until free from carbon. Crucible was cooled, kept in a desiccator and weighed. Same procedure was repeated to arrive at constant weight. The percentage of total ash obtained was calculated with reference to the air dried drug. Total ash values of powdered crude drugs were recorded.
c) **Alcohol soluble extractive value:** About 5 g of coarse powder (60-80 mesh) of the crude drug (shade dried) was weighed and macerated in iodine flask with 100 ml of 70 % v/v alcohol, for a duration of 24 hours, with frequent shaking. Solution was filtered rapidly; taking precaution against loss of alcohol, 25 ml of filtered solution was evaporated to dryness at 105^0C in a tarred flat bottomed Petridis. The percentage of alcohol soluble extract was determined with reference to the shade dried drug.
d) **Water soluble extractive value:** About 5 g of coarse powder (60-80 mesh) of the crude drug (shade dried) was weighed and macerated in iodine flask with 100 ml of chloroform-water, for a duration of 24 hours with frequent shaking and finally allowed to stand for 18 hrs. Solution was filtered rapidly; 25 ml of filtered solution was evaporated to dryness at 105^0C in a tarred flat bottomed Petridish. The percentage of alcohol soluble extract was determined with reference to the shade dried drug.

2.2.4. Preparation of extracts

Successive solvent extraction of plant materials: Fruits of *Aegle marmelos*, Stems and leaves of *Psidium guajava* and seeds of *Manilkara zapota* were air dried at room temperature, powdered in blender and passed through sieve size # 12. Approximately 50 gm of sieved powder was weighed accurately and subjected to extraction by successive solvent extraction method using Soxhlet apparatus. The extracts prepared were used for phytochemical evaluation and bioassays.

Procedure: A known quantity of powdered drug was extracted in a soxhlet apparatus successively with solvents of increasing polarity; viz. petroleum ether (60-80ºC), Chloroform and 95% alcohol and refluxation with water for 8 hours. The extract was collected and each time before successive extraction with next solvent the powdered material was air dried. All the extracts were filtered through Whattmann filter paper number 1 and then concentrated at low temperature (40-50°C). The extracts were then air dried. The percentage yield of the extracts was calculated. The yield, colour and consistency of the extracts were noted. The results are tabulated in **Table no. 5**. A graphical representation of yield and nature of extracts used is shown in **Graph no. 2**.

3. ANTIOXIDANT ACTIVITY

3.1 Antioxidant activity of individual extracts: Antioxidant activity of all the individual extracts was carried out by DPPH [2, 2(diphenyl-1-picryl hydrazyl)] reduction method in duplicate.

Procedure: 2.36mg of the DPPH was dissolved in 100ml of methanol to get $6x10^{-5}$m methanolic solution of DPPH. Different concentrations of plant extract of 50, 100 and 150µg/ml were prepared by diluting with methanol. 1ml of the diluted test samples were mixed with 3ml of DPPH solution in each test tube. Test tubes were covered with aluminium foil to protect from light and kept in dark place. Methanol was used as a blank and absorbance was taken at 517nm on UV-Visible Spectrophotometer

(Shimadzu, UV-1601, Japan). The % of inhibition was calculated by using following formula and compared with the values of standard ascorbic acid. Mensor LL et al. (2001) **[Table no. 5 to 7, Graph no. 3 to 5]**

$$\% \text{ inhibition of DPPH activity} = \{\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\} \times 100$$

4. *IN-VITRO* ANTHELMINTIC ACTIVITY OF EXTRACTS

4.1 Preparation of Standard solution: Reference standard of Piperazine citrate was obtained from Adani pharmaceuticals along with Certificate of Analysis. Concentration of 15mg/ ml of Standard was prepared by dissolving with distilled water.

4.2 Preparation of Sample solution: Ethanolic extracts of *Aegle marmelos, Psidium guajava* and *Manilkara zapota* with better anti-oxidant activity were taken and different concentrations (25, 50, 100mg/ml) of crude ethanolic extracts were prepared by triturating in distilled water. Bhawana S et al. (2011)

4.3 Anthelmintic Assay for individual extracts: Anthelmintic assay was carried as per the method of Ajaiyeoba. Assay was performed on earthworms, due to its anatomical and physiological resemblance with the intestinal roundworms parasite of human being. Easy availability of earthworms have made wide usage for initial evaluation of anthelmintic compounds *in-vitro*. Petri dishes of equal sizes were taken and numbered. 50ml of different concentrations of the plant extracts were placed in nine Petri dishes and 50ml of standard was placed in tenth petridish. Each group having 6 earthworms of similar sizes were placed in Petri dishes. All the Petri dishes were placed at room temperature. Time of paralysis was noted when no moment of any sort could be observed except when the worms were shaken vigorously and the time for death recorded after ascertaining that worms neither moved when shaken vigorously, nor when they were dipped in warm water (50^0C). **(Table no. 8, Graph no. 6.)**

5. ANTIOXIDANT ACTIVITY OF HERBAL MIXTURE COMPRISING ETHANOLIC EXTRACT OF *AEGLE MARMELOS, PSIDIUM GUAJAVA* AND *MANILKARA ZAPOTA*

Antioxidant activity of herbal mixture comprising ethanolic extract of *Aegle marmelos, Psidium guajava* and *Manilkara zapota* was carried out by DPPH (2,2diphenyl-1-picryl hydrazyl) reduction method in duplicate. **(Table no. 9, Graph no. 7)**

Preparation of mixture: Selected extracts were accurately weighed according to CCD ratios by using design expert 6.05 versions (Stat-ease Ind., Minneapolis, Minnesota), as given in Table no.2. Different concentrations of 18 combinations were prepared by triturating the mixture. Further procedure was followed as described earlier for antioxidant activity by DPPH method. Mensor LL et al. (2001)

The analysis of variance (ANOVA) information for the combination of *Aegle marmelos, Psidium guajava* and *Manilkara zapota* was generated. The response surface and the contour plots were obtained by the statistical experimental method for the different combinations, which reveals the effect of different factors i.e., *Aegle marmelos, Psidium guajava* and *Manilkara zapota* on the percentage of inhibition and hence the antioxidant activity.

6. *IN-VITRO* ANTHELMINTIC ACTIVITY OF EXTRACTS–COMBINED MIXTURES

6.1. Preparation of solution of extract mixture: Optimized combination of extracts for antioxidant activity was taken in three different concentrations (25, 50, 100mg/ml). Anthelmintic assay was carried out for combined extracts as explained above. Time of paralysis and time of death were recorded. Prakash V et al. (1980)

6.2. Anthelmintic Assay for combined extracts: Petridish of equal sizes were taken and numbered. 50ml of combined mixture with concentrations of the plant extracts and standard were placed in respective Petri dishes having 6 earthworms of similar sizes were placed. Petri dishes were placed at room temperature. Time of paralysis was noted when no moment of any sort could be observed except

when the worms were shaken vigorously and the time for death recorded after ascertaining that worms neither moved when shaken vigorously, nor when they were dipped in warm water (50^0C). **(Table no. 10)**

7. FORMULATION DEVELOPMENT OF POLYHERBAL SYRUP:

7.1. Method of preparation of simple syrup (USP): 666.7 g of Sucrose was weighed and added to purified water and heated until it dissolved with occasional stirring. Sufficient boiling water was added to produce 1000 ml.

7.2. Method of preparation of final herbal syrup: one part of decoction was mixed with five parts of simple syrup (1:5). Required quantity of Methyl paraben was added as Preservative, to the above mixture. Solubility was checked by observing the clarity of solution visually. The final herbal syrup was then subjected for evaluation.

Table no: 1

Ingredients of formulation

SN	Ingredients	Formulation
1	Sugar Syrup	66.66%
2	Methyl Paraben	0.18%
3	Propyl Paraben	0.02%
4	Herbal Mixture	1.00%
5	Distilled Water	QS

PHYSICOCHEMICAL EVALUATION OF THE DEVELOPED HERBAL FORMULATION: Standardization is a system that ensures a predefined amount of quantity, quality & therapeutic effect of ingredients in each dose. Herbal product cannot be considered scientifically valid if the drug tested has not been authenticated and characterized in order to ensure reproducibility in the manufacturing of the product. Moreover, many dangerous and lethal side effects have recently been reported, including direct toxic effects, allergic reactions, effects from contaminants, and interactions with herbal drugs. Therapeutic activity of an herbal formulation depends on its phytochemical constituents hence final herbal syrup was subjected for evaluation. Parameters such as physical appearance (colour, odour, and taste), pH and specific gravity were evaluated. Fatima R et al. (2013), Neeraj C (2011), Vandana G et al. (2012)

a) Colour examination: Five ml final syrup was taken into watch glasses and placed against white back ground in white tube light. It was observed for its colour by naked eye.

b) Odour examination: Two ml of final syrup was smelled individually. The time interval among two sample smelling was kept 2 minutes to nullify the effect of previous smelling.

c) Taste examination: A pinch of final syrup was taken and examined for its taste on taste buds of the tongue.

d) Determination of pH: Placed an accurately measured amount 10 ml of the final syrup in a 100 ml volumetric flask and made up the volume up to 100 ml with distilled water. The solution was sonicated for about 10 minutes. pH was measured with the help of digital pH meter.

e) Specific gravity at 25^0C: A thoroughly clean and dry specific gravity bottle was selected and calibrated by filling it with recently boiled and cooled water and weighing the contents at 25^0C. Assuming that the weight of 1 ml of water at 25^0C when weighed in air of density 0.0012 g/ml was 0.99602g. The capacity of the specific gravity bottle was calculated. Adjusting the temperature of the final syrup to about 20^0C and the specific gravity bottle was filled with it. Then the temperature of the filled specific gravity bottle was adjusted to 25^0C, any excess syrup was removed and weight was

taken. The tare weight of the Specific gravity bottle was subtracted from the filled weight. The weight per millilitre was determined by dividing the weight in air, expressed in g, of the quantity of syrup which fills the specific gravity bottle at the specified temperature, by the capacity expressed in ml, of the specific gravity bottle at the same temperature. Specific gravity of the final syrup was obtained by dividing the weight of the syrup contained in the specific gravity bottle by the weight of water contained, both determined at 25^0C

g) Stability testing: Stability testing of the prepared poly-herbal syrup was performed on keeping the samples at accelerated temperature conditions. Portions of the final syrup were taken in amber coloured glass bottles and were kept at accelerated temperature at 4^0C, room temperature and 47^0C.

8 RESULTS

PHARMACOGNOSTIC EVALUATION OF SELECTED PARTS OF MEDICINAL PLANTS

8.1 Macroscopic examination: Macroscopic characters of the dried fruits of *Aegle marmelos*, leaves and stems of *Psidium guajava* and seeds of *Manilkara zapota*: such as colour, odour, size, shape, surface characters and texture were examined. **(Table no. 2)**

Table no 2: Macroscopic characters of selected medicinal plants

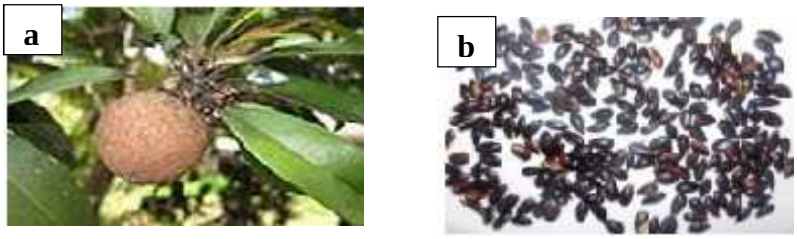
SN	Macroscopy	*Aegle marmelos*	*Psidium guajava*	*Manilkara zapota*
1	Colour	Brown	Green pale yellow	Deep brown- black
2	Odour	Aromatic odour	Slightly sweet aroma	Characterisitc
3	Taste	Muciligeneous	Astringent and has a refreshing taste	Bitter
4	Size (cm)	5-6	9-15	2-2.5
5	Shape	Round	Dorsiventral, Oblong-elliptic shape, short petiole	Hard, Bean shaped, shiny, hook at one end, flattened
6	Texture	Rough	Rough	Smooth

Photographs of selected medicinal plants

Fig no. 1 Photographs of *Aegle marmelos*: a) Fruits b) Dried Fruits

Fig no. 2 Photographs of *Psidium guajava*: a) Leaves b) Dried leaves c) Dried stems

Fig no. 3 Photographs of *Manilkara zapota* a) Aerial part b) Dried seeds



8.2 Microscopic examination: Powdered fruits of *A. marmelos*, Leaves of *P. guajava* and seeds of *M. zapota* were observed individually under microscope.

Aegle marmelos: Powdered dried fruits of A. marmelos exhibited presence of parenchyma of the mesocarp with an oil cell, endocarp, and layers of the testa.

Psidium guajava: Dried powder of the leaves contained paracytic stomata, layers of wide rectangular cells, xylem, phloem, secretory cavity, parenchymal cells and fragment of palisade mesophyll.

Manilkara zapota: Analysis of dried powder of seed exhibited crystals like structure, parenchymal cells.

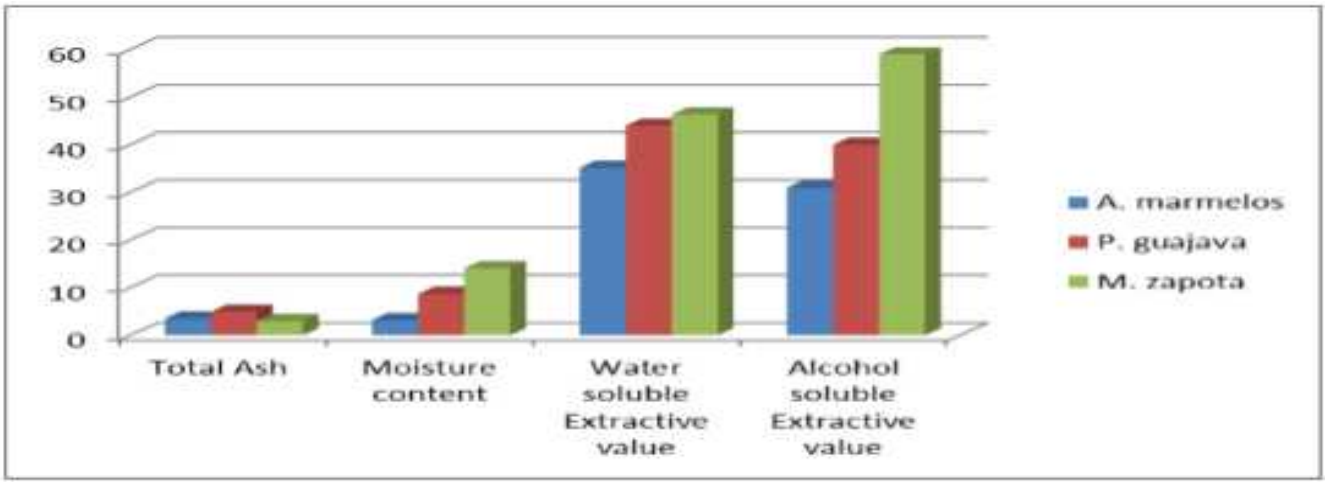
Proximate analysis: The results of proximate analysis such as loss on drying, total ash, alcohol soluble extractive value and water soluble extractive values are shown in Table no 3.

Table no 3: Proximate analysis of aerial parts of selected medicinal plants

SN	Parameters	*Aegle marmelos*	*Psidium guajava*	*Manilkara zapota*
1	**Total Ash**	3.52±0.15%	4.94±0.12%	3.00±0.17%
2	**Moisture content**	3.21±0.19%	8.70±0.16%	14.04±0.18%
3	**Water soluble extractive value**	35.16±0.2%	44.00±0.21%	46.40±0.18%
4	**Alcohol soluble extractive value**	31.01±0.2%	40.02±0.22%	50.04±0.19%

Values are expressed in terms of Mean± SEM of results done in triplicate.

Graph no. 1: Proximate analysis of *Aegle marmelos*, *Psidium guajava* and *Manilkara zapota*



TA: Total Ash, WSEV: Water Soluble Extractive Value, ASEV: Alcohol Soluble Extractive Value, MC: Moisture Content

Among the three samples studied, *Psidium guajava* exhibited highest ash content followed by *Aegle marmelos* and *Manilkara zapota*. Value for all the three plant materials were within the permissible limits. Moisture content of all the three samples were within the limits of guidelines. Alcohol soluble and Water soluble extractive values are highest in *Manilkara zapota*, followed by *Psidium guajava* and *Aegle marmelos*.

8.3 EXTRACTION OF PLANT MATERIALS

***Aegle marmelos*:** Extracts of dried fruits of Aegle marmelos with solvent Pet. Ether was yellow colour, oily in consistency with 3.95% yield. Chloroform extract and Ethanol extract were brown and sticky in consistency with yield of 0.88% 7.76% respectively. Water extract with yield of 22.44 % was in powder form and brown in colour shown in Table no. 4

Table no 4: Yield and nature of extracts of *Aegle marmelos, Psidium guajava*, *Manilkara zapota*

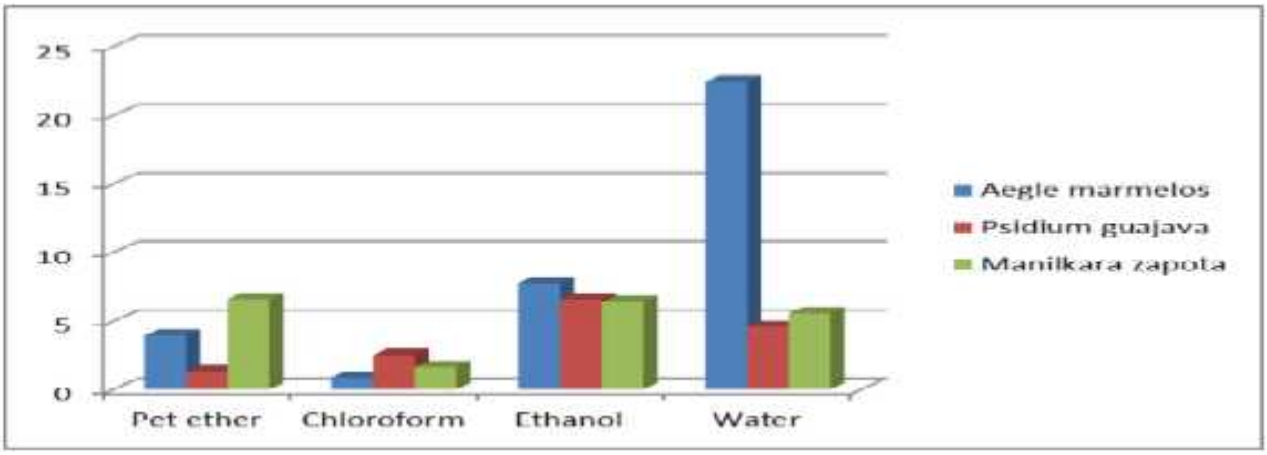
SN	Solvent used	*Aegle marmelos*		
		% yield (g)	Consistency	Colour
1	Pet.ether	3.95±0.05	Oily	Yellow
2	Chloroform	0.88±0.27	Sticky	Brown
3	Ethanol	7.76±0.46	Sticky	Brown
4	Water	22.44±0.9	Powder	Brown
		Psidium guajava		
1	Pet.ether	1.29 ±0.05	Sticky	Green
2	Chloroform	2.51±0.27	Sticky	Blackish green
3	Ethanol	6.58±0.46	Powder	Blackish green
4	Water	4.56±0.43	Powder	Blackish green
		Manilkara zapota		
1	Pet.ether	6.59 ±0.54	Oily	Cream
2	Chloroform	1.61±0.07	Sticky	Brown
3	Ethanol	6.38±0.41	Sticky	Brown
4	Water	5.55±0.23	Powder	Brown

Values are expressed in terms of Mean± SEM of results done in triplicate.

***Psidium guajava*:** Extracts of Psidium guajava dried leaves and stems with solvent Pet. Ether with 1.29% yield was green and sticky. Chloroform extract was sticky with yield of 2.51% and ethanol extract was powder with 6.58% yield. Water extract with yield of 4.56% was powder in consistency. Chloroform, Ethanol and water extracts were green in colour shown in Table no. 4

Manilkara zapota: Different extracts of Manilkara zapota dried seeds with selected solvents Pet. Ether, Chloroform and Ethanol were oily and sticky in nature. Water extract was in form of powder upon drying. Highest percentage yield was obtained with Pet. Ether (6.59 ±0.54%) and lowest yield with Chloroform (1.61 ±0.05%) shown in Table no. 4

Graph no. 2: Percentage yield of *Aegle marmelos, Psidium guajava* and *Manilkara zapota*



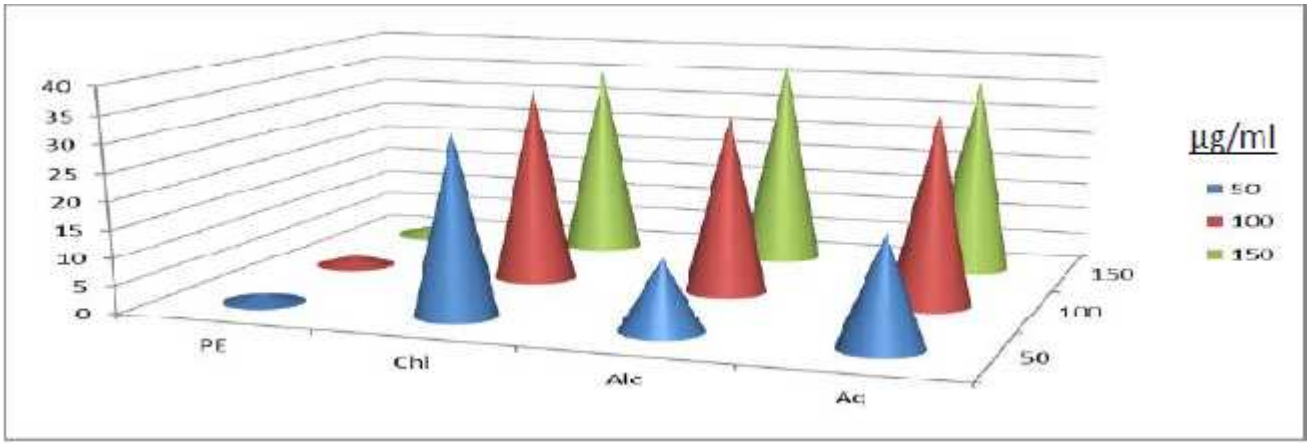
8.4. ANTIOXIDANT ACTIVITY FOR EXTRACTS:

Antioxidant activity of extracts of *Aegle marmelos, Psidium guajava* and *Manilkara zapota* was carried out by DPPH (2, 2-diphenyl -1-picryl hydrazyl) method. [16]

Table no 5: Antioxidant activity of *Aegle marmelos* extracts by DPPH assay

SN	Conc. (µg/ml)	% of inhibition			
		PE	Chl	Alc	Aq
1	50	1.56±0.25	32.58±0.12	12.84±0.15	19.5±0.66
2	100	1.87±0.32	35.91±0.56	32.67±0.18	34.42±0.53
3	150	1.68±0.09	35.52±0.85	37.93±0.51	36.40±0.61

Graph no. 3: Antioxidant activity of *Aegle marmelos* extracts



PE=Petroleum ether, Chl=Chloroform, Alc=alcohol, Aq=Water.

Values are expressed in terms of Mean± SEM of results done in triplicate.

Table no. 6: Antioxidant activity of *Psidium guajava* extracts by DPPH assay

SN	Conc. (µg/ml)	% of inhibition			
		PE	Chl	Alc	Aq
1	50	12.86±0.32	6.19 ±0.05	67.62±1.05	5.25±0.55
2	100	15.54±0.42	7.24±0.07	82.5±1.51	9.04±0.52
3	150	15.6±0.33	7.91±0.05	93.27±1.92	27.41±0.33

PE=Petroleum ether, Chl=Chloroform, Alc=alcohol, Aq=Water.

Values are expressed in terms of Mean± SEM of results done in triplicate.

Graph no. 4: Antioxidant activity of *Psidium guajava* extracts

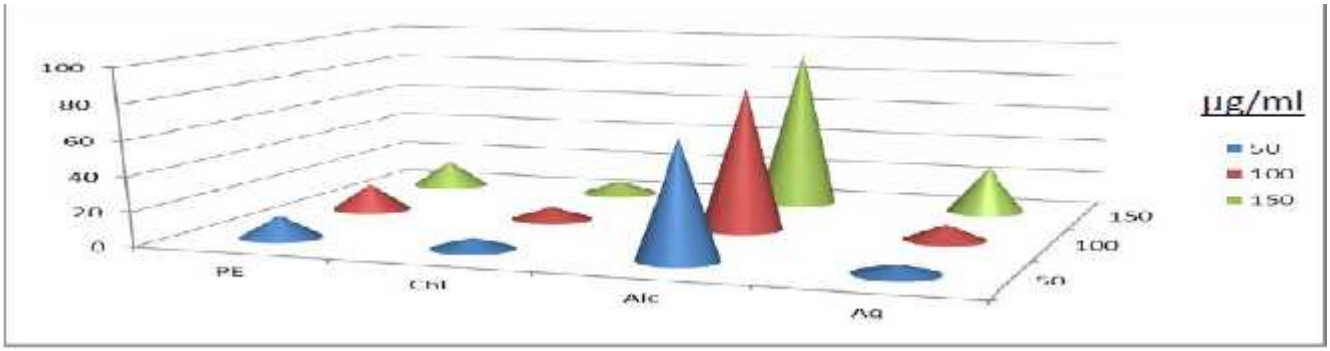
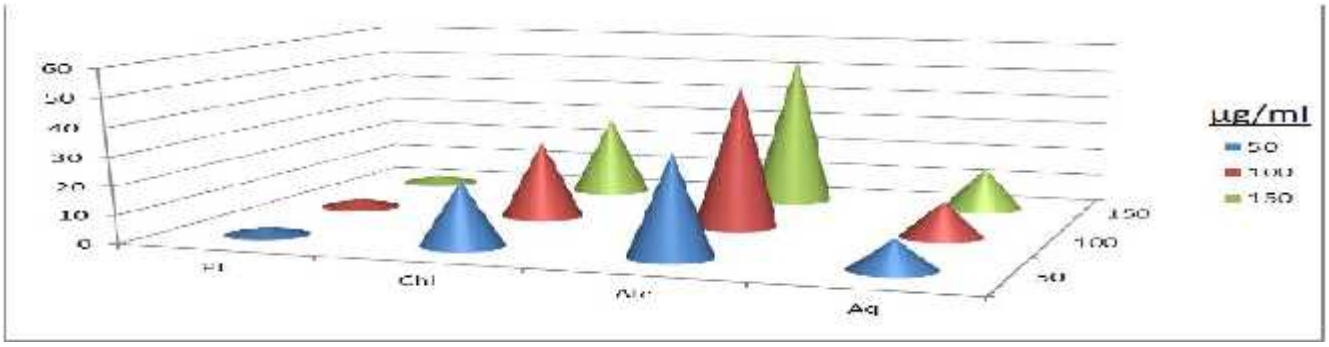


Table no. 7: Antioxidant activity of *Manilkara zapota* extract by DPPH assay

SN	Conc. (µg/ml)	% of inhibition			
		PE	Chl	Alc	Aq
1	50	2.53±0.06	22.29±0.62	35.22±0.55	10.78±0.53
2	100	2.96±0.05	27.46±0.51	50.73±0.63	12.65±0.61
3	150	2.63±0.15	28.44±0.51	54.43±0.75	15.21±0.51

PE=Petroleum ether, Chl=Chloroform, Alc=alcohol, Aq=Water Values are expressed in terms of Mean± SEM of results done in triplicate.

Graph no. 5: Antioxidant activity of *Manilkara zapota* extract



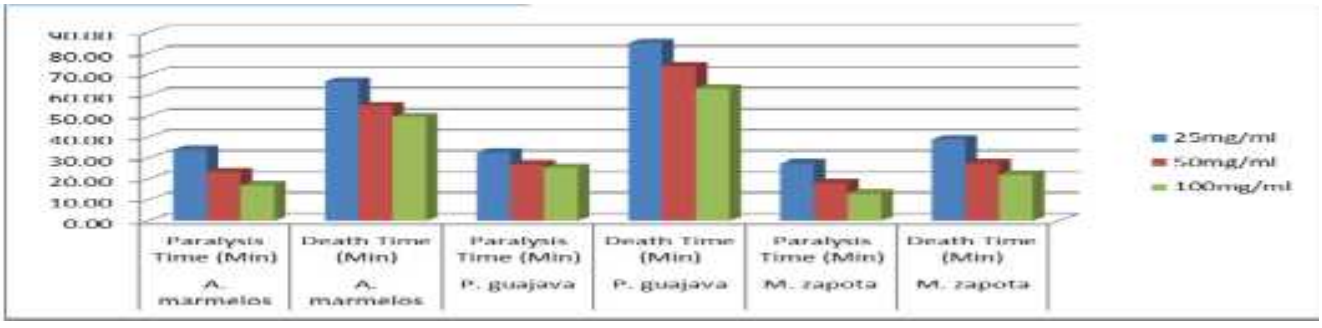
8.5. Anthelmintic Assay for individual extracts:

Anthelmintic assay was carried as on earthworms, due to its anatomical and physiological resemblance with the intestinal roundworms parasite of human being. **(Table no.8)**

Table no. 8: Anthelmintic activity of ethanolic extracts of *Aegle marmelos*, *Psidium guajava*, and *Manilkara zapota.*

SN	Treatment	Dose (mg/ml)	Paralysis time (Min)	Death time (Min)
1	Dist. Water	2	Nil	
2	Piperazine citrate-	15	35	85
3	***Aegle marmelos***	25	34.0	66.8
		50	23.2	55.0
		100	16.9	49.5
4	***Psidium guajava*,**	25	32.6	85.0
		50	27.0	74.0
		100	25.4	63.2
5	***Manilkara zapota***	25	27.5	38.8
		50	18.0	27.5
		100	13.1	22.0

Graph no. 6: Anthelmintic activity of ethanolic extracts of *Aegle marmelos, Psidium guajava,* and *Manilkara zapota.*



Ethanolic extract of *Aegle marmelos* exhibited antihelmintic activity with paralysis time of 16mins and death time of 50 mins at concentration of 150mcg/ml. Ethanolic extract of *Psidium guajava* at 150mcg/ml exhibited anthelmintic activity with paralysis time at 25 mins and death time at 63 mins at 150mcg/ml of concentration. Ethanolic extract of *Manilkara zapota* exhibited anthelmintic activity with paralysis time of 14mins and death time of 23 mins. Among three ethanolic extracts *Manilkara zapota* exhibited highest activity as Anthelmintic followed by *Aegle marmelos* and *Psidium guajava*. It was evident by antioxidant and anthelmintic evaluation. Among various extracts ethanolic extract of *Psidium guajava* had a maximum antioxidant activity. Further in order to select suitable dose of each drug to be incorporated in the intended formulation, experiment was designed.

8.6: Selection of extracts based on antioxidant activity by CCD

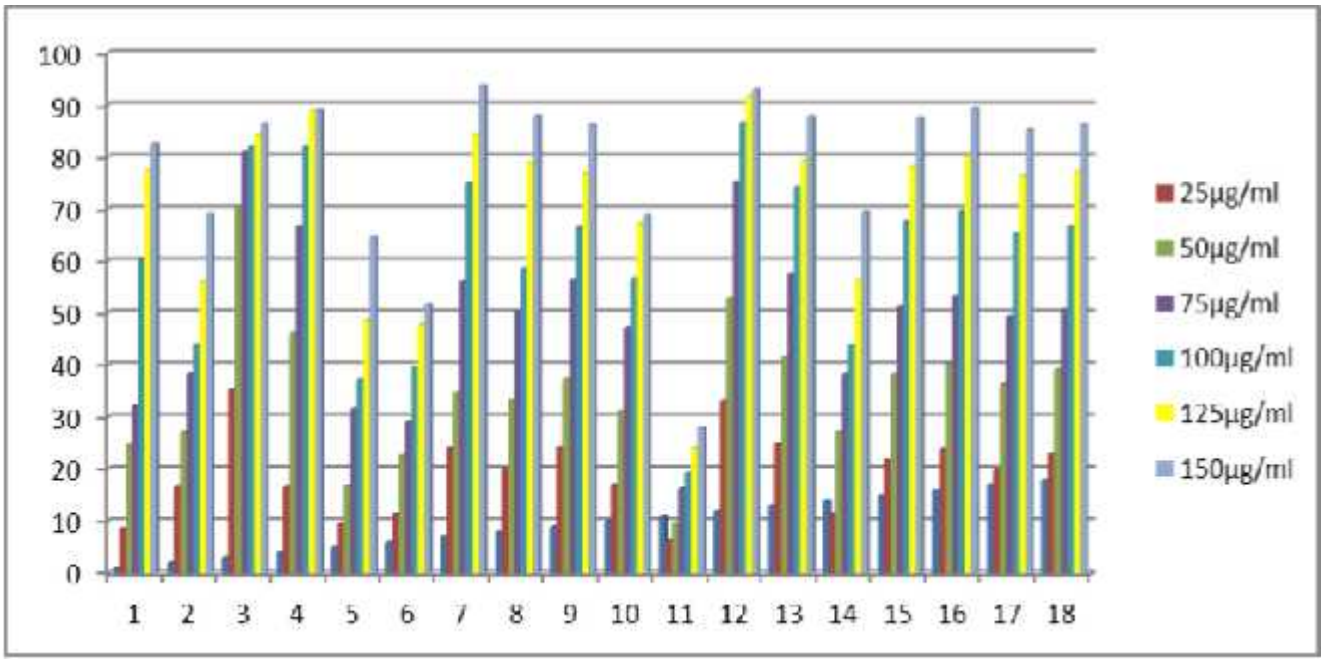
As the present study was intended to develop an herbal formulation comprising extracts of all the three selected plants, in order to detect the dose of each extract to be incorporated; the technique of Central Composite Design (CCD) was employed. Instead of selecting extracts by trial and error manner, a sophisticated robust, formula based programme was adopted. This design has given 18 combinations comprising of above extracts of *A. marmelos, P. guajava* and *M. zapota* combinations were prepared as per the design and antioxidant activity determined for each combination in duplicate. As per the results, increasing antioxidant activity was observed with increasing concentration. Hence the maximum concentration of 150µg/ml was considered and data with respect to the concentration 150µg/ml of each extract was analysed by CCD. **(Table no. 9)**

The results obtained from the experiments were analysed for response variables by using Design expert 6.05 version (State-Ease Ind., Minneapolis, Minnesota). The analysis of variance (ANOVA) information shown in **Table no. 10**

Table no. 9: Central composite design matrix defining composition of *A.marmelos, P.guajava* and *M.zapota* and their antioxidant activity in terms of percentage of inhibition.

SN	Type	*A.marmelos* D1 Mg	*P.guajava* D2 Mg	*M.zapota* D3 mg	Concentration of mixture and % of inhibition* by DPPH assay					
					25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	125 µg/ml	150 µg/ml
1	Fact	2000	2000	2000	8.7	24.58	32.19	60.37	77.4	82.58
2	Fact	6000	2000	2000	16.55	26.99	38.3	43.93	56.12	69
3	Fact	2000	6000	2000	35.3	70.55	80.92	82.11	84.32	86.45
4	Fact	6000	6000	2000	16.52	46.23	66.66	82.08	88.86	89.03
5	Fact	2000	2000	6000	9.6	16.91	31.64	37.18	48.56	64.64
6	Fact	6000	2000	6000	11.51	22.65	29.04	39.56	47.74	51.58
7	Fact	2000	6000	6000	24.05	31.69	56.14	74.95	84.38	93.8
8	Fact	6000	6000	6000	20.06	33.1	50.21	58.59	78.98	87.89
9	Axial	636.41	4000	4000	24.28	37.23	56.43	66.54	76.92	86.19
10	Axial	7363.59	4000	4000	17	31.29	47.18	56.63	67.21	68.88
11	Axial	4000	636.41	4000	6.32	9.69	16.33	19.29	24.08	28.13
12	Axial	4000	7363.59	4000	33.04	52.71	75.13	86.57	91.66	93.06
13	Axial	4000	4000	636.41	24.87	41.62	57.52	74.2	79.33	87.74
14	Axial	4000	4000	7363.59	11.34	27.1	38.22	43.82	56.41	69.36
15	Centre	4000	4000	4000	21.86	38.17	51.31	67.6	78.22	87.52
16	Centre	4000	4000	4000	23.76	40.23	53.21	69.51	80.34	89.46
17	Centre	4000	4000	4000	19.78	36.24	49.32	65.31	76.43	85.21
18	Centre	4000	4000	4000	22.95	39.12	50.63	66.65	77.21	86.39

Graph no. 7: Antioxidant activity of 18 combinations of ethanolic extracts mixture of *Aegle marmelos, Psidium guajava,* and *Manilkara zapota*



1-18 - Combination of herbal extracts as per the central composite design (Table.11)

Table no. 10: Analysis of variance table for dependent variables for the antioxidant activity of *A.marmelos, P.guajava* and *M.zapota* by central composite design.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4910.9989	9	545.6665	105.3852	< 0.0001	
A	129.8839	1	129.8839	25.0846	0.0010	
B	3490.7690	1	3490.7690	674.1762	< 0.0001	
C	1024.8018	1	1024.8018	197.9211	< 0.0001	
A2	53.7678	1	53.7678	10.3842	0.0122	
B2	127.6869	1	127.6869	24.6603	0.0011	Significant
C2	111.7365	1	111.7365	21.5798	0.0017	
AB	6.7161	1	6.7161	1.2971	0.2877	
AC	42.8275	1	42.8275	8.2713	0.0206	
BC	4.3660	1	4.3660	0.8432	0.3853	
Residual	41.4226	8	5.1778			
Lack of Fit	32.0702	5	6.4140	2.0574	0.2933	Not significant
Pure Error	9.3525	3	3.1175			
Cor Total	4952.4215	17				
Std. Dev.	2.2755		R-Squared	0.9916359		
Mean	61.0494		Adj R-Squared	0.9822263		
C.V.	3.7273		Pred R-Squared	0.9394895		
PRESS	299.6734		Adeq Precision	33.007898		

Note: Values of "prob>F' less than 0.05 indicate the model terms are significant

8.7. Antioxidant activity of Herbal mixture

From ANOVA, it is observed that the model was found to be significant ($p<0.05$), with an F- value 105.38. Further ANOVA analysis depicts that the factor A, B, C, B2 and C2 was found to be significant. Hence, this is the factor which actually influence or affect the antioxidant activity when present in combination. Also inter-reaction between A and C was found to be significant (< 0.0001) with F-value of 674.17. The "Lack of Fit F-value" of 2.05 indicates there is a 0.29% chance that a "Lack of Fit F-value" of this large could occur due to noise **(Table No.9)**

Final Equation in Terms of Coded Factors:	
% Inhibition	
87.0002	
-4.69228	* A
12.68935	* B
-4.76401	* C
-2.7497	* A2
-5.55602	* B2
-2.30084	* C2
3.53875	* A * B
-1.62125	* A * C
5.82125	* B * C

Based on the above equation following conclusions were drawn. Positive (+ve) values indicate positive effect on the % of inhibition. Negative (-ve) values indicate the negative effect on the % of inhibition and hence on the antioxidant activity. *Aegle marmelos* and *Psidium guajava* have positive effect on antioxidant activity whereas *Manilkara zapota* has negative effect. Interaction effects are seen between *Aegle marmelos* and *Manilkara zapota* and between *Psidium guava* and *Manilkara zapota.*

Table no.11: Optimized formula for the combination of *A. marmelos, P. guajava* and *M. zapota,* predicted % of inhibition and experimental results.

Solutions	*A.marmelos* (mg)	*P. guajava* (mg)	*M. zapota* (mg)	% inhibition	
				Predicted	Experimental
1	4152.93	5974.24	3305.92	93.2±0.12	91.7±0.30

Table no. 12: Anthelmintic activity of ethanolic extracts in combined mixture at various concentrations.

SN	Treatment	Dose (mg/ml)	Paralysis time (Min)	Death time (Min)
1	Dist. Water	2	Nil	
2	Piperazine citrate	15	33	90
3	Combined mixture	25	25.5	65.80
		50	20.5	50.00
		100	12.0	41.50

To evaluate the efficacy of individual extracts and the extract mixture, anthelmintic evaluation was carried out against drugs by technique. As these helminthes infections leads to other health problems

such as malnutrition and anaemia. Mixture of three extracts showed synergistic effect against earthworms. *Manilkara zapota* exhibited better activity both in individual concentration and also in combined mixture.

PHYSICOCHEMICAL EVALUATION OF THE DEVELOPED POLYHERBAL ORAL FORMULATION:

The developed oral polyherbal syrup was subjected to evaluation for various physicochemical parameters. **Formulation:** As per the observations, herbal syrup was light brown in color, transparent, slightly viscous in appearance and had pleasant odor, characteristic in taste. pH was constant throughout the study which was found to be 1.6 (Table no: 13). It did not contain any particles and air bubbles. Stability studies were carried out for one month and results revealed that, herbal formulation was stable with respect to colour, appearance, pH and specific gravity. There was not much variation after testing at different temperature conditions.

Table no. 13: Evaluation of Herbal syrup for its physicochemical properties

SN	Colour	Odour	pH	Specific Gravity	Taste
1	Dark Brown	Characteristic	1.6	1.06	Slightly acrid
2	Brown	Characteristic	1.7	1.15	Slightly acrid
3	Brown	Characteristic	1.7	1.09	Slightly acrid

Table no. 14: Evaluation of Syrup for Anthelmintic activity

SN	Treatment	Paralysis Time (Min)	Death Time (Min)
1	Dist. Water	Nil	
2	Piperazine citrate-15mg/ml	36	98
3	Formulation	26	51

Polyherbal syrup evaluated for anthelmintic activity exhibited paralysis time of 26mins and death time of 51mins.

DISCUSSION

Natural remedies are more acceptable with belief that they are safer or have fewer side effects than the synthetic ones. Herbal formulations have growing demand in the world market. This study attempt was successful in establishing safety and efficacy of a new polyherbal oral formulation comprising extracts of *Aegle marmelos*, *Psidium guajava* and *Manilkara zapota*. The studies revealed that the herbal extract mixture selected for formulation was effective with *in-vitro* anthelmintic activity. Developed polyherbal oral formulation consisting of extracts mixture of three plants was also found to be effective as anthelmintic agent by *in-vitro*.

ACKNOWLEDGEMENTS

We thank the support given by our institution to carry out the above research work.

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