EVIDENCE BASED HERBAL MEDICINE PART - VI

(Scientific validation of Pharmacological action of AYUSH drugs)

Edited by

Dr. P. Selvam., M. Pharm., Ph.D, FNABS, FISNS

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R. ARAVINDH

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PREFACE

India is one of the richest biodiversity country in the world and its nature gifted medicinal plants which are essential for treatment of human diseases. 80% world human population depends on herbal medicine for human wellness. Natural medicinal herbs enriched with novel bioactive molecules, nutraceuticals and micronutrients are responsible for therapeutic and medicinal benefits. AYUSH is leading herbal based therapeutic system for treatment of many diseases practiced in India for more than 2000 years, Siddha and Ayurveda system of medicine is oldest practice by ancestors. Scientific validation of therapeutic actions of plants and poly herbal formulation is essential for authentication of pharmacological action and therapeutic efficacy. Evidence based herbal medicine is significant for proof of concept to verify medicinal plants therapeutic potential and medicinal values. This text book of “Evidence Based Herbal Medicine - Part V” is a collection of research articles of scientific validations of pharmacological actions of selected medicinal plants and poly herbal formulations helps to distinguish and discover the therapeutic benefits by modern scientific methods.

Contents

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Aravindh Herbal Labs (P) Limited
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EVIDENCE BASED HERBAL MEDICINE - PART - VI

In-vitro anti-oxidant, anti-inflammatory and antimicrobial activity of Feverex polyherbal formulations

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Abstract *–Herbofeverox is a polyherbal Ayurvedic formulation that contains 10 ingredients developed in ancient times. It is a good therapeutic and dietary medicine for women, which may be easily prepared at home and used in the treatment of complications that arise during menstrual problems. It is very effective as a uterine tonic and in the treatment of any uterus-related disorders. In the current study, the ability of Feverox in scavenging the free radicals was assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, and the in-vitro anti-inflammatory studies by protein denaturation assay for aqueous crude extract, in-vitro antibacterial activity also performed against human pathogenic bacteria like staphylococcus aureus, klebsiella pneumonia, E. coli, Pseudomonas aeruginous. The results proved that the Herbofeverox aqueous extract and syrup were investigated for in vitro antioxidant activity at the concentration of 1 mg/mL by the DPPH method and showed significant antioxidant activity, as compared to standard Vitamin C (ascorbic acid) under similar conditions. Moreover, the antibacterial study of Herebofeverox revealed the formulation possesses improved inhibitory activity against pathogenic bacterial strains and anti-inflammatory activity. The study suggests that*

 ISBN: 978-93-5408-448-5

Herbofeverox hasa good source of natural therapeutic components and is considered useful in combating the advances of oxidative stress, infections, and inflammation thereby preventing diseases that are caused by various disease-causing agents.

Keywords *–Herbofeverox, antioxidant activity, DPPH, in-vitro anti-inflammatory activity, antibacterial activity*

1. INTRODUCTION

Herbofeverox is a polyherbal formulation, composed of versatile medicinal plants such as *Rungiarepens* (L) nees (Parpadagam) [1], *Cyperus rotundus* L (Koraikilangu) [2], *Withania somnifera* (Ammukkara) [3,4], Akil Kattai [5], Chukku, Milagu, Thippli [6], *Andrographis paniculata* (Sriyanangai) [7], Kadukkai, Thandrikai, *Emblica officinalis* (Nellikai) Gaertn, *Terminalia belerica* Linn and *Terminalia chebula* Retz in equal proportions (1:1:1) [8],*Curcuma longa*(Kasthuri Manjal) [9], Padikaram [10], *Linga chendooram*, *Annabethi chendooram* [11]. Herbofeverox polyherbal formulation is considered an important Siddha herbal medicine with excellent pharmacological and medicinal value due to the presence of enriched phytochemicals. The phytochemical contents of the polyherbal formulation Herbofeverox are responsible for antioxidant activity, which leads to many therapeutic and medicinal values.The antioxidant potential of bioactive molecules of herbs is responsible for the recommendation of treating many diseases. The present work is envisaged to study the in-vitro antioxidant activity by using the DPPH assay to explore the level of antioxidant potential. The antibacterial activity is tested against human pathogenic bacteria like staphylococcus aureus, *Klebsiella pneumonia*, *E. coli*, and *Pseudomonas aeruginousa* and also investigated for in-vitro anti-inflammatory activity by protein denaturation assay. The results suggested that Herbofeverox possesses notable antioxidant, antibacterial and anti-inflammatory activity.Polyherbal formulation with antioxidant activity is essential for the treatment of many human diseases. Free radicals are mainly

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responsible for uterine disorders condition and Herbofeverox containing excellent herbal combinations is used as a potential antipyretic activity.

2. MATERIALS & METHODS

2.1. In vitro antioxidant activity

The Feverox was investigated for in vitro antioxidant activity by DPPH, ABTS, FRAP, and NO for the estimation of the anti-oxidant potential of Rhumatigo aqueous extract [5].

2.2. Determination of DPPH radical scavenging activity

Antioxidant activity in the samples was estimated for their free radical scavengingactivity by using DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals (George *et al*., 1996) [12,13]. 100µL of the extract was taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark conditions. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively and read the plate on Elisa plate reader at 490nm Standard ascorbic acid was used as reference. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

2.3. In vitro anti-inflammatory activity-inhibition of albumin denaturation

The reaction mixture of 0.5ml aqueous extractcontains drug 0.05ml in the concentration (1mg/ml) with 0.45 ml aqueous solution of bovine albumin fraction (5%). The pH (6.3) of the solution was adjusted using a small amount of 0.1N HCl at 37ºC for 20 min, then heat to 57 0C for 30 min. Cool the solution and transfer it to the 96-well plate and measure the absorbance at

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660nm. Standard was used as Diclofenac sodium (1000µg/ml) and the control contain 0.05ml distilled water [14].

The percentage of inhibition of albumin denaturation was calculated by the following formula,

Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.

A sample - absorbance reaction mixture with Sample)

2.4. Antibacterial activity

The antibacterial activity of evaluation ofHerofeverox extracts against *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli*, and *Pseudomonas aeruginousa* was performed by well diffusion methods [15]. The inoculum of themicroorganism was prepared from the bacterial cultures. 15 ml of nutrient agar (Hi media) medium was poured into clean sterilized Petri plates and allowed to cool and solidify. 100 µl of broth of bacterial strain was pipette out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 6 mm in diameter were bored using a sterile cork borer. Solutions of all the extracts (1mg/ml) in DMSO were prepared. 100µl of extracts solutions was added to the wells. The Petri plates were incubated at 37°C for 24 h. streptomycin (1mg/ml) was prepared as a positive control DMSO was taken as the negative control. Antibacterial activity was evaluated by measuring the diameters of the zone of inhibitions (ZI) all the determinations were performed in triplicates.herbofeverox extract was also tested for antibacterial activity against human pathogens like *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli*, and *Pseudomonas aeruginousa*.

3. RESULTS & DISCUSSION

Herbofeverox is a polyherbal formulation that had a significant antioxidant activity of 90% when compared with standard Vit-C ascorbic acid of91% under similar conditions, and also had dose dependant activity (85-69% at 1-5 mg). Herbofeverox exhibits significant anti-inflammatory

 ISBN: 978-93-5408-448-5

activity of 78% inhibition by protein denaturation when compared with standard diclofenac sodium of 98% at 100 mg/ml under similar conditions.

FEVEREX CAPSULE:

***IN VITRO* ANTIOXIDANT ACTIVITY BY DPPH ASSAY**

S.NO	COD	SOD	% inhibition	Average
1	1.57	0.13	91.71	
2	1.57	0.17	89.17	
3	1.55	0.16	89.96	
4	1.55	0.13	91.61	
5	1.55	0.21	86.45	
6	1.55	0.15	90.32	89.87%

STANDARD- ASCORBIC ACID (VITAMIN C):

***IN VITRO* ANTIOXIDANT ACTIVITY BY DPPH ASSAY**

S.NO	COD	SOD	% inhibition	Average
1	0.98	0.02	97.90	
2	0.99	0.09	90.90	
3	0.99	0.10	89.80	
4	0.98	0.09	90.80	
5	0.99	0.09	90.90	
6	0.98	0.10	89.70	91.60%

FEVEREX CAPSULE-(0.1)

S. NO.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.22	78.0%
2	520	1.00	0.12	88.0%
3	520	1.00	0.15	85.0%
				85.6%

FEVEREX CAPSULE-(0.2)

S. NO.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.14	86.0%
2	520	1.00	0.18	82.0%
3	520	1.00	0.14	86.0%
				84.6%

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FEVEREX CAPSULE-(0.3)

S. NO.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.22	78.0%
2	520	1.00	0.21	79.0%
3	520	1.00	0.18	82.0%
				79.6%

FEVEREX CAPSULE-(0.4)

S. NO.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.25	74.4%
2	520	1.00	0.28	71.7%
3	520	1.00	0.22	77.5%
				74.5%

FEVEREX CAPSULE-(0.5)

S. NO.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.27	72.4%
2	520	1.00	0.32	67.3%
3	520	1.00	0.33	67.8%
				% 69.1

FEVEREX CAPSULE

***In-vitro* anti-inflammatory activity**

S.NO	COD	SOD1	SOD2	% inhibition	Average
1	0.89	0.02	0.20	77.52	
2	0.89	0.02	0.19	78.65	
3	0.91	0.01	0.18	75.25	77.14%

STANDARD- DICLOFENAC

***In-vitro* anti-inflammatory activity**

NM-640

S.NO	COD	SOD	% inhibition	Average
1	0.89	0.02	97.75	
2	0.89	0.02	97.75	
3	0.91	0.01	98.87	98.13%

 ISBN: 978-93-5408-448-5

***In-vitro* antibacterialactivity of Feverex capsule**

S.NO.	ORGANISM	60 µl	80 µl	CONTROLSTANDARD
1	*Staphylococcus aureus*	22mm	25mm	penicillin19mm
2	*Klebsiella pneumoniae*	24mm	28mm	streptomycin 17mm
3	*E. coli*	25mm	28mm	ciprofloxacin 24mm
4	*Pseudomonas aeruginosa*	28mm	28mm	kanamycin 11mm

Staphylococcus aureus *Klebsiella pneumoniae*

E. coli *Pseudomonas aeruginosa*

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The antibacterial activity is tested against human pathogenic bacteria such as *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli*, and *Pseudomonas aeruginousa*. Herbofeverox demonstrated for potent antibacterial activity than standard antibiotics under similar conditions. Herbofeverox is documented for significant antioxidant, anti-inflammatory, and antibacterial activity due to their versatile poly-herbal combinations and their enriched phytochemicals content in their constituent's plants.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

In-vitro evaluation of anti-oxidant and anti-cancer potential of Herbuterin syrup to evidence against uterine diseases and cervical cancer

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Abstract *–Herbuterin is a polyherbal Ayurvedic formulation contains 10 ingredients, among them Asoka pattai (SaracaAsoca) is one of its chief constituents. It is a good therapeutic and dietary medicine for women, which may be easily prepared at home and used in the treatment of complications that arise during menstrual problems. It is very effective as a uterine tonic and in the treatment of any uterus related disorders. In the current study, the ability of Herbuterin syrup in scavenging the free radicals was assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, and the cytotoxicity studies tested in human cervical cancer (HeLa) cells by MTT assay for both crude extract and the final formulations. The results proved Herbuterinwith notable antioxidant activity. Herbuterin aqueous extract and syrup were investigated for invitro antioxidant activity at the concentration of 1 mg/mL by DPPH method.Herbuterinraw material extract and syrup product had significant antioxidant activity,as compared to standard Vitamin C (ascorbic acid) under similar conditions.Herbuterin extract and syrup had significant inhibitory activity against human cervical cancer cells. The study suggests that, Herbuterin possess good source of natural antioxidants, and is considered useful in combating the advances of*

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oxidative stress, thereby preventing from the diseases that are caused by the free radicals.

Keywords *–Herbuterin, antioxidant, anticancer, DPPH assay, MTT assay, HeLa cell*

1. INTRODUCTION

Herbuterin is polyherbal formulation, composed of versatile medicinal plants such as Saracaasoca [1],Ocimum sanctum[2], Santalum album [3],Ficus religiosa [4], Glycyrrhizha glabra [5], Piper nigrum [6],Argemone Mexicana L. [7], Terminalia arjuna [8], Withaniasomnifera [9] with excellent pharmacological and medicinal value due to the presence of enriched bioactive molecules and nutraceuticals. The phytochemical contents of poly herbal formulation Herbuterinis responsible for antioxidant activity, which leads to many therapeutic and medicinal values.Antioxidant potential of bioactive molecules of herbs is responsible for the recommendation of treatment many diseases. Present work is envisaged to study the in vitro antioxidant activity by using DPPH assay method to explore the level of antioxidant potential.The cytotoxicity is tested in human cervical cancer cells (HeLa cell lines) by MTT assay for both the crude extract and their formulation. The results suggested that Herbuterin possess notable antioxidant activity.Poly herbal formulation with antioxidant activity essential for the treatment of many human diseases. Free radicals are mainly responsible for uterine disorders condition and Herbuterin containing the excellent herbal combinations is used as uterine tonic [10,11].

2. MATERIALS & METHODS

2.1. Solvents and reagents

The Ayurvedic formulation Herbuterin used for evaluating antioxidant activity were procured from Aravindh Herbal Labs (P) Ltd.,Rajapalayam, Virudhunagar (District), Tamil Nadu, India. Analytical grade of chemicals only was used in all the experiments. Analytical grade solvents

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methanol (99.9%)purchased from Merck Specialties' Pvt. Ltd., Mumbai, Indiaand petroleum ether (99%) was purchased from Thermo Fisher Scientific India Pvt. Ltd.,Mumbai, India. The other reagents like standard ascorbic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH),hydrogen peroxide, potassium ferricyanide, trichloroacetic acid and ferric chloride were acquired from Sigma Aldrich, India Pvt. Ltd. Milli-Q water (Milli-Q10 TS) was used to prepare all reagents in the laboratory during the analysis.

2.2. Apparatus used

UV spectrophotometer (Lab India Instruments Pvt. Ltd.), Rotary evaporator (BucchiLabor Technik, Switzerland) and Centrifuge machine (Remi instruments-C24) were used in the analysis.

2.3. Experimental methods

Preparation of aqueous extract of Herbuterin: About 50mg of the Ayurvedic formulation Herbuterin was ground into fine powder. The powdered sample was defatted with petroleum ether and the homogenate was added to 50 mL methanol for extraction.The mixture was shaken intermittently and then filtered using Whatmanfilter paper No. 1. The obtained filtrate was processed on a rotary evaporator at 40°C for slow evaporation of the solvent, thereby forming the concentrated / dried product.The final concentrated material was separated and stored in desiccator for the in-vitro studies. **Cellline:** HeLacancercelllines were selected to evidence the effect of Herbuterin formulation against cervical cancer [12]. **Media:** DMEM with high glucose (Cat No-11965-092), FBS (Gibco, Invitrogen Cat. No.-10270106) Antibiotic–Antimycotic100Xsolution (Thermo Fisher Scientific Cat. No.-15240062).

2.4. Experimental procedure of MTT assay

HeLa cells were cultured and incubated at a concentration of 1 x 104cells/mL in culture medium for 24h at 37°C and 5% CO2.The cells were seeded at 1 x 104cells/well in 100µL culture

 ISBN: 978-93-5408-448-5

medium andthen, 100µL of samples at three different concentrationsof 10, 40, 100µLwere added into micro plates (Tissue culture grade - 96 wells).Triplicate wells were reserved for control, wherein the cells were incubated along with DMSO (0.2% inPBS). The cells were checked for normal survival and the percentage of live cells was measured.All the test samples were also tested in triplicate (n=3). Cellcultures were incubatedfor 24 h at37°Cand5% CO_2in CO_2 incubator (ThermoscientificBB150).Afterthe incubation,themediumwascompletelyremovedand, then 20µLof MTT reagent (5mg/mLin PBS) was added to all the wells. After addition of MTT, the cells were again incubated for 4 h at 37oCin the CO_2incubator.The wells were observed for formazan crystal formation under microscope.The yellowish MTT was reduced to dark colored formazan by viable cells only. Afterremoving the medium completely, 200µL of DMSO was added, kept for 10 min under incubation at 37oC (wrapped with aluminium foil). Triplicate samples were analyzed by measuring the absorbance of each sample using a microplate reader (Benesphera E21) at a wavelength of 550 nm [13, 14].

2.5. In vitro antioxidant activity

Ability of a polyherbal product in scavenging free radicals can be assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), nitric oxide (NO) radical scavenging assay and nitric oxide (NO) assay [15-18]. In vitro antioxidant study was performed for Herbuterin, after defatted with petroleum ether by DPPHassay to ensure the antioxidant potentialat the concentration of 1.0 mg/mL and compared with standard ascorbic acid under similar conditions.

2.6. Determination of total antioxidant activity

Free radical scavenging activity (DPPH assay)

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the formulation Herbuterin[15-18].Different concentrations of herbal extract were added, at an equal volume, to methanolic solution of DPPH (100 M). After

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15 min incubation at room temperature, the absorbance was recorded at 517 nm using a UV-Visible spectrophotometer. The experiment was repeated for three times using Ascorbic acid as the standard reference. The IC_{50} value is denoted as the concentration of sample, which is required to scavenge50% of DPPH free radicals.

3. RESULTS & DISCUSSION

The physico-chemical properties like pH, loss on drying, refractive index, etc. of the syrup formulations produced in different batches are compared to assess the uniformity standards (Table 1). Two batches of Herbuterin samples were investigated for invitro antioxidant activity at the concentration of 1 mg/mL by DPPH method to ensure the antioxidant activity.The results obtained from the antioxidant study indicated that Herbuterin exhibit significant free radical scavenging potential. The study suggests that Herbuterin has a good origin of natural antioxidants and might be beneficial in impeding the oxidative stress.The results suggested that both extract and syrup possess notable antioxidant activity (Tables 2 and 3).They had exhibited significant antioxidant activity compared to standard ascorbic acid under similar conditions(Table 4).In the MTT assay against Human cervical cancer cells (HeLa cell lines), both aqueous extract and syrup formulation had significant anti-cancer activity by inhibiting the cell proliferation of HeLa cell lines (Table 5).

Table 1: Physico-chemical properties of Herbuterin syrup

Sample Mfg. Year	Colour	Odour	Taste	LOD	SP. G	pH	Sugar	Sugar (Br)	pH Paper	RI
Jan18	Reddish Brown	Pleasant	SweetAstringent	33.01 %	1.261	3.84	64%	1.5%	4.0	1.440
Jun18	Reddis	Pleasa	SweetAstringe	33.33	1.25	3.8	64%	1.5%	4.0	1.44

 ISBN: 978-93-5408-448-5

	h Brown	nt	nt	%	4	7				2
Jul19	Reddish Brown	Pleasant	SweetAstringent	38.73 %	1.263	3.91	60%	1.5%	4.0	1.442
Nov19	Reddish Brown	Pleasant	SweetAstringent	36.32 %	1.270	3.90	60%	1.5%	4.0	1.445
Jun20	Reddish Brown	Pleasant	SweetAstringent	38.69 %	1.264	3.94	60%	1.5%	4.0	1.446
Nov20	Reddish Brown	Pleasant	SweetAstringent	37.75 %	1.269	4.00	60%	1.5%	4.0	1.445

Table 2:*Invitro* antioxidant activity of Herbuterin extract

S.No.	NM	C.OD	S.OD	% Inhibition
1	517	3.215	0.426	86.7%
2	517	3.215	0.396	87.6%
3	517	3.215	0.293	90.8%
4	517	3.215	0.449	86.0%
5	517	3.215	0.491	84.7%
6	517	3.215	0.551	82.8%
				86.43%

 ISBN: 978-93-5408-448-5

Table 3:*Invitro* antioxidant activity of Herbuterin syrup

S.No.	NM	C.OD	S.OD	% Inhibition
1	517	3.215	0.869	72.9%
2	517	3.215	1.127	64.9%
3	517	3.215	1.444	55.0%
4	517	3.215	0.983	69.4%
5	517	3.215	0.999	68.9%
6	517	3.215	0.788	75.4%
				67.75%

Table 4:*Invitro* antioxidant activity of ascorbic acid (Vitamin-C) standard

S.No.	NM	C.OD	S.OD	% Inhibition
1	520	0.84	0.12	85.7%
2	520	0.82	0.16	80.4%
3	520	0.82	0.16	80.4%
4	520	0.89	0.18	79.7%
5	520	0.90	0.25	72.2%
6	520	0.84	0.12	85.7%
				80.68%

Table5: EffectsofHerbuterin samples againstHeLacancercelllinebyMTTassay

S. No.	Sample	Concentration	OD	%Inhibition
1	Control		0.975	
2	Herbuterinextracts	10µg/mL	0.565	42.15
		40µg/mL	0.436	55.28
		100µg/mL	0.423	56.61

 ISBN: 978-93-5408-448-5

3	HerbuterinSyrup	10µL	0.658	32.51
		40µL	0.556	42.97
		100µL	0.512	49.46

About ≥50% inhibition of cell growth was found at the concentration of 40 µg/mL and 100 µL sample of Herbuterin extract and syrup formulation, respectively.The antioxidant potential and the anti-cancer efficiency of Herbuterinformulation could aid for both prophylactic and therapeutic tool against uterine diseases including uterus cancer.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Physico chemical characterization and *invitro* activities of Kasthuri karuppu

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Abstract *–Standardization and analytical characterization of traditional medicinal preparations are required to provide scientific evidence and validation for their global acceptance.The presentstudyfocuses on the characterization of a mercury and sulphur based polyherbomineralSiddha medicine preparation, Kasthuri Karuppu,throughmodernanalyticaltechniques.Particlessize and surface morphology was identified usingzeta sizer and scanningelectronmicroscopy(SEM), which showed polydisperse clusters form due combination of herbs and inorganic compounds. The functional groups were observed by the peaks using Fouriertransforminfraredspectroscopy (FTIR), which showedβ –HgSalong with other functional groups. XRD analysis confirmed the crystalline nature and the presence of Hg and S according to JCPDS data file. The in vitro assays are performed to assess the anti-inflammatory activity and anti-oxidant activityoftheformulation.Kasthuri Karuppu showed maximum anti-inflammation properties at 600µg/mL concentration with inhibition percentage 90.1% and significant anti-oxidant properties at 100µg/mL concentration with inhibition percentage 64.7%. The study showed potent therapeutic effects of Kasthuri Karuppu, which could help to standardize the product specifications and quality.*

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Keywords *–Kasthuri karuppu, polyherbo-mineral formulation, siddha medicine, analytical characterization, anti-oxidant, anti-inflammatory*

1. INTRODUCTION

Kasthuri Karuppu is categorized under mercury-based herbo-mineral formulations, and used for the treatment like fever, coughs, bronchitis, cold, and other respiratory infections[1].It is a classical siddha medicine with prominent therapeutic effect and longer shelf life.The ingredients present in Kasthuri Karuppu includes sudhiseidha (purified/detoxified) lingam (mercuric sulphide), sudhiseidharasam (elemental mercury), sudhiseidhagandhakam (elemental sulphur), sudhiseidhapooram (mercurous (I) chloride), sudhiseidharasachendooram (artificially processed mercuric sulphide), sudhiseidhapathirathaalakam (yellow arsenic trisulphide), sudhiseidhamanosilai (arsenic disulphide),kasthoori (Moschus moschiferous), ommam (Trachyspermumammi), thippili (Piper longum), kostum (Saussurealappa),korasanai (Calculus bovis), pachaikarpooram (Cinnamomum camphora) andkunkumapoo (Crocus sativus).

Figure 1: Kasthuri Karuppu

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The processed form of mercuric sulphide of black is termed as Kajjali in Siddha medicine.The preparation protocol was followed as per the Sidhhavaithyathirattu (a traditional medicinal encyclopedia) and Indian standard monographs as Ayurveda & Siddha Pharmacopeia. The formulation procedure begins with Kajjali preparation by mixing mercury and sulphur, followed by the addition of remaining ingredients as individual grinded form to obtain smooth fine powder.The aim of the present study is scientific validation of Kasthuri Karuppu using modern analytical technique and in vitro assays. Standardization tools for evaluating the quality of these medicines are required to meet global acceptance [2, 3]. Compatibility, interoperability, safety, repeatability, and quality are improved by standardization and validation. The modern analytical techniques like Zeta sizer, Scanning Electron Microscopy (SEM), Fourier Transform Infra-Red (FTIR) studies andX-Ray Diffraction (XRD) analysis are utilized to assess the physico-chemical characteristics of the sample [4, 5]. The in-vitro studies like anti-inflammatory assay and anti-oxidant assay are carried out to provide the basic scientific evidence for intended therapeutic uses.

2. MATERIALS & METHODS

Kasthuri Karuppu manufactured by Aravind Herbals Pvt. Ltd., Rajapalayam was used in the experiment. The chemicals for in-vitro analysis like DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and L-ascorbic acid were commercially purchased from Sigma-Aldrich, Mumbai.

2.1. Particle size analysis

The particle size and zeta potential of Kasthuri Karuppu was analyzed using the Dynamic Light Scattering (DLS) approach [6].Zeta sizer is used to measure the particle size of dispersed system from sub-nanometer to several micrometres in diameter.The mean particle size in a constrained size range can be determined using straightforward DLS sensors that measure at a fixed angle.

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2.2. SEM analysis

The morphological characterizationwas performed by scanning electron microscopy (SEM) [7]. The signals that result from electron-sample interactions provide details about the sample surface morphology (texture).Using the SEM objective lens, the electron beam is concentrated to a narrow area on the sample surface and the sample was visualized at different magnifications like 10X, 15X, 30X, 50X and 100X.

2.3. FTIR analysis

The KBr pellet technique was used to perform FTIR analysis. The FTIR instrument emits infrared radiation at a wavenumber ranging from 10,000 to 100 cm-1, which is passed through the material. The IR radiation causes vibrations leading to stretching and bending of different functional groups of the molecules.FTIR analysis is an appropriate method for identifying the chemical nature of substances, because each molecule or chemical composition will have a unique spectral fingerprint. The sample's FTIR spectra was taken at wavelengths ranging from 400 to 4000 cm-1. The spectrum was used to identify the organic functional groups, chemical composition and nature of herbominerals.

2.4. XRD analysis

XRD analysis was carried out using Bruker D8-Advance X-ray diffractometer using Cu-Ka radiation with a wavelength of 1.5405 A◦.The XRD pattern was recorded at an angle of 2-theta.This technique for determining a substance's crystallographic structure. XRD first bombards the material with incident X-rays in order to quantify the frequencies and dispersion angles of the X-rays that depart it. In order to scatter incident X-rays, crystal atoms connect to a network with their electrons. This phenomenon, known as elastic scattering, involves the electron as the scatterer. A regular array of scatterers yields a regular array of circular waves. According to Bragg's law, these waves add constructively in a small number of specified directions, but cancel each other out in most directions through destructive interference.

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2.5. In-vitro anti-inflammatory activity

Pure extract of Kasthuri Karuppu was prepared by macerating 1g of sample in 20mL of distilled water at room temperature for 15 min.The mixture was heated to 80°C for 20 min and then, cooled down to room temperature and finally, filtered using filter paper. The obtained extract was at the concentration of 1mg/mL, which was further diluted to get 100, 200, 400 and 600µg/mL. Aqueous solution of bovine serum albumin fraction was prepared at 1% w/v and phosphate buffer was prepared with pH 6.7. Aspirin was taken as standard reference sample. About 10mg of Aspirin was dissolved in 10mL of ethanol to get 1mg/mL concentration, which was further diluted to obtain 100, 200, 400 and 600 µg/mL concentration solutions. To 2mL of the test samples and standard drug Aspirin solutions at varying concentrations, about 0.2 mL of BSA and 2.8 mL of phosphate buffer were added and heated for 20 min. Then the absorbance of the samples was observed under UV-Visible spectroscopy at the wavelength of 660 nm [8].

2.6. In-vitro anti-oxidant activity

The crude extract sample of Kasthuri Karuppu was prepared with 2.5g of sample in 50mL of ethanol. It was occasionally shaken for 8 h and kept undisturbed for 16 h. Then the samples were kept in the water bath to evaporate till dryness and obtain the powder.About 10mg of the obtained extract dry powder was dissolved in 10mL of methanol to get the concentration of 1mg/mL, and further diluted to 10, 20, 40, 60, 80, and 100 µg/mL concentration solutions. For the standard solution, 10mg of Ascorbic acid was taken in 10mL of methanol and then, diluted to obtain 10, 20, 40, 60, 80, and 100 µg/mL concentrations. About 0.004% DPPH solution was prepared by taking 0.004g of DPPH in 100mL of methanol and incubated in the dark condition for 5 h. To the 3mL of test and standard samples at different dilutions, about 3mL of DPPH was added and incubated for 1 h. Then, the absorbance of the solutions was observed under UV-Visible spectroscopy at the wavelength of 517 nm [9].

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3. RESULTS& DISCUSSION

3.1. Zeta sizer analysis

The particle size distribution and zeta potential of Kasthuri Karuppu observed using Zeta sizer is shown in the Table 1 and Figure 2& 3, respectively.

Table 1: Zeta sizer results of Kasthuri Karuppu

S. No.	Parameters	Kasthuri karuppu
1	Average Particle size	269 nm
2	Poly dispersity index	0.279
3	Zeta potential	-8.6 mV

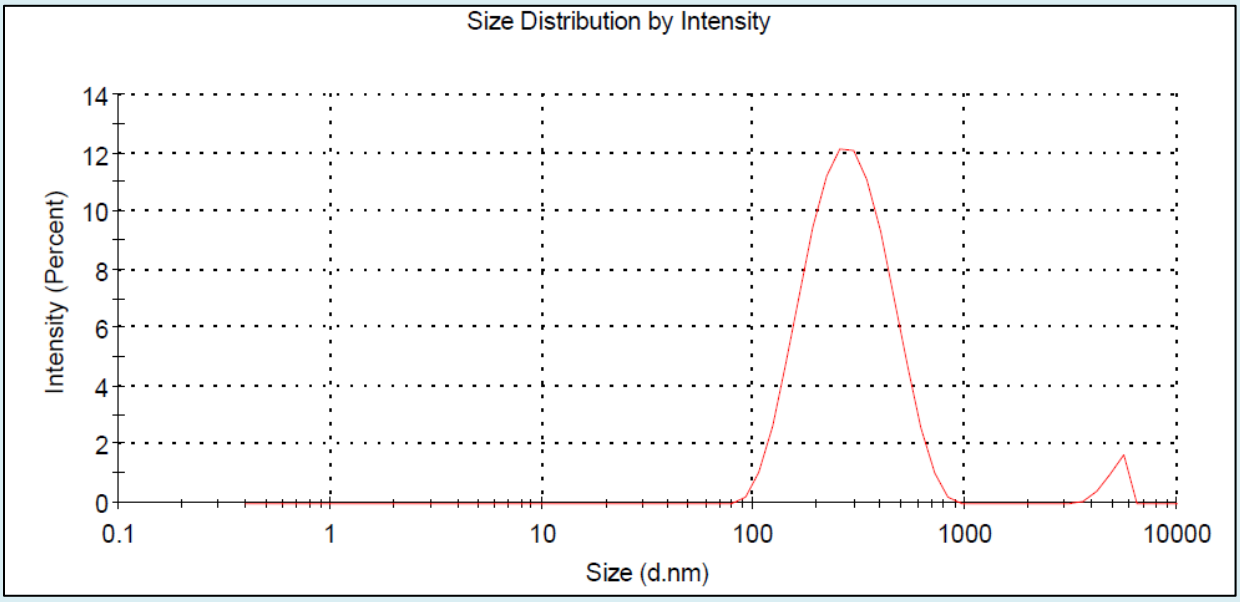

Figure 2: Particle size distribution of Kasthuri Karuppu

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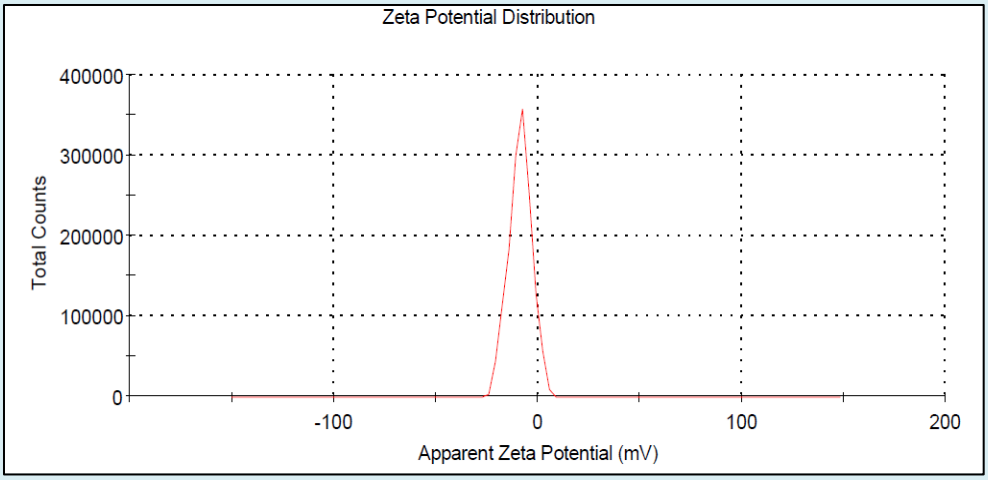

Figure 3: Zeta potential of Kasthuri Karuppu

3.2. SEM analysis

The surface morphology analysis of the Kasthuri Karuppu was carried out using scanning electron microscopy. The SEM image of the sample (Figure 4) showed cluster particles with irregular shape and size, indicating the polydisperse nature due to the presence of herbs and metals/minerals combination.

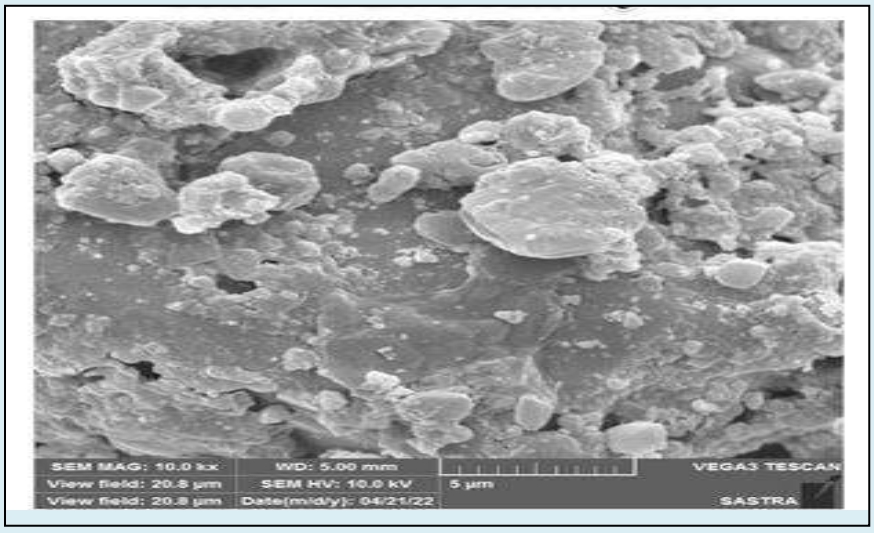

Figure 4: SEM image of Kasthuri Karuppu

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3.3. FTIR analysis

In FTIR spectra (Figure 5) analysis, the sample Kasthuri Karupu exhibited peaks at 3524 cm^{-1}, 3340 cm^{-1}, 2834 cm^{-1}, 2930 cm^{-1}, 2900 cm^{-1} and 1646 cm^{-1} showing O-H stretching, N-H stretching and C=N stretching. This indicated the presence of functional groups such alcohol, aliphatic primary amine and imine/oxime in the polyherbal composition (Table 2).The characteristic peak of beta-HgS was identified clearly in Kathuri Karuppu at 1020 cm^{-1}, which indicated the existence of Hg-S bond.This confirmed the presence of black mercuric sulphide (*kajjali*) in Kasthuri Karuppu[10].

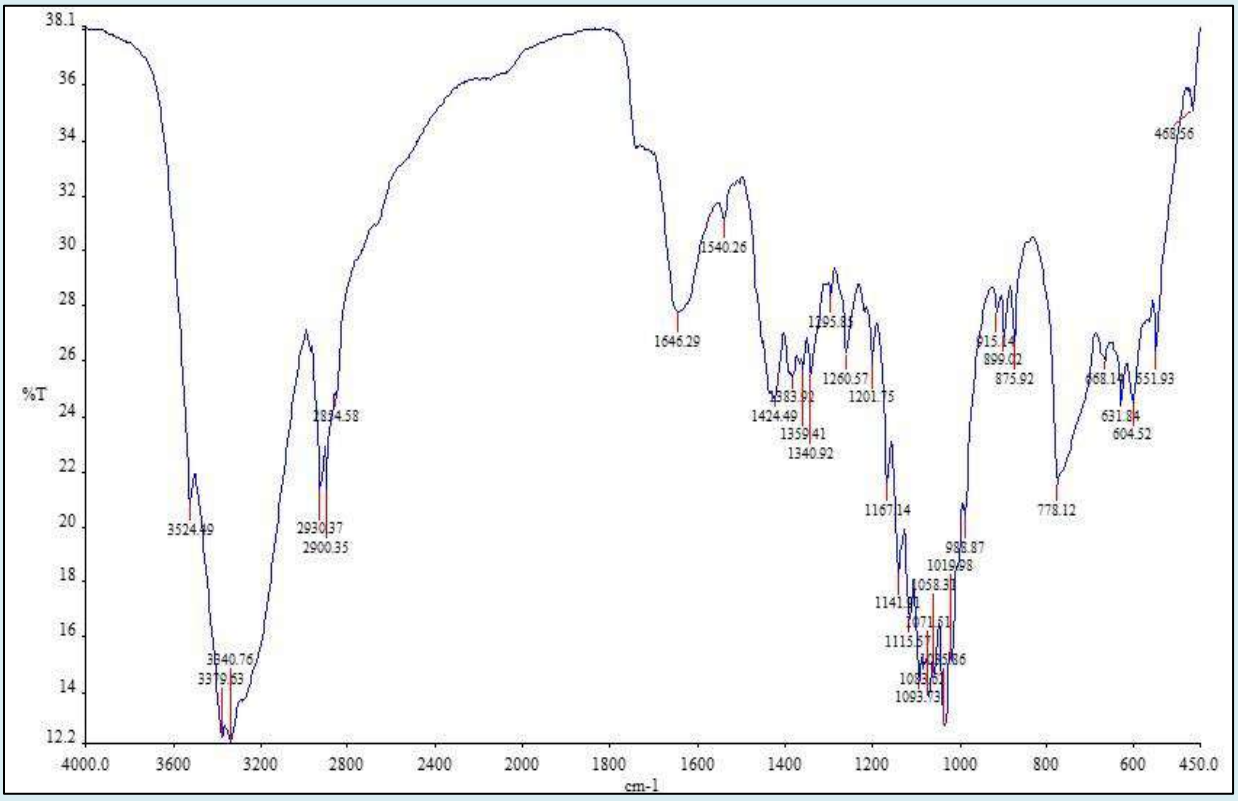

Figure 5: FTIR of KasthuriKaruppu

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Table 2: FTIR Vibrational peaks and functional group of Kasthuri Karuppu

Wavenumber (cm^{-1})	Functional group
3524	O-H stretching
3340	N-H stretching
2834	O-H stretching
1646	C=N stretching
1020	β-HgS

3.4. XRD analysis

The XRD pattern of Kathuri Karuppu exhibited characteristic peaks due to the presence of free sulphur, mercury oxide, and mercury sulphide (HgS). The data base value of the peaks was identified from the files with the numbers 20-1227, 01-0896, and 02-461 by the Joint Committee on Powder Diffraction Standards [JCPDS].

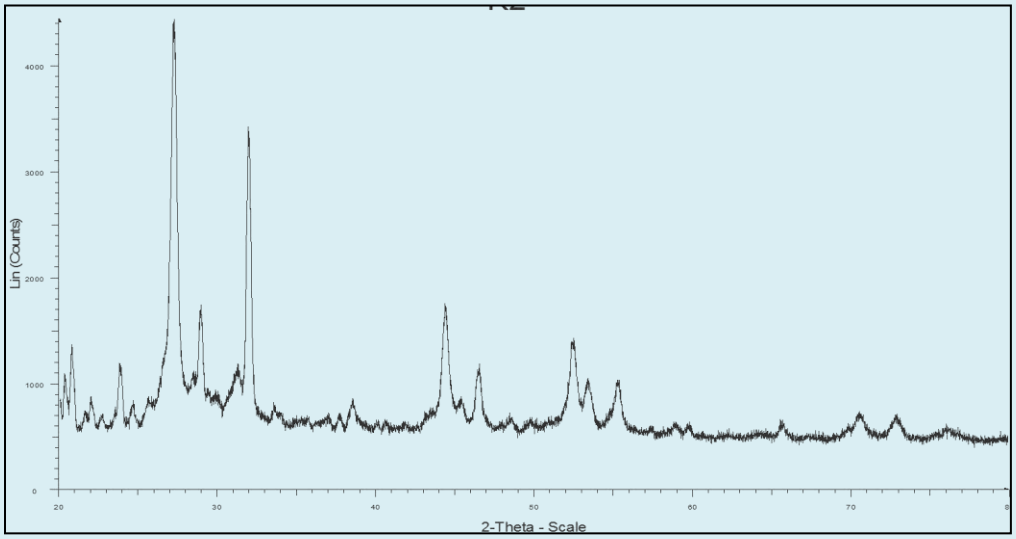

Figure 6: XRD pattern of Kasthuri Karuppu

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The HgS peak was observed at 35º for Kasthuri Karuppu. The Kasthuri Karuppu showed sharp peaks, which confirmed the crystalline nature of the minerals.The XRD pattern also confirmed the presence of beta HgS peaks at 30.5º for metacinnabar by comparing the result with Miguel *et al.* (2014) [11], which was the best identity option to trace the phase transition.The rest of the peaks had extremely low intensities and showed the contributions of other compounds in the polyherbomineral mixtures.

3.5. In-vitro anti-inflammatory activity

The *in-vitro* anti-inflammatory activities of Kasthuri Karuppu were evaluated through bovine serum albumin denaturation method. The % inhibitions of standard sample aspirin were 96.25%, 98.5%, 99% and 99.33% at the concentration of 100, 200, 400 and 600 µg/mL, respectively.The % inhibitions of Kasthuri Karuppu were 68.75%, 85.5%, 88.75% and 90.16% at the concentration of 100, 200, 400 and 600µg/mL, respectively. The Kasthuri Karuppu possessed moderate and lesser anti-inflammatory activity compared to the standard sample aspirin.

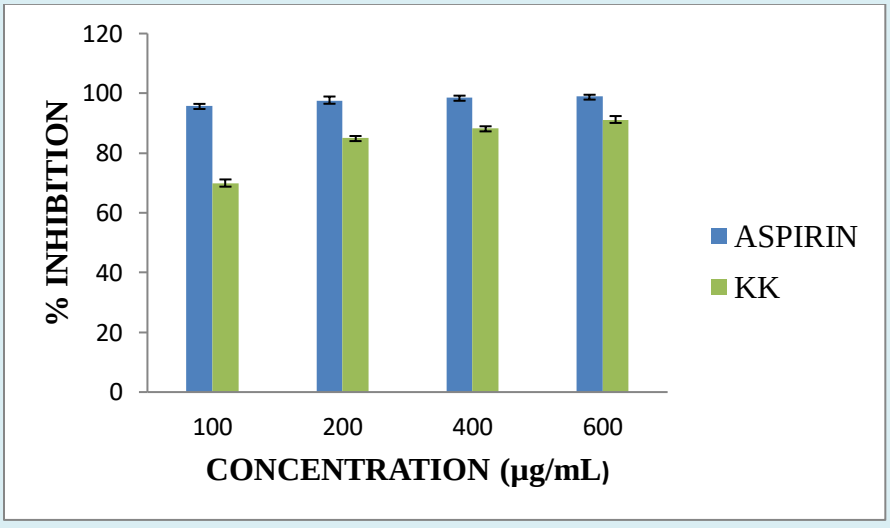

Figure 7: Anti- inflammatory activity for Kasthuri Karuppu

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*3.6. In-vitro*anti-oxidant activity

The *in-vitro* anti-oxidant activity of Kasthuri Karuppu was evaluated against DPPH radical scavenging method. The % inhibitions of standard sample ascorbic acid were 53.71%, 60.53%, 80.03%, 97.42% and 99.4 %at the concentration of 20, 40, 60, 80 and 100µg/mL, respectively.The % inhibitions of Kasthuri Karuppu were 61.18%, 62.38%, 62.91%, 63.67%and 64.71% at the concentration of 20, 40, 60, 80 and 100µg/mL, respectively. The Kasthuri Karuppu possessed significant anti-inflammatory activity as compared to the standard sample ascorbic acid[12].

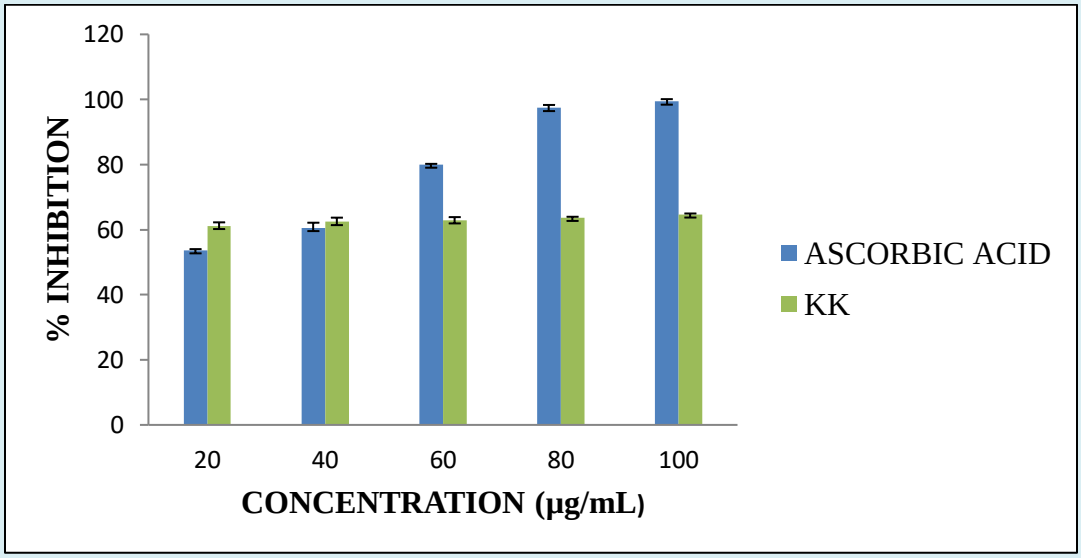

Figure 8: Anti-oxidant activity for Kasthuri Karuppu

CONCLUSIONS

The traditional Siddha medicine preparation Kasthuri Karuppu has been characterized through modern analytical techniques.The results of zeta sizer and scanning electron microscopy confirmed the particles with polydisperse nature and non-uniform surface morphology due combination of herbs and inorganic compounds.The FTIR results confirmed the presence of characteristic peaks, especially for the*β*-HgSinKasthuri Karuppu.The XRD analysis showed the

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formulation was crystalline in nature because of the presence of Hg and S, based on the JCPDS data reference file.Further, *in vitro* assays showed that Kasthuri Karuppu had maximum anti-inflammatory activity at 600µg/mL concentration with inhibition percentage of 90.1% and significant anti-oxidant property at 100µg/mL concentration with inhibition percentage 64.7%.The characterization studies and *in vitro* activities could be the scientific tool to assess the quality and potentialtherapeutic effects.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

In vitro anti-oxidant and anti-inflammatory activity of Painex poly herbal formulation

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Abstract *–Painex is a polyherbal Ayurvedic formulation prepared with ten different herbal ingredients, each one containing chief phytoconstituents. It is a good therapeutic and dietary medicine for women, which may be easily prepared at home.It is used in the treatment of complications that arise during pain, as an immediate effective pain reliever.In the current study, the ability of Painex in scavenging the free radicals was assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, and the invitro anti-inflammatory studies by protein denaturation assay.Painex aqueous extract and syrup were investigated for in vitro antioxidant activity at the concentration of 1 mg/mL by DPPH method.Painex extract had significant antioxidant activity, as compared to standard Vitamin C (ascorbic acid) under similar conditions.Painex had notable anti-inflammatory activity. The study suggests that, Painex possess good source of natural antioxidants, and is considered useful in combating the advances of oxidative stress, thereby preventing from the diseases that are caused by the free radicals.*

Keywords *–Painex, DPPH assay, anti-oxidant activity, invitro anti-inflammatory activity*

1. INTRODUCTION

Painex is a polyherbal formulation composed of versatile medicinal plants such asVasambu (Acorus calamus) [1,2], Omum (Trachyspermumammi) [3,4], Lavangam (Syzygiumaromaticum) [5,6], Milagu(Piper nigrum) [7,8], Chukku (Zingiber officinale) [9,10], Thippili(piper longum) [11,12].Painex poly herbal formulation exhibits excellent pharmacological and medicinal value due to the presence of enriched bioactive molecules.The phytochemical contents of poly herbal formulation Painex is responsible for its notable antioxidant activity, which leads to many therapeutic and medicinal values.Antioxidant potential of bioactive molecules of herbs is responsible for the recommendation of treatment many diseases. Present work is envisaged to study the in vitro antioxidant activity by using DPPH assay method to explore the level of antioxidant potential compared to standard reference and also to investigate the invitro anti-inflammatory activity by protein denaturation assay. The results suggested that Painex, possess notable antioxidant, and anti-inflammatory activity.Free radicals are mainly responsible for several internal organ diseases including hepatic, kidney, cardiovascular and uterine disorders.The poly herbal formulation with high antioxidant activity is essential for the treatment of many such human diseases.

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2. MATERIALS & METHODS

2.1. Method of preparation of sample

10 g of *PAINEX* powderwas heated with 100 mL of ethanol separately for 5 hours under reflux condenser in water bath, then cooled and filtered.The filtrate was evaporated under vacuum to get the concentrated ethanolic extract.

2.2. Pharmacological evaluation

***In vitro* antioxidant activity**

Painex alcohol extract was investigated for *in vitro* antioxidant activity by DPPH for theestimation of anti-oxidant potential of poly herbal formulation.

Determination of *DPPH radical scavenging activity*

Antioxidant activity in the sample was estimated by their free radical scavengingactivity through DPPH assay using (1, 1-Diphenyl-2, Picryl-Hydrazyl) reagent[13-15].About 100 µL of extract sample was taken in the microtiter plate. Then, 100µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration from purple to yellow and pale pink were considered as strong and weak positive, respectively.The plate was read using Elisa plate reader at 490nm.Pure ascorbic acid was used as standard reference sample. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

Table 1: In vitro antioxidant activity of Painex by DPPH assay

S. No.	COD	SOD	% inhibition	Average
1	1.35	0.06	95.55	
2	1.41	0.05	96.45	
3	1.35	0.05	96.29	

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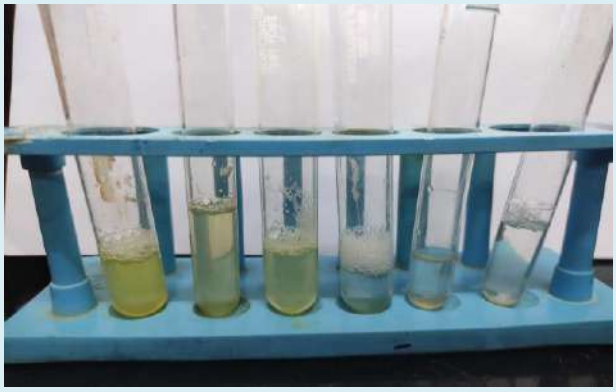
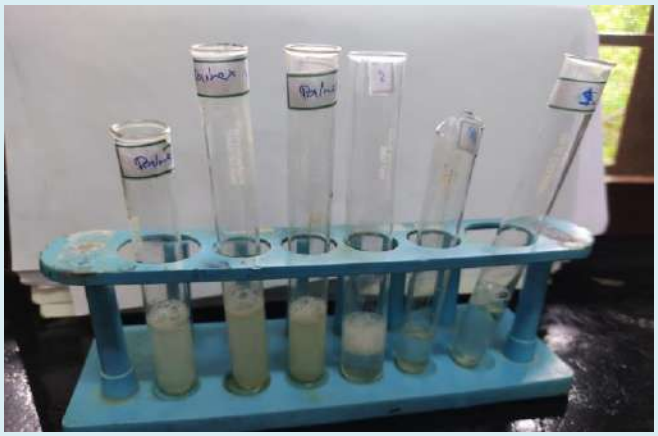
4	1.34	0.06	95.52	
5	1.40	0.05	96.42	
6	1.40	0.05	96.42	96.10%

Table 2: In vitro antioxidant activity of standard ascorbic acid by DPPH assay

S. No.	COD	SOD	% inhibition	Average
1	0.98	0.02	97.90	
2	0.99	0.09	90.90	
3	0.99	0.10	89.80	
4	0.98	0.09	90.80	
5	0.99	0.09	90.90	
6	0.98	0.10	89.70	91.60%





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***In vitro* anti-inflammatory activity-Inhibition of albumin denaturation**

The reaction mixture 0.5 mL aqueous extract sample contains drug 0.05 mL in the concentration (1 mg/mL) with 0.45 mL aqueous solution of bovine albumin fraction (5%). The pH (6.3) of solution was adjusted using small amount of 0.1N HCl at 37 ^{0}C for 20 min, then heated to 57 ^{0}C for 30 min.The solution was cooled and transferred to the 96 well plate and the absorbance was measured at 660nm. Standard was used as Diclofenac sodium (1000µg/mL) and control contain 0.05mL distilled water [16,17]. The percentage of inhibition of albumin denaturation was calculated by the following formula, Percentage of inhibition (%) =[($A_{control}$ – A_{sample}) / $A_{control}$] x 100. Where $A_{control}$ - Absorbance above all mixture except drug and A_{sample} - absorbance reaction mixture with Sample)

Table 3: In vitro anti-inflammatory activity of Painex

S. No.	COD	SOD1	SOD2	% inhibition	Average
1	0.89	0.02	0.28	68.53	
2	0.89	0.02	0.34	61.79	
3	0.91	0.01	0.40	56.04	62.12%

Table 4: In vitro anti-inflammatory activity standard Diclofenac

S. No.	COD	SOD	% inhibition	Average
1	0.89	0.02	97.75	
2	0.89	0.02	97.75	
3	0.91	0.01	98.87	98.13%

PAINEX CAPSULE:

PAINEX CAPSULE-(0.1)

S. No.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.21	78.5%
2	520	1.00	0.22	77.7%
3	520	1.00	0.20	79.5%
				78.5%

PAINEX CAPSULE-(0.2)

S. No.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.18	78 %
2	520	1.00	0.20	78.9%
3	520	1.00	0.22	77.5%

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	79.0%

PAINEX CAPSULE-(0.3)

S. No.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.25	74.4%
2	520	1.00	0.13	86.7%
3	520	1.00	0.17	82.6%
				81.2%

PAINEX CAPSULE-(0.4)

S. No.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.14	85.8%
2	520	1.00	0.16	83.6%
3	520	1.00	0.18	81.6%
				83.6%

PAINEX CAPSULE-(0.5)

S. No.	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.19	93.8%
2	520	1.00	0.16	83.6%
3	520	1.00	0.17	83.6%
				83.4%

3. RESULTS& DISCUSSION

Painex is polyherbal formulation which had demonstrated significant antioxidant activity of 96% when compared with standard ascorbic acid (Vitamin C) of 92 % under similar conditions. It also exhibited dose dependent activity (79-83 % at 1-5 mg).Painex exhibited moderately good anti-inflammatory activity of 62% inhibition of protein denaturation when compared to the standard diclofenac sodium of 98% at 100 mg/mL concentration under similar conditions.Painex documented for significant antioxidant and anti-inflammatory due to their versatile poly herbal combinations and their enriched phytochemicals content.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Physico chemical characterization and *in vitro* activities of Sivanar amirtham

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Abstract *–Polyherbomineral formulations are the higher order medicines which possess high therapeutic efficacy than the general herbal formulations. Even though the formulations are in clinical practice since many hundred years ago, but it lacks scientific evidence which is considered to be the crucial one for the global acceptance of these medicines. So, the aim of the study is to develop the analytical profile for a polyherbomineral formulation, Sivanar Amirtham. Different physicochemical characterization studies includingparticle size distribution, surface morphology, chemical nature, crystalline properties are studied.From the results the average particle size distribution was found to be 448nm with polydispersity index of 0.57. FTIR spectrum showed the peaks of chemical compounds' functional groups present, and XRD confirmed the presence of metacinnabar with its crystalline peak.The in-vitro assays wereperformedfor Sivanar Amirtham to acquire supportive evidence for its anti-inflammatory and anti-oxidant activity using protein denaturation method and DPPH assay, respectively.*

Keywords *–Sivanar amirtham, polyherbomineral, analytical characterization, anti-inflammatory, anti-oxidant*

1. INTRODUCTION

Sivanar Amirtham isa Polyherbomineral formulation which is categorized under Karuppu in Siddha system of medicine. Sulphur and Mercury are the main ingredients present along with the other herbals.It is used to treat 80 types of vatha diseases, 40 types of pitha diseases, 20 types of kapha diseases, 5 types of respiratory diseases, leucoderma, gastric ulcer, fits, piles, tuberculosis and liver problems. The ingredients present in Sivanar Amirtham includes sudhiseidharasam (elemental mercury), sudhiseidhagandhakam (elemental sulphur), sudhiseidha manosilai (arsenic trisulphide), sudhiseidha venkaram (borax), sukku (*Zingiber officinale*), karunabi (*Aconitum ferox*), thippili (*Piper longum*), iruvi (*Dryopteris filixmas*) and milagu (*Piper nigrum*) [1, 2, 3]. The processed form of mercuric sulphide of black is termed as *Kajjali* in Siddha medicine. The preparation protocol was followed as per the Siddha Formulary of India (1993) and Siddha vaithya thirattu (a traditional medicinal encyclopedia) and Indian standard monographs as Ayurveda & Siddha Pharmacopeia. The formulation procedure begins with *Kajjali* preparation by mixing mercury and sulphur [4, 5], followed by the addition of remaining ingredients as individual grinded form to obtain smooth black fine powder.

Figure 1: Sivanar amirtham

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The aim of the present study is validating Sivanar Amirtham scientifically using modern analytical techniques and *in vitro* assays. Standardization of these drugs are crucial for analyzing the quality of drug as well as essential to meet global acceptance. The modern analytical techniques like Zeta sizer, Scanning Electron Microscopy (SEM), Fourier Transform Infra-Red (FTIR) studies and X-Ray Diffraction (XRD) analysis are utilized to develop analytical profile for Sivanar Amirtham. The *in-vitro* studies like anti-inflammatory assay and anti-oxidant assay are carried out to provide the basic scientific evidence for intended therapeutic uses.

2. MATERIALS & METHODS

2.1. Materials

Sivanar Amirtham manufactured by Aravind Herbals Pvt. Ltd., Rajapalayam was used in the experiment. The chemicals for *in-vitro* analysis like DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and L-ascorbic acid were commercially purchased from Sigma-Aldrich, Mumbai.

2.2. Particle size analysis

Zeta sizer was used to determine the average particle size and zeta potential of Sivanar Amirtham using Dynamic Light Scattering (DLS) technique. This helps to measure the distribution of particles from sub nanometer to micrometer level. The mean particle size range can be determined at fixed angle using DLS sensors [6].

2.3. SEM analysis

Surface morphology of Sivanar Amirtham was characterized using scanning electron microscopy (SEM).Passing a high energy electron beam over the sample results in the signals, due to interactions between them, which provides the textural details. The samples were visualized at different magnifications of 10X, 15X, 30X, 50X and 100X.

2.4. FTIR analysis

Fourier Transform Infrared Spectroscopy (FTIR) spectrum for Sivanar Amirtham was recorded between 4000- 400 cm^{-1}.About 50 mg of sample was pelletized with KBr for analysis. Irradiating the sample with IR causes vibrational changes of the chemical bonds like stretching or bending, resulting in the characteristic peaks. The samplewas scanned and screened for the identification of different functional groups present in the compound [7,8].

2.5. XRD analysis

X-ray diffraction patterns helps in determining the crystalline nature of the samples.Sivanar Amirtham was characterized using X-ray Diffractometer (Bruker D8-Advance X-ray diffractometer) by using Cu-Ka radiation with a wavelength of 1.5405Å. Angular range was measured at the rate of 0.01°/ sec [9].

2.6. In-vitro anti-inflammatory activity

About 1g of Sivanar Amirthamwas taken in 20mL of distilled water and kept at room temperature for 15 min. Then, it was heated at 80°C for 20 min and then, let to cool down to room temperature and filtered using filter paper. The crude extract sample was taken at the concentration of 1mg/mL and further diluted to get 100, 200, 400 and 600µg/mL. The 1% aqueous solution of bovine serum albumin (BSA) fraction was prepared. Phosphate buffer with pH 6.7 was prepared. Aspirin was taken as standard reference sample. About 10mg of Aspirin in 10ml of ethanol was prepared to get 1mg/mL concentration, which was further diluted to obtain 100, 200, 400 and 600 µg/mL concentration solutions. To 2mL of the test sample extracts and the standard drug Aspirin solutions at different concentrations, about 0.2 mL of BSA and 2.8 mL of phosphate buffer were added and heated for 20 min. Then, the absorbance of all the samples was observed under UV-Visible spectroscopy at the wavelength of 660 nm[9].

2.7. In-vitro anti-oxidant activity

About 2.5g of Sivanar Amirtham was taken in 50mL of ethanol. It was occasionally shaken for 8 h and kept undisturbed for 16h. Then, the sample was kept in water bath to evaporate till dryness and obtain the powder.About 10mg of dried concentrate extract powder was taken in 10mL of methanol to get the concentration of 1mg/mL and further diluted to 10, 20, 40, 60, 80, and 100 µg/mL concentration solutions. For the standard solution, 10mg of Ascorbic acid in 10mL of methanol was taken and diluted to obtain 10, 20, 40, 60, 80, and 100 µg/mL concentrations. About 0.004% DPPH solution was prepared by taking 0.004g of DPPH in 100mL of methanol and incubated in the dark for 5 h. To the 3mL of test and standard samples (at different dilutions), 3mL of DPPH was added and incubated for 1 h. Then, the absorbance of all the solutions was observed under UV-Visible spectroscopy at the wavelength of 517 nm [10, 11].

3. RESULTS& DISCUSSION

3.1. Zeta sizer analysis

The particle size distribution and zeta potential of Sivanar Amirtham observed using Zeta sizer is shown in the Table 1 and Figures 2 & 3, respectively.

Table 1: Zeta sizer results of Sivanar Amirtham

S.No.	Parameters	Sivanar amirtham
1	Average Particle size	448.4 nm
2	Poly dispersity index	0.570
3	Zeta potential	-18 mV

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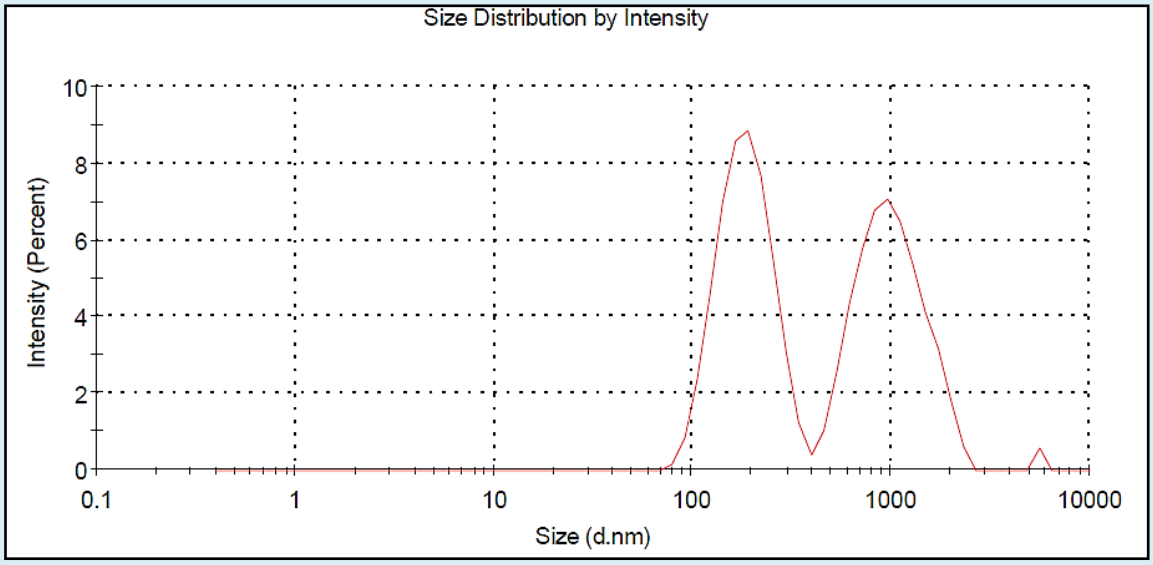

Figure 2: Particle size distribution of Sivanar Amirtham

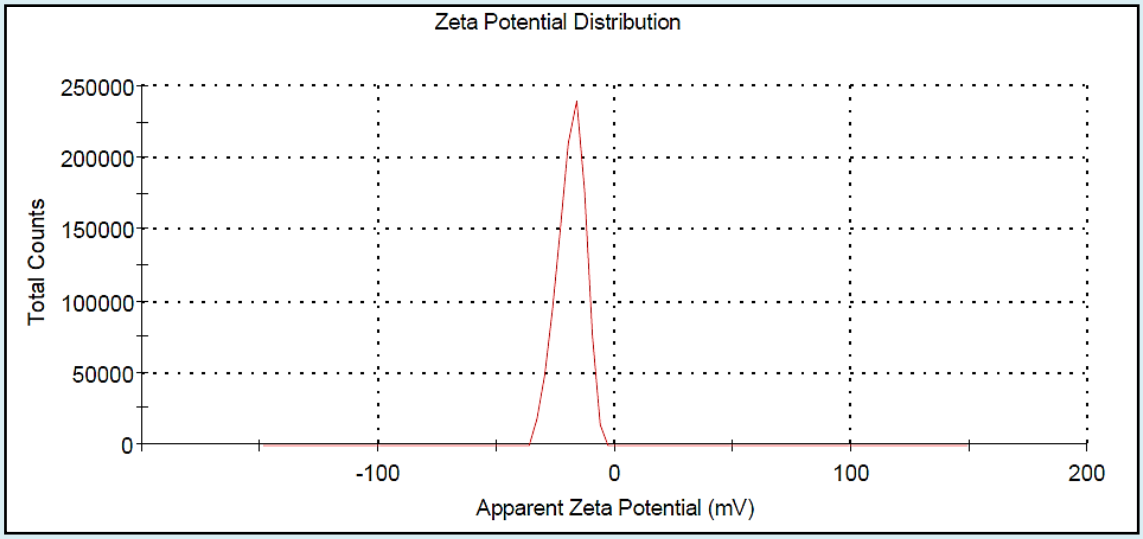

Figure 3: Zeta potential ofSivanar Amirtham

3.2. SEM analysis

The surface morphology analysis of the Sivanar Amirtham was carried out using scanning electron microscopy. The SEM image of the sample(Figure 4) showed irregular size and shaped particles (both in micron and nano dimension), indicating the polydisperse nature due to the presence of herbs and metals/minerals combination.

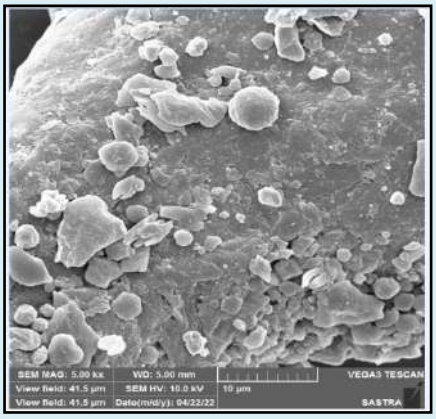

Figure 4: SEM image of Sivanar Amirtham

3.3. FTIR analysis

In FTIR spectra (Figure 5) analysis, the sample Sivanar Amirtham exhibited peaks at 3360cm^{-1}, 2924 cm^{-1}, 1635 cm^{-1} and1446 cm^{-1}showing O-H stretching, N-H stretching, C=C stretching and C-H bending. This indicated the presence of functional groups such alcohol, aliphatic primary amine, conjugated alkenes and alkanes in the polyherbal composition (Table 2).The characteristic peak of beta-HgSwas identified clearly in Sivanar Amirtham at 1031 cm^{-1}, which indicated the existence of Hg-S bond.This confirmed the presence of black mercuric sulphide (*Kajjali*) in Sivanar Amirtham.

Table 2: FTIR Vibrational peaks and functional group of Sivanar Amirtham

Wavenumber (cm^{-1})	**Functional group**
3360	O-H stretching
2924	N-H stretching
1635	C=C stretching
1446	C-H bending
1031	β-HgS

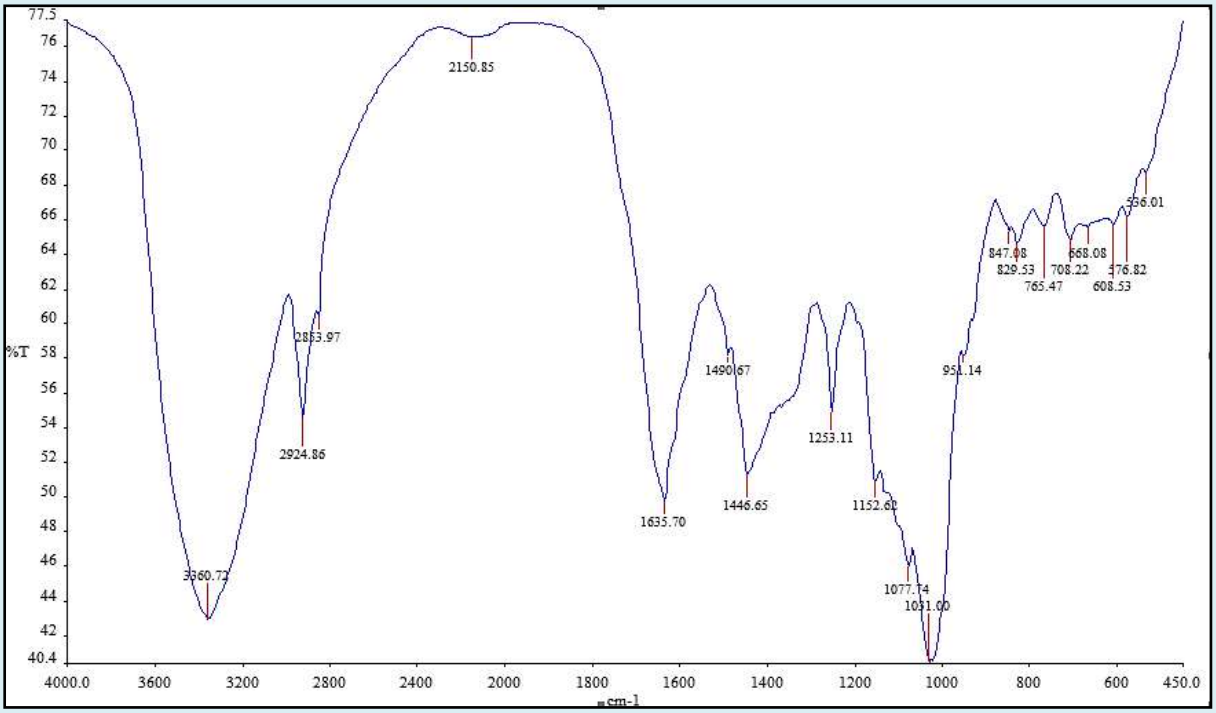

Figure 5: FTIR of Sivanar Amirtham

3.4. XRD analysis

The XRD pattern of Sivanar Amirtham exhibited characteristic peaks due to the presence of free sulphurand mercury sulphide (HgS). The data base value of the peaks was identified from the files with the numbers 08-0247 and 75-1538 by the Joint Committee on Powder Diffraction Standards [JCPDS]. The HgS peak was observed at 26º for Sivanar Amirtham. The Sivanar Amirtham showed sharp peaks, which confirmed the crystalline nature of the minerals.The XRD pattern also confirmed the presence of beta HgS peaks at 26º for metacinnabar and free sulphur at 23º by comparing the result with Miguel *et al.*[12] which was the best identity option to trace the phase transition.The XRD pattern also confirmed the presence of beta HgS peaks at 26º for metacinnabarand free sulphur at 23ºby comparing the result with Bandari Srinivasulu*et al.*[13] which was the best identity option to trace the phase transition.The rest of the peaks had extremely low intensities and showed the contributions of other compounds in the polyherbomineral mixtures.

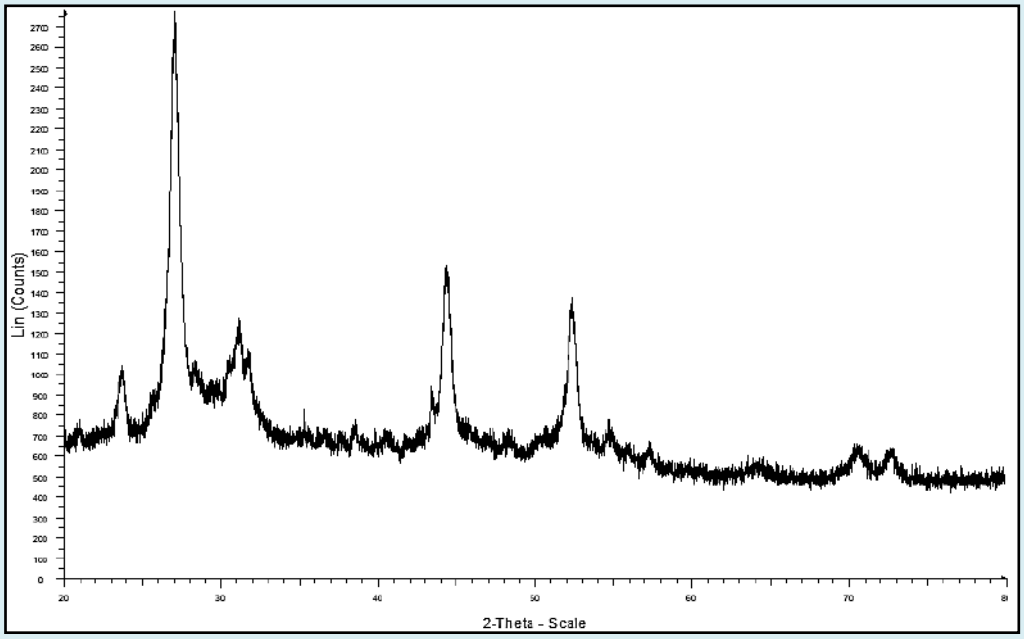

Figure 6: XRD pattern of Sivanar Amirtham

3.5. In-vitro anti-inflammatory activity

Anti-inflammatory activities of Sivanar Amirtham were evaluated through protein denaturation method. The percentage inhibition of standard sample aspirin was 96.45%, 96.62%, 95.38% and 95.66%at the concentration of 100, 200, 400 and 600µg/mL, respectively. The percentage inhibition of Sivanar Amirtham were 71.54%, 76.48%, 83.73% and 87.13% at the concentration of 100, 200, 400 and 600µg/mL, respectively. The Sivanar Amirtham possessed lesser anti-inflammatory activity compared to the standard sample aspirin.

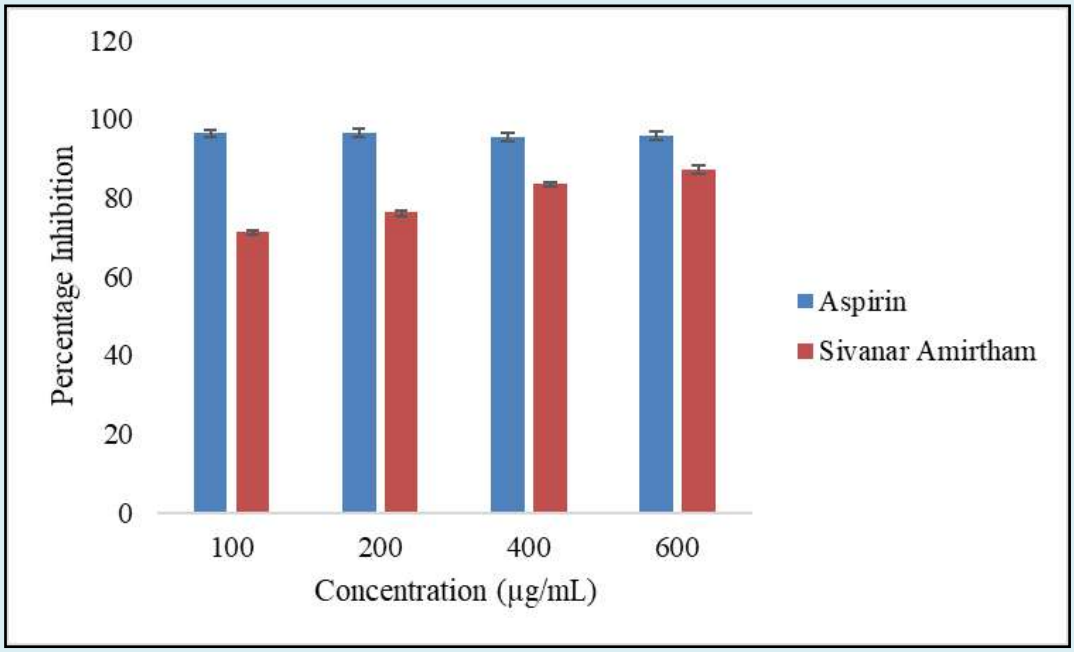

Figure 7: Anti- inflammatory activity for Sivanar Amirtham

3.6. In-vitro anti-oxidant activity

Anti-oxidant activity of Sivanar Amirtham was evaluated against DPPH radical scavenging method. The percentage inhibition of standard sample ascorbic acid was 53.71%, 60.53%, 80.03%, 97.42% and 99.4 %at the concentration of 20, 40, 60, 80 and 100µg/mL, respectively. The percentage inhibition of Sivanar Amirtham were 92.9%, 92.29%, 95.14%, 97.76%and 98.25% at the concentration of 20, 40, 60, 80 and 100µg/mL, respectively. The Sivanar Amirtham possessed significant anti-inflammatory activity as compared to the standard sample ascorbic acid.

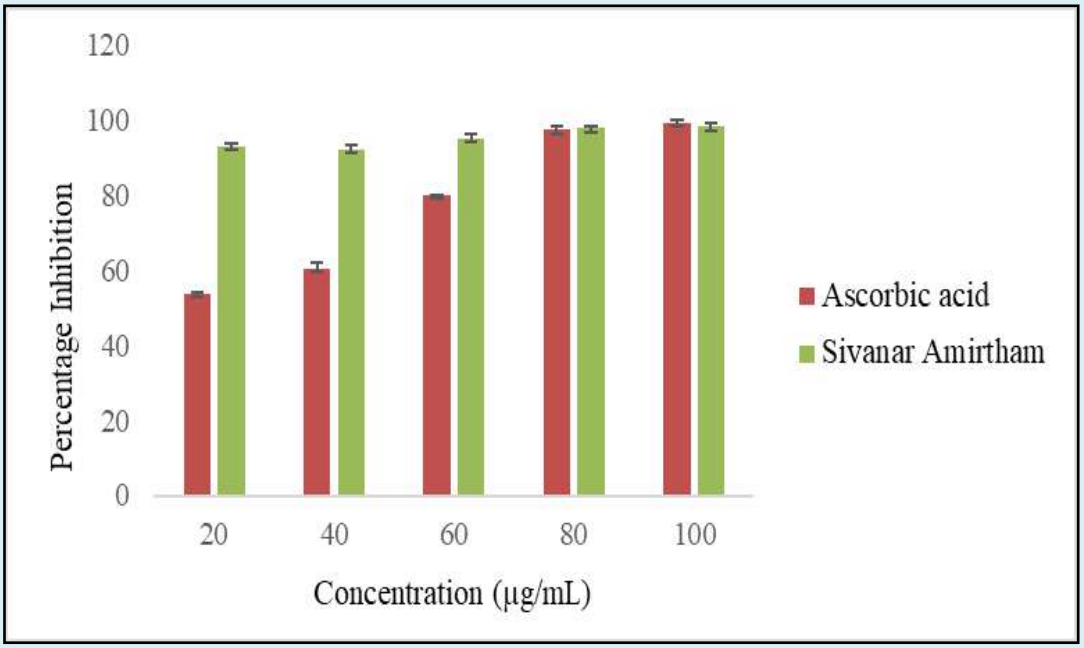

Figure 8: Anti-oxidant activity for Sivanar Amirtham

CONCLUSIONS

The present study involved characterization of the traditional Siddha preparations Sivanar Amirthamthrough modern analytical techniques. Particles size analyzed using zeta sizer and e surface morphology identified using scanning electron microscopy (SEM) confirmed the polydisperse nature of the Herbo-mineral formulation due to the combination of herbs and inorganic compounds.The FTIR spectrum of the formulation showed characteristic functional group peak for*β*-HgS along with other functional groups. The XRD analysis showed the formulations were crystalline in nature.Also, the presence of Hg and S was confirmed by X-Ray diffraction (XRD) data analysisusing JCPDS data file.Sivanar Amirtham showed maximum anti-inflammation activity at 600µg/mL concentration with inhibition percentage of 87.13%.And, it showed significant anti-oxidant properties at 100µg/mL concentration with inhibition percentage of98.25%. The characterization studies will help in developing the analytical profile for the polyherbomineral sample.Also, this could be the scientific tool to assess the quality of the sample. The *in-vitro* activity studies help to evaluate the potential therapeutic properties.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Phytochemical analysis and *in vitro* antioxidant activity and anti-inflammatory activity of under eye gel

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Abstract *–Under Eye Gelis a polyherbal Ayurvedic cosmetic formulation contains ingredients Azadirachta Indica (NEEM) , Smilax China* ***(Paranki patai****), Oleaeuropaea (olive oil), Aloe vera L (aloes), among them aloe vera is one of its chief constituents and base for gel. It is a good therapeutic medicine for under eye skin disease, which may be easily prepared at home and used in the treatment of complications that arise during skin problems. In the current study, the ability of UEG in scavenging the free radicals was assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, and the in vitro anti-inflammatory studies tested by protein denaturation assay MTT assay. The results proved UEG with notable antioxidant activity. UEG investigated for invitro antioxidant activity at the concentration of 1 mg/mL by DPPH method. UEG had significant antioxidant activity, as compared to standard Vitamin C (ascorbic acid) under similar conditions UEG had significant anti-inflammatory activity when compared with standard diclofenac sodium under similar conditions.Invitro antimicrobial activity of UEG tested against staphylococcus aureus, streptococcus epidermis and candida albicans by well plate method and compared with standard streptomycin under similar condition. UEG had significant activity against staphylococcus aureus and mild activity against other organism. The study suggests that, this herbal composition*

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possess good source of natural antioxidants, and is considered useful in combating the advances of oxidative stress, thereby preventing from the diseases that are caused by the free radicals.

Keywords *–Aloe vera gel, antioxidant activity, DPPH assay, anti-inflammatory assay, Azadirachta indica (neem), Smilax china, Oleaeuropaea (olive oil), Aloe vera L (aloes)*

1. INTRODUCTION

Under Eye gel is herbal formulation, composed of versatile medicinal plants such as *Azadirachta Indica* (NEEM) [1], *Smilax China* (Paranki patai) [2], *Oleaeuropaea* (olive oil) [3], *Aloe vera* L (aloes) [4], with excellent pharmacological and medicinal value due to the presence of enriched bioactive molecules and nutraceuticals. The phytochemical contents of herbal formulation of UEG is responsible for antioxidant activity, which leads to many therapeutic and medicinal values. Antioxidant potential of bioactive molecules of herbs is responsible for the recommendation of treatment many diseases. Present work is envisaged to study the *in vitro* antioxidant activity by using DPPH assay method to explore the level of antioxidant potential. invitro anti-inflammatory activity also investigated by protein denaturation assay using diclofenac sodium. Invitro antimicrobial activity of PAG tested against *staphylococcus aureus, streptococcus epidermis* and *candida albicans* by well plate method and compared with standard streptomycin under similar condition. Herbal formulation with antioxidant activity essential for the treatment of many human diseases. Free radicals are mainly responsible for various disorders and containing the excellent herbal combinations is used as skin [10,11].

2. MATERIALS & METHODS

2.1. In vitro antioxidant activity

UEG is investigated for *in vitro* antioxidant activity by DPPH, ABTS, FRAP and NO for the estimation of anti-oxidant potential of Under eye gel [5].

2.2. Determination of DPPH Radical scavenging activity

Antioxidant activity in the sample UEG were estimated for their free radical scavengingactivity by using DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals (George *et al.*, 1996) [6].

100µL of UEG extract was taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively and read the plate on Elisa plate reader at 490nm Standard ascorbic acid was used as reference. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

2.3. In vitro anti-inflammatory activity-Inhibition of albumin denaturation

Reaction mixture 0.5ml aqueous extract of UEG contains drug 0.05ml in the concentration (1mg/ml) with 0.45 ml aqueous solution of bovine albumin fraction (5%). The pH (6.3) of solution was adjusted using small amount of 0.1N HCl at 37 ^{0}C for 20 min, then heat to 57^{0}C for 30 min. Cool the solution and transfer to the 96 well plate and measure the absorbance at 660nm. Standard was used as Diclofenac sodium (1000µg/ml) and control contain 0.05ml distilled water [7].The percentage of inhibition of albumin denaturation was calculated by the following formula, Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.

A sample - absorbance reaction mixture with Sample)

3. RESULTS& DISCUSSION

Under Eye Gel (UEG) were investigated for *invitro* antioxidant activity at the concentration of 1 mg/mL by DPPH method to ensure the antioxidant activity. The results obtained from the antioxidant study indicated that UEG exhibit significant free radical scavenging potential. The study suggests that UEG has a good origin of natural antioxidants and might be beneficial in impeding the oxidative stress. The results suggested that UEG possess notable antioxidant activity (Table 1, and 2). They had exhibited significant antioxidant activity compared to standard ascorbic acid under similar conditions (Table 3). In the *invitro* anti-inflammatory activity UEG had significant anti-inflammatory activity when compared with standard diclofenac sodium (Table 5). Invitro antimicrobial activity of PAG tested against *staphylococcus aureus, streptococcus epidermis* and *candida albicans* by well plate method and compared with standard streptomycin under similar condition. PAG had significant activity against human pathogenic bacteria *staphylococcus aureus* and mild activity against other organism. The antioxidant potential and the anti-inflammatory efficiency of UEG formulation could aid for both prophylactic and therapeutic tool against skin diseases including inflammatory.

Table 1:*In vitro* anti-oxidant activity of UEG by DPPH assay

S. No.	COD	SOD	% inhibition	Average
1	0.36	0.14	61.11%	**57.41 %**
2	0.36	0.14	61.11%	
3	0.36	0.18	50.00%	

Table 2:*In vitro* anti-oxidant activity of UEG by DPPH assay

S. No.	COD	SOD	% inhibition	Average
1	0.36	0.19	47.22%	**47.05 %**
2	0.36	0.19	47.22%	
3	0.37	0.20	45.95%	
4	0.37	0.20	45.95%	
5	0.36	0.18	50.00%	
6	0.37	0.20	45.95%	

Table 3:*In vitro* anti-oxidant activity of Vit C (STD) by DPPH assay

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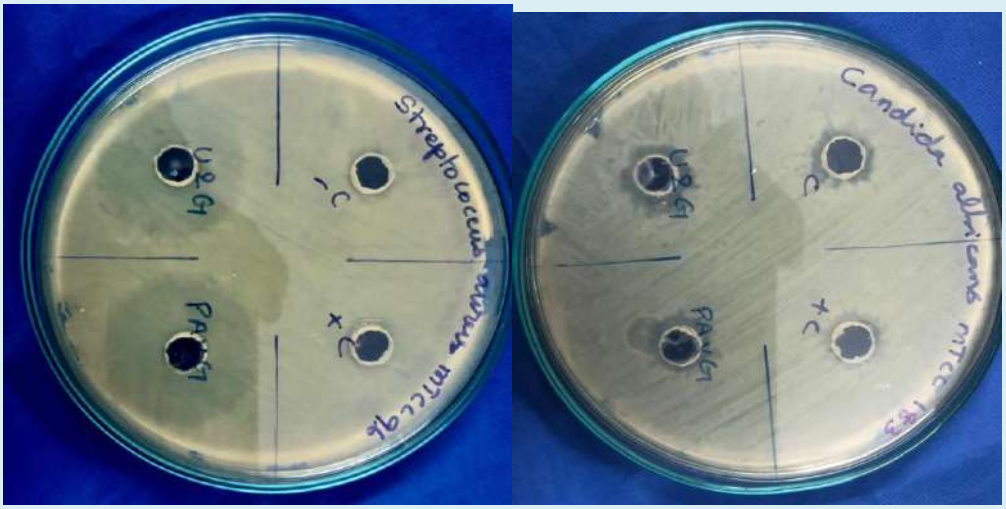
S. No.	COD	SOD	% inhibition	Average
1	0.36	0.06	83.30	**81.45 %**
2	0.36	0.07	80.50	
3	0.36	0.06	80.50	

Table 4:*In vitro* anti-inflammatory activity of UEG

S. No.	COD	SOD	% inhibition	Average
1	0.34	0.16	52.94	
2	0.34	0.15	55.88	**51.95%**
3	0.34	0.18	47.05	

Table 5:*In vitro* anti-inflammatory activity of Diclofenac sodium

S. No.	COD	SOD	% inhibition	Average
1	0.34	0.05	82.29	**81.37%**
2	0.34	0.07	79.41	
3	0.34	0.07	79.41	



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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Investigation of Pharmacological actions of *Clitoria ternatea* ethanolic extract (CT-ET)

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Abstract –Phytochemicals are ecologically derived plant secondary metabolites produced by the plants to protect them against environmental stress and invasions against pathogenic microbes.These Phyto ingredients are known to elicit beneficial as well as pharmacological actions against human ailments. It is an established fact that the active principles present in the medicinal plants act synergistically to alleviate the primary and secondary complications of several diseases.*Clitoria ternatea* is widely used as a therapeutic agent as well as dietary supplement for the treatment of various diseases. In the present study, the ability of the CT in scavenging free radicals was assessed by using DPPH assays, *invitro* anti-inflammatory activity, and the antibacterial activity of evaluation of CT extracts against multidrug resistance (MDR) *staphylococcus aureus, Klebsiella pneumoniae, E. coli performed* by well diffusion methods. The results obtained evidenced that the ethanol extract possesses significant antioxidant properties, anti-inflammatory activity and anti-bacterial activity. The data presented provide scientific evidence for the antioxidant and other therapeutic efficacy of the polyherbal preparation which in turn may be due to the presence of biologically active molecules present in the herbal preparation.

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Keywords *–Clitoria ternatea*, *invitro* antioxidant assays, DPPH, *invitro* anti-inflammatory

1. INTRODUCTION

Butterfly Pea (*Clitoria ternatea*) L. belongs to family Fabaceae generally known as Katarolu (Sinhala), Kokkattam (Tamil), Aparajitha (Bengali), and Asian Pigeon wing (English), is a perennial twinning medicinal plant, which has been widely used in traditional and Ayurveda systems of medicine in many parts of the world(1). Butterfly Pea is well adapted to a variety of soil types (pH 5.5–8.9) including calcareous soil and It exhibit excellent re growth after cutting or grazing within a short period and produce high yield(2). Moreover, it survives in both the extended rainfall regions and prolonged periods of drought. Butterfly Pea has pinnate leaves with 5 or 7 leaflets. Flowers auxiliary, single or paired; color range from white, dark blue to purple. Seeds 8–11/pod, oblong, somewhat flattened, 4.5–7 mm long, 3–4 mm wide, olive-brown to, almost black, shiny, often mottled, minutely pitted. Since this is a multipurpose plant, this has been used in traditional systems of medicine, herbal tea, cover crop, green manure, animal feed, nitrogen fixation crop, and as a weed-suppressing purpose (3). At the same time, phytochemical profiles of different plant parts like, root, leaves, flower extracts are widely used in Ayurveda and traditional systems of medicine to cure a number of diseases, including asthma, burning sensation, ascites, inflation, leucoderma, leprosy, hemicranias, amentia, pulmonary tuberculosis, ophthalmology, insect bites snakebite, scorpion sting and skin diseases (4). Moreover, the plant is used in a number of ailments including body-aches, infections, urinogenital disorders as antihelmintic and antidote to animal stings. They are considered safe for colic, dropsy, and enlargement of abdominal viscera (5).

The extracts from butterfly pea flower lowered the serum glucose levels of diabetic rats and increased their body weight. Further, the anti-diabetic effect was comparable to the diabetes drug glibenclamide (6). Since it not made from the camellia sinensisplant, butterfly pea tea is naturally caffeine-free, making it an ideal beverage for people with caffeine sensitivity. At the

same time the flower popular in Thailand, Malaysia, and Philippines as an edible dye. The petals are used to prepare ice creams and soups. At the same time, it can use as tea (7). Even though the plant is used for diverse purposes and widely distrusted in many parts of the country, it has not been yet systematically explored for its actual potential as an industrial crop (8). Therefore, the present study was undertaken to explore different C. ternatea species by means of taxonomic, phytochemical, and antioxidant capacity (6).

Biological properties of *Clitoria ternatea*

- Analgesic
- Antipyretic
- Anti-inflammatory properties
- Antioxidant
- Antidiabetic
- Antimicrobial
- Antihelminthic
- Hepaprotective
- Antiasthmatic
- Antipyretic
- Analgesic (9)

Chemical analysis

- Major constituents
- Flavonoids
- Anthocyanins
- Alkaloids
- Ternatins
- Saponins
- Tannins

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- Taraxerol
- Taraxerone (9)

Compounds:

- Quercetin 3-(2G - rhamnosylrutinoside),
- Kaempferol 3-(2G - rhamnosylrutinoside),
- Kaempferol 3-neohesperidoside,
- Quercetin 3-neohesperidoside,
- Myricetin 3- neohesperidoside,
- Kaempferol 3-rutinoside,
- Quercetin 3- rutinoside,
- Myricetin 3-rutinoside,
- Kaempferol 3- glucoside,
- Quercetin 3-glucoside,
- Myricetin 3-glucoside,
- Kaempferol 3-O-(2″-O- α-rhamnosyl-6″-O-malonyl)- βglucoside,
- Quercetin 3-O-(2″-O- α-rhamnosyl-6″-O-malonyl)- β-glucoside
- Myricetin 3-O-(2″-O- αrhamnosyl-6″-O-malonyl)- β-glucoside. Moreover,
- Kaempferol 3-glucoside,
- Quercetin 3-glucoside
- Myricetin 3-glucoside (10)

2. MATERIALS & METHODS

Extraction of Clitoria ternatea

Ethanol extract of Clitoria ternatea areal part prepared by hot continuous extraction process. 10 gm of CT powder and 100 ml methanol taken in round bottomed flask and refluxed in water bath

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for 6 hours and filter. The filtrate is dried under vacuum and dried extract used for pharmacological activity.

DPPH for Antioxidant activity

The free radical method using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) is a well-established assay for the in vitro determination of antioxidant activity in food and biological extracts. The standard DPPH assay uses methanol or ethanol as solvents, or buffered alcoholic solutions in a ratio of 40%/60% (buffer/alcohol, v/v) to keep the hydrophobic hydrazyl radical and phenolic test compounds soluble while offering sufficient buffering capacity at different pHs tested. Following this protocol, we were unable to keep proteinaceous antioxidants soluble at different pHs to test for their antioxidant activity(11).

Testing DPPH scavenging by control antioxidants in detergent buffer

As internal standard and for comparison to published methods, we recommend to test the antioxidant activity of the control compounds ascorbic acid (vitamin C) and the water-soluble derivative of vitamin E (TROLOX, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) in the detergent-based buffer (12)

Procedure

2.5, 5, 10, 15, and 20Mm of the control antioxidant compound were directly reconstituted in 950ml assay buffer. The reaction was started by adding 50ml of 2 Mm freshly prepared methanolic DPPH solution to the buffer to achieve a final DPPH concentration of 100 Mm. The solution was gently mixed by pipetting half the reaction volume (500 ml) 3–5 times. Note that a carry-over effect at higher ratios of antioxidant/DPPH can be observed using the same pipette tip in mixing the triplicate samples. This leads to higher standard deviations at higher antioxidant concentrations and needs to be considered during data analysis. The solution mix was incubated

for a total of 120 min at RT and the absorbance at 515 nm of each sample was measured in triplicate after 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min(13).

Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control – absorbance of DPPH

A sample – absorbance reaction mixture (DPPH with Sample)

Anti-inflammatory activity

Protein denaturation has been identified as the cause of inflammation. Indications are that when living tissues are injured, inflammation results. This is characterized by redness, pain, heat, swelling, as well as loss of function in the affected area. Disruption of the electrostatic, hydrogen, hydrophobic and disulphide bonds in the protein structure occurs. In addition, a complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair, occur, causing the protein to lose its molecular conformation and functions or become denatured (14). Clitoria ternatea is a well-known bioactive plant used to treat several inflammatory ailments in Ayurvedic system of medicine in India. The present investigation aimed to determine the anti-inflammatory and anti-arthritic activity of ethanolic extract of Clitoria ternatea roots (EECT) in animal models(15).

Procedure:

Reaction mixture 0.5ml contains drug 0.05ml in the concentration (1mg/ml) with 5% aqueous solution of bovine albumin fraction (0.45ml). The pH (6.3) of solution was adjusted using small amount of 0.1N HCL at 37 0C for 20 min. then heat to 57 0C for 30 min. Cool the solution and transfer to the 96 well plate and measure the absorbance at 660nm. Standard was used as Diclofenac sodium (1000µg/ml) and control contain 0.05ml distilled water.

Bovine Serum Albumin Assay (BSA):

The anti-inflammatory activities of the crude and fractionated plant extracts were determined using a modified version of the BSA assay reported by Williams et al.11 BSA solution (0.4%, w/v) was prepared in Tris Buffered Saline (one tablet is dissolved in 15 mL of deionized water to yield 0.05M Tris and 0.15M sodium chloride, pH 7.6 at 25 o C). The pH was

adjusted to 6.4 with glacial acetic acid. Stock solutions of each plant extract were prepared in methanol at a concentration of 50 µg/mL or 0.005%, w/v. Respective aliquots of 5.0 µL, 10 µL and 20 µL representing concentrations of 0.25 µg/mL, 0.50 µg/mL and 1.00 µg/mL of the stock solutions were added to test tubes containing 1 mL of 0.4%, w/v BSA buffer solution. Both negative (methanol) and positive (aspirin) controls were assayed in a similar manner. The solutions were then heated in a water bath at 72 °C for 10 minutes, and cooled for 20 minutes under laboratory conditions. The turbidity of the solutions (level of protein precipitation) was measured at 660 nm in a Hach Spectrophotometer using an air blank. The experiments were conducted in duplicate and the mean absorbance values were recorded. The percentage inhibition of precipitation (protein denaturation) was determined on a percentage basis, relative to the negative control using the following equation.

Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.

A sample - absorbance reaction mixture with Sample)

3. RESULTS& DISCUSSION

Among the various in vitro methods to measure the activity of antioxidants in food and biological samples, the substratefree DPPH assay has become quite popular due to its simplicity and speed of analysis (for reviews on antioxidant assays refer to). a combination of test procedures with a careful selection of antioxidant assays based on the system under study and question to be addressed have been proposed. We selected the substrate-free DPPH assay for our studies because its interaction kinetics with polyphenolic and non-phenolic compounds have been extensively characterized in the last two decades. The optimized DPPH assay protocol described above (see section ‘‘Determining DPPH scavenging by protein antioxidants in detergent buffer’’) could be a complementary, rapid and simple in vitro method to identify total antioxidant activity of soluble and possibly membrane proteins or peptides that would otherwise denature and precipitate in the conventional methanol and ethanol-based buffer systems. By

using control antioxidants (ascorbic acid, TROLOX) as standards we were able to determine absolute parameters of antioxidant activity (EC50) that were identical to what was published with the standard ethanol- or methanol-based buffer system. For example, EC50 values of 0.24 (ascorbic acid) and 0.33 (TROLOX) mole antioxidant per mole DPPH radical were obtained. We then calculated relative antioxidant activity values (VCEAC, TEAC) for the respective proteins to provide a second measure for total antioxidant activity of proteins relative to ascorbic acid and TROLOX. Preliminary data in our lab further indicated that thiol-containing compounds show a different kinetic with the DPPH radical than hydroxyl groupcontaining compounds using this new buffer system, potentially offering a convenient method for future studies to discriminate antioxidant-active residues involved in the total antioxidant activity of a given peptide, protein or protein mixtures under study.

Table 1: In vitro anti-oxidant activity by DPPH assay

CT- INVITRO ANTIOXIDANT ASSAY- DPPH METHOD

1 mg/ml:

S.NO	COD	SOD	% Inhibition	Average
1	0.36	0.09	75.00	
2	0.36	0.10	72.22	
3	0.36	0.08	77.77	74.99

2 mg/ml:

S.NO	COD	SOD	% Inhibition	Average
1	0.36	0.05	86.11	
2	0.36	0.12	66.66	
3	0.36	0.10	72.22	74.99

3 mg/ml:

S.NO	COD	SOD	% Inhibition	Average
1	0.36	0.04	88.88	
2	0.36	0.10	72.22	
3	0.36	0.09	75.00	78.77

4 mg/ml:

S.NO	COD	SOD	% Inhibition	Average
1	0.36	0.08	77.77	
2	0.36	0.11	69.44	
3	0.36	0.03	91.66	79.62

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5 mg/ml:

S.NO	COD	SOD	% Inhibition	Average
1	0.36	0.11	69.44	
2	0.36	0.07	80.55	
3	0.36	0.12	66.66	72.21

Table 2: Anti-inflammatory activity by protein denaturation assay

CT- In-vitro anti-inflammatory activity by protein denaturation assay

STANDARD:

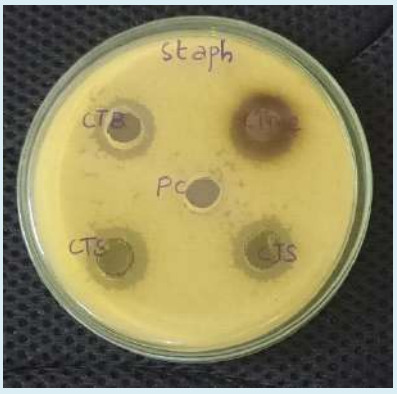
S.NO	COD	SOD	% Inhibition	Average
1	0.32	0.02	93.73	
2	0.32	0.02	93.73	
3	0.32	0.01	96.87	94.78

EXTRACT:

S.NO	COD	SOD1	SOD2	% Inhibition	Average
1	0.34	0.02	0.26	23.52	
2	0.30	0.02	0.24	20.00	
3	0.32	0.01	0.22	31.25	24.92

Antimicrobial activity of *clitoria ternatus* against MDR bacteria at 250 ml

S.NO	Organism	ZOI mm CTS	CTS ME	CTB
1	*Staphylococcus aureus* MRSA	17	19	18
2	*Klebsiella pneumoniae* MDR	20	18	20
3	*E. coli* MDR	0	20	0



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Adoption of the BSA protein denaturation assay for the in vitro evaluation of anti-inflammatory potential of ethanolic plant extracts circumvented the ethical issues associated with the use of animals, especially in the early stages of screening for plants with potential lead anti-inflammatory compounds. In addition, protein denaturation has been described as a pathological process which involves the loss of configuration, and as a result, loss of functionality. 4 This makes the reduction in protein denaturation, and by extension the BSA protein denaturation assay, ideal for the determination of anti-inflammatory potential. It should be noted that the experiments were conducted at pH 6.4, which represents the pathological pH (6.2 - 6.5) at which, reportedly heat treated BSA is stabilized (denaturation is inhibited) by several NSAIDs, 11 lending further credence to the choice of method.

This plant is a natural product that may provide a new source of antimicrobial agents which has an enormous therapeutic potential to heal a significant number of infectious diseases. Examples of microorganisms that have gained resistance to antimicrobials include Staphylococcus aureus, Candida albicans, Shigella dysenteriae, Streptococcus faecalis, Salmonella enterica serovar Typhi, S. enterica serovar Enteritidis and Escherichia coli(16).

The methanolic and petroleum ether extracts of the CT leaves are the greatest protection against Bacillus cereus and S. enterica serovar Typhi, respectively, each with a larger inhibition zone. Besides that, an earlier study carried out by highlighted that methanolic extraction of Clitoria ternatea showed the maximum zone of inhibition against bacterial species such as Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa and S. enterica serovar Typhi with zone of inhibition at 10, 12, 16 and 13 mm, respectively(17).

Moreover, the antifungal activity of Clitoria ternatea methanol extract also showed that maximum zone of inhibition against the fungal species such as Aspergillus niger, Penicillium chrysogenum, Aspergillus flavous and Fusarium oxiporum with inhibition zone at 10, 7, 12 and 11 mm, respectively.

Conclusion

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Clitoria ternatea ethanolic extract (CT ET) investigated for invitro antioxidant activity by DPPH method using ascorbic acid as standard. CT-ET had significant antioxidant activity when compared with ascorbic acid under similar conditions.CT-ET tested for invitro anti-inflammatory activity by protein denaturation method using diclofenac sodium as standard and exhibits moderate anti-inflammatory activity when compared with standard diclofenac sodium. The antibacterial activity of evaluation of CT extracts againstmulticx drug resistance (MDR) staphylococcus aureus, Klebsiella pneumoniae, E. coli performed by well diffusion methods.CT-ET and CT-ET had significant antibacterial activity at 100 µg/ml.

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Evaluation of Herbo green and Dermo herb ointments

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Abstract – *Dermo herb is a polyherbal formulation. Mercury (II) sulfide, Zinc oxide, Copper sulphate, Lead (II) oxide, Chaulmoogra seeds, Psoralea corylifolia, Papaver somniferum, Sesbania grandiflora, Ruellia patula, Cocos nucifera, and Bee's wax-yellow used to heal skin disorders and venereal diseases. This ointment is made of effective herbs which have more medicinal properties for a speedy recovery from skin disorders. In the present study, we have investigated the antioxidant activity of the Dermo herb. Phytoconstituents of Dermo herb is responsible for the antioxidant activities that can be evaluated with suitable in vivo studies. Herbo green ointment is a polyherbal formulation. Lead (II, IV) oxide, Lead (II) oxide, Copper sulphate, Psoralea corylifolia, Semecarpus anacardium, Bee's wax (Yellow), and Cocos nucifera used to heal skin disorders and venereal diseases. This ointment is made of effective herbs which have more medicinal properties for a speedy recovery from skin disorders. In the present study, we have investigated the antioxidant activity of Herbo green. Phytoconstituents of Herbo green is responsible for the antioxidant activities that can be evaluated with suitable in vivo studies.*

Keywords *- Dermo herb, Herbo green, antioxidant activity, skin disorders, venereal diseases*

1. INTRODUCTION

Dermo herb ointment is a Siddha proprietary medicine, made of pure Indian herbs that help to heal skin disorders and venereal diseases. This ointment is made of effective herbs which have more medicinal properties for a speedy recovery from skin disorders. Dermo herb ointment contains a combination of versatile medicinal plants such as Mercury (II) sulfide, Zinc oxide, Copper sulphate, Lead (II) oxide, Chaulmoogra seeds, *Psoralea corylifolia*, *Papaver somniferum*, *Sesbania grandiflora*, *Ruellia patula*, *Cocos nucifera* and Bee's wax-yellow with high therapeutic values and pharmacological actions due to their enriched phytochemical constituents, which is used for the treatment of skin and venereal diseases [1-15]. Polyherbal formulations with antioxidant, anti-inflammatory, and anti-bacterial activity are an essential requirement for the treatment of skin and venereal diseases [13-16]. The current study involves the preparation of an aqueous extract from Dermo herb polyherbal formulation by using the hot continuous extraction method. The present work involves the study of the antioxidant activity of the aqueous extract. Antioxidant activity by the DPPH method was compared with standard ascorbic acid under similar conditions. The antibacterial activity of the extract was assessed against *Streptococcus epidermidis* (MTCC 435) and *Pseudomonas aeroginosa* (MTCC424). Mercury sulfide can exist in several crystal structures, one of which mercury sulfide red occurs in hexagonal structure. It can be precipitated from hydrogen sulfide and mercury salt solution [1]. Zinc oxide is an inorganic compound with the formula ZnO. It is a white powder that is insoluble in water. ZnO is present in the Earth's crust as the mineral zincite. Most ZnO used commercially is synthetic. Zinc oxide is commonly found in medical ointments where it is used to treat skin irritations [2]. Copper (II) sulfate, also known as copper sulphate, is an inorganic compound with the chemical formula $CuSO_4(H_2O)_x$, where x can range from 0 to 5. The pentahydrate (x = 5) is the most common form. Copper sulphate is widely used as a fungicide, either alone or in association with other fungicides (maneb, zineb), for the treatment of orchards and vineyards. Lead (II) oxide, also called lead monoxide, is an inorganic compound with the

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molecular formula PbO. PbO occurs in two polymorphs: litharge having a tetragonal crystal structure, and massicot having an orthorhombic crystal structure. Modern applications for PbO are mostly in lead-based industrial glass and industrial ceramics, including computer components. It is an amphoteric oxide [3]. Chaulmoogra (*Hydnocarpus wightianus*) is an herb. Chaulmoogra powder, oil, emulsion, or ointment on the skin to treat skin problems including psoriasis and eczema. Chaulmoogra is given intravenously for leprosy [1-2]. *Psoralea corylifolia* is a plant used in Indian and Chinese traditional medicine. The seeds of this plant contain a variety of coumarins, including psoralen [3-5]. The opium poppy (*P. somniferum*) is grown as an ornamental and for its edible seeds. It is an important source of opiate drugs, including heroin, morphine, and codeine [6-8]. *Sesbania grandiflora*, is commonly known as the vegetable hummingbird. The leaf extract may inhibit the formation of advanced glycation end-products. The leaf extract contains linolenic acid and aspartic acid, which were found to be the major compounds responsible for the anti-glycation potential of the leaf extract [9-11]. Ruellia is used in the treatment of gonorrhea, syphilis, eye sore, renal infection, cough, wounds, scalds, toothache, stomach ache, and kidney stones [12-13]. *Cocos nucifera* is considered to be antipyretic and antidiuretic. Milk from a young coconut is a diuretic, laxative, anti-diarrhoeic and counteracts the effects of poison. The oil is used to treat diseased skin and teeth and mixed with other medicines to make embrocations. Bee's wax is used for lowering cholesterol and for relieving pain. It is also used for swelling (inflammation), ulcers, diarrhea, and hiccups [14-15].

Herbo green ointment is a Siddha proprietary medicine, made of pure Indian herbs that help to heal skin disorders and venereal diseases. This ointment is made of effective herbs which have more medicinal properties for a speedy recovery from skin disorders. Herbo green ointment contains a combination of versatile medicinal plants such as Lead (II, IV) oxide, Lead (II) oxide, Copper Sulphate, *Psoralea corylifolia*, *Semecarpus anacardium*, Bee's wax (Yellow), and *Cocos nucifera* (Figure 1) with high therapeutic values and pharmacological actions due to their enriched phytochemical constituents, which is used for the treatment of skin and venereal diseases [1-15]. Polyherbal formulations with antioxidant, anti-inflammatory, and anti-bacterial

activity are essential requirements for the treatment of skin and venereal diseases [13-16]. The current study involves the preparation of an aqueous extract from Herbo green polyherbal formulation by using the hot continuous extraction method. The present work involves the study of the antioxidant activity of the aqueous extract. Antioxidant activity by the DPPH method was compared with standard ascorbic acid under similar conditions. The antibacterial activity of the extract was assessed against *Streptococcus epidermidis* (MTCC 435) and *Pseudomonas aeroginosa* (MTCC424). Lead (II, IV) oxide, also called lead monoxide, is an inorganic compound with the molecular formula PbO. PbO occurs in two polymorphs: litharge having a tetragonal crystal structure, and massicot having an orthorhombic crystal structure. Modern applications for PbO are mostly in lead-based industrial glass and industrial ceramics, including computer components. It is an amphoteric oxide [3]. Copper (II) sulfate, also known as copper sulphate, is an inorganic compound with the chemical formula $CuSO_4(H_2O)_x$, where x can range from 0 to 5. The pentahydrate (x = 5) is the most common form. Copper sulphate is widely used as a fungicide, either alone or in association with other fungicides (maneb, zineb), for the treatment of orchards and vineyards. *Psoralea corylifolia* is a plant used in Indian and Chinese traditional medicine. The seeds of this plant contain a variety of coumarins, including psoralen [3-5]. *Cocos nucifera* is considered antipyretic and diuretic properties. Milk from a young coconut is a diuretic, laxative, anti-diarrhoeic and counteracts the effects of poison. The oil is used to treat diseased skin and teeth and mixed with other medicines to make embrocations. Beeswax is used for lowering cholesterol and for relieving pain. It is also used for swelling (inflammation), ulcers, diarrhea, and hiccups [14-15].

2. MATERIALS & METHODS

Organoleptic characteristics

The formulation was tested for physical appearance, colour, texture, and homogeneity. These characteristics were evaluated by visual observation. Homogeneity and texture were tested by pressing a small quantity of the ointment between the thumb and index finger. The consistency

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of the formulations and the presence of coarse particles were used to evaluate the texture and homogeneity of the formulations.

Spreadability

The spreadability of the formulation was determined by measuring the spreading diameter of 1 g of the sample between two horizontal glass plates (10 cm×20 cm) after one minute. The standard weight applied to the upper plate was 25 g. The procedure was performed in triplicates.

pH

One gram of formulation was dispersed in 25 mL of chloroform, and the pH was determined using a pH meter. Measurements were made in triplicate. The pH meter was calibrated with standard buffer solutions (pH 4, 7, and 10) before each use.

Washability

Washability was determined by rubbing a little amount of formulation on the hand and washing it off with water without using soap.

Irritancy test

An area of 1 sq. cm on the dorsal surface of the hand was marked. The ointment was applied to the specified area and time was noted. Irritancy, erythema, and edema were checked if any after 10 minutes and reported.

Release study

The experiments were conducted in the Franz diffusion cell apparatus (Electrolab, Mumbai, India) with a nominal volume of the receptor compartment of 12 mL filled with 7.4 PBS (Phosphate Buffer Saline). For the donor compartment, an amount of 0.25 g of each formulation was initially set. The experiments were conducted in triplicate, carried out at 37ºC and 100 rpm

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for 4 h. Samples were evaluated at different time points, by withdrawing 2 ml of sample, and data analysis was performed. The sample was spectrophotometrically determined at 235 nm using a UV-Visible spectrophotometer.

3. RESULTS & DISCUSSION

HERBO GREEN

Organoleptic characteristics

S. No.	Parameter	Inference
1	Physical appearance	Opaque
2	Colour	Green
3	Texture	Greasy
4	Homogeneity	Homogenous

Spreadability

S. No.	Length (cm)	Time (Sec)	Spreadability (S=m*l/t)	Diameter (cm)
1	4.2	1.47	71.42	2.8
2	4.5	1.55	72.58	3.5
3	4.7	1.58	77.53	3.2
Average			73.84	3.16 cm

m – weight tied on upper slide
l – length of glass slide
t – time in seconds

pH

S. No.	pH
1	5.36
2	5.41
3	5.45
Average	5.41

Washability

The ointment was greasy and not easily washable.

Irritability test

The ointment shown no signs of redness, edema, irritation and inflammation during the study.

 ISBN: 978-93-5408-448-5

Release study

Time (mins)	Absorbance
0	0
5	0.0441
10	0.0304
20	0.0627
30	0.0167
45	0.0932
60	0.0774
90	0.0939
120	0.0591
150	0.0449
180	0.0394
210	0.1457
240	0.1945

DERMO HERB

Organoleptic characteristics

S. No.	Parameter	Inference
1	Physical appearance	Opaque
2	Colour	Red
3	Texture	Greasy
4	Homogeneity	Homogenous

Spreadability

S. No.	Length (cm)	Time (Sec)	Spreadability (S=m*l/t)	Diameter (cm)
1	4.1	1.35	75.92	2.5
2	4.7	1.60	73.43	3.7
3	4.5	1.65	68.18	3.1
Average			72.51	3.1 cm

m – weight tied on upper slide
l – length of glass slide
t – time in seconds

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pH

S. No.	pH
1	5.16
2	5.20
3	5.12
Average	5.16

Washability

The ointment was greasy and not easily washable.

Irritability test

The ointment shown no signs of redness, edema, irritation, and inflammation during the study.

Release study

Time (mins)	Absorbance
0	0
5	0.0158
10	0.0512
20	0.0162
30	0.1135
45	0.045
60	0.097
90	0.121
120	0.0274
150	0.0523
180	0.0396
210	0.2136
240	0.2654

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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Pharmacological, antimicrobial and anticancer activities of *Vitex negundo* leaf extract

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Abstract *–Phytochemicals are environmentally derived plant secondary metabolites were plants make to protect themselves against environmental stress and pathogenic microbial invasion. These phytochemicals have been shown to have both positive and pharmacological effects in the treatment of human illnesses. It is well known that the active ingredients found in medicinal plants work together to reduce the primary and secondary difficulties of a variety of ailments. Vitex negundo (Family: Lamiaceae) is widely used as a therapeutic agent. In the present study, extract is subjected to Assay of Amylase Inhibition, Anti-diabetic (alpha amylase inhibition), Antimicrobial activity and MTT Assay (Colon Cancer cell line). The data presented provide scientific evidence for the anticancer & antidiabetic activity is responsible for therapeutic efficacy of Vitex negundo. Which in turn may be due to the presence of biologically active molecules present in the Vitex.*

Keywords *–Vitex, anticancer activity, antidiabetic activity*

1. INTRODUCTION

Vitex negundo belongs to Family Lamiaceae is a versatile medicinal plant, their excellent pharmacological and therapeutic benefits is due their enriched phytochemical constituents [1]. Vitex reported for broad spectrum of pharmacological activity due their chemical compounds and combination of medicinal plants of high therapeutic values [2] and documented for good therapeutic effect [3-7].

Figure 1: Photograph showing *Vitex negundo* L.

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In humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of polymeric substrates into shorter oligomers. Later on in the gut these are further hydrolyzed by pancreatic alpha amylases into maltose, maltotriose and small malto-oligosaccharides. The digestive enzyme (alpha-amylase) is responsible for hydrolyzing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of alpha amylase can lead to reduction in post prandial hyperglycemia in diabetic condition. Treatment of diabetes include: improvement of the activity of insulin at the objective tissues, with the utilization of sensitizers (biguanides, thiozolidinediones); incitement of endogenous insulin discharge with the utilization of sulfonylureas (glibenclamide, glimepiride), and decrease of the interest for insulin utilizing particular enzyme inhibitors (acarbose, miglitol). In the present study, the ability of the Assay of Amylase Inhibition, Anti-diabetic (alpha amylase inhibition), Antimicrobial activity and MTT Assay (Colon Cancer cell line) are tested.

2. MATERIALS & METHODS

2.1. Assay of amylase inhibition

In vitro amylase inhibition was studied by the method of Bernfeld [14]. In brief, 100 µLof the test extract was allowed to react with 200 µL of α-amylase enzyme (Hi media Rm 638) and 100 µL of 2 mM of phosphate buffer (pH-6.9). After 20-minute incubation, 100 µL of 1% starch solution was added. The same was performed for the controls where 200 µLof the enzyme was replaced by buffer. After incubation for 5 minutes, 500 µL of dinitrosalicylic acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula:

Inhibition (%) = Abs 540 (control) – Abs 540 (extract) * 100

Abs 540(control)

Suitable reagent blank and inhibitor controls were simultaneously carried out.

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2.2. Antibacterial activity

The antibacterial activity of evaluation of Vitex extracts against Staphylococcus aureus, Klebsiella pneumonia, E. coli, and Pseudomonas aeruginousa was performed by well diffusion methods [15]. The inoculum of the microorganism was prepared from the bacterial cultures. 15 ml of nutrient agar (Hi media) medium was poured into clean sterilized Petri plates and allowed to cool and solidify. 100 µl of broth of bacterial strain was pipette out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 6 mm in diameter were bored using a sterile cork borer. Solutions of all the extracts (1mg/ml) in DMSO were prepared. 100µl of extracts solutions was added to the wells. The Petri plates were incubated at 37°C for 24 h. streptomycin (1mg/ml) was prepared as a positive control DMSO was taken as the negative control. Antibacterial activity was evaluated by measuring the diameters of the zone of inhibitions (ZI) all the determinations were performed in triplicates. herbofeverox extract was also tested for antibacterial activity against human pathogens like Staphylococcus aureus, Klebsiella pneumonia, E. coli, and Pseudomonas aeruginousa.

2.3. MTT assay

Cell line: Colo-205 (Colon Cancer cell line)

Media: DMEM with high glucose (Cat No-11965-092), FBS (Gibco, Invitrogen)Cat No - 10270106 Antibiotic – Antimycotic 100X solution (Thermo fisher Scientific)-Cat No-15240062

Experimental procedure:

- Cells were incubated at a concentration of 1 × 104cells/ml in culture medium for 24 h at 37°C and 5% CO2.
- Cells were seeded at a concentration (70µl) 104cells/well in 100 µl culture medium and 100µl WTP and VNA Sample in (10, 20, 40, 80, 100 µg/ml) into micro plates respectively (tissue culture grade, and 96 wells).

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- Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture.
- Cell cultures were incubated for 24 h at 37°C and 5% CO2 in CO2 incubator (Thermo scientific BB150)
- After incubation, the medium was completely removed and Added 20 µl of MTT reagent
- (5mg/min PBS).
- After addition of MTT, cells incubated for 4 hrs at 37oC in CO2incubator.
- Observed the wells for formazan crystal formation under microscope. The yellowish MTT was reduced to dark coloured formazan by viable cells only.
- After removing the medium completely.Added 200µl of DMSO (kept for 10 min) and incubate at 370C (wrapped with aluminium foil).
- Triplicate samples were analyzed by measuring the absorbance of each microplate reader (Benesphera E21) at a wavelength of 570 nm.

3. RESULTS & DISCUSSION

The given sample VNA, which was utilized for the activity of anti-diabetic by using alpha amylase enzyme. The given sample taken in the different concentration, 100 to 1000 µg/ml. The results showed that the VNA showed good percent inhibition of the amylase enzyme. At the Concentration 10, 20, 40, 80, 100 µg/ml, Sample Code VNA and WTP, showed good percent inhibition Colo-205 (colon cancer cell line) cell line as compared to standard drug. The extract is against bacterial strains such as Staphylococcus aureus MRSA, Klebsiella pneumoniae MDR and E. coli MDR.

Table 1: Anti-diabetic (alpha amylase inhibition)

S. No.	Sample Code	ABS at 540 nm
1	Blank	0.51
2	Standard - 100	0.31
3	Standard - 200	0.29
4	Standard - 400	0.27

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5	Standard - 800	0.17
6	Standard - 1000	0.13
22	VNA II - 100	0.36
23	VNA II - 200	0.31
24	VNA II - 400	0.28
25	VNA II - 800	0.24
26	VNA II - 1000	0.21

S. No.	Sample Code(µg/ml)	Activity(%)
1	Standard - 100	39.21
2	Standard - 200	43.13
3	Standard - 400	47.05
4	Standard - 800	66.66
5	Standard - 1000	74.50
21	VNA - 100	29.41
22	VNA - 200	39.21
23	VNA - 400	45.09
24	VNA - 800	52.94
25	VNA - 1000	58.82

Table 2: Antimicrobial activity of VNA Vitex negundo (VN) against MDR bacteria at 250 ul

S. No.	Organism	ZOI mm VNA	PC	EA Me
1	*Staphylococcus aureus* MRSA	17	0	24
2	*Klebsiella pneumoniae* MDR	20	0	20
3	*E. coli* MDR	18	0	17

 ISBN: 978-93-5408-448-5

Table 3: Effects of compound against Colo-205 Cell line (colon cancer cell line) by MTT assay

S.No	Sample	Concentration(µg/ml)	OD	% inhibition	IC 50 (µg/ml))
1	Control		0.589		
2	Std. 5 FU	10	0.209	64.51	02.02
		20	0.131	77.75	
		40	0.119	79.79	
		80	0.081	86.24	
		100	0.044	92.52	
4	VNA	10	0.367	37.69	60.03
		20	0.318	46.01	
		40	0.310	47.36	
		80	0.283	51.95	
		100	0.248	57.89	

 ISBN: 978-93-5408-448-5

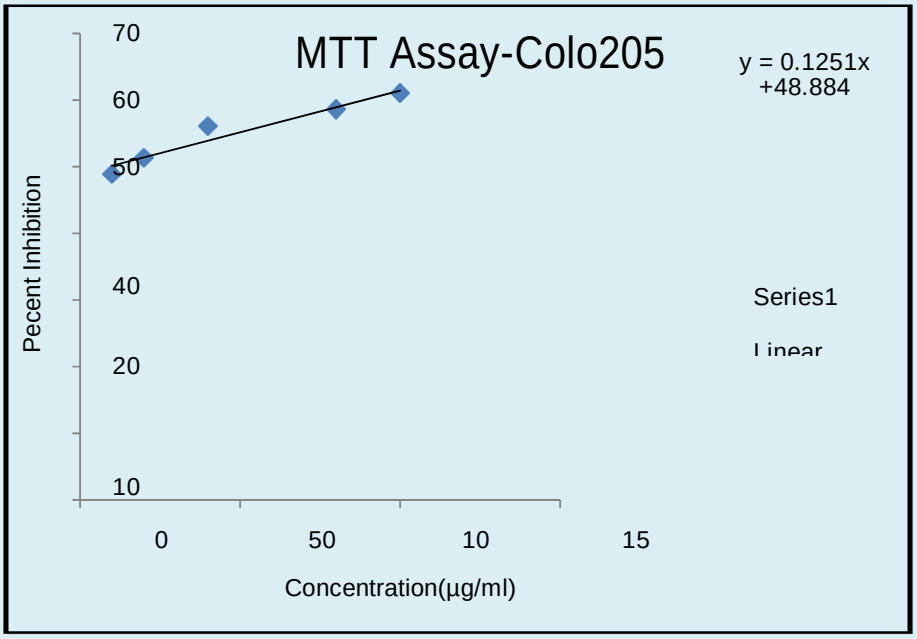
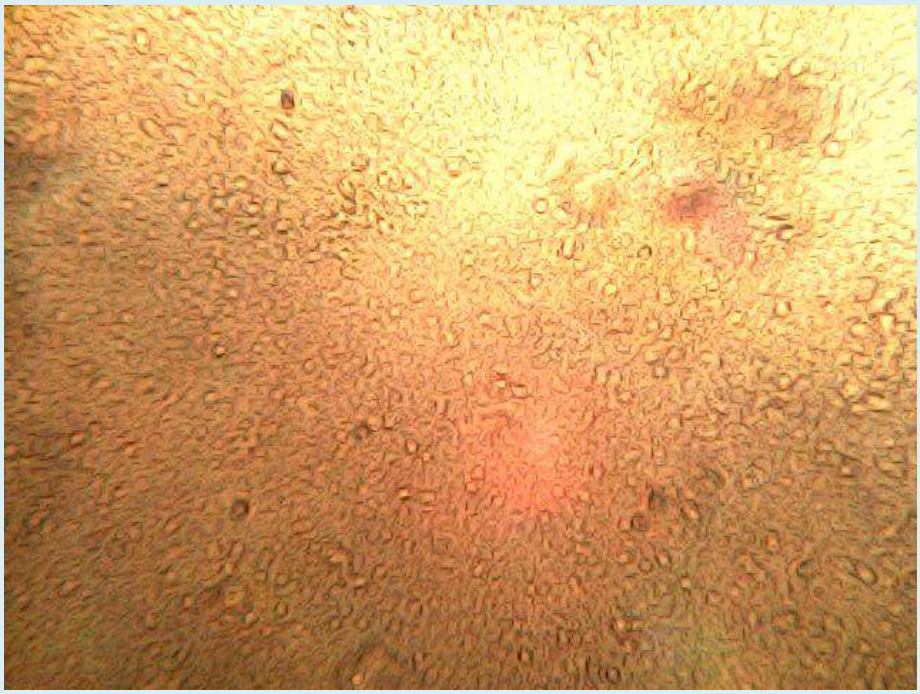


Figure 2: Anticancer activity of VNA extract by using colo 205 cell line.



Control

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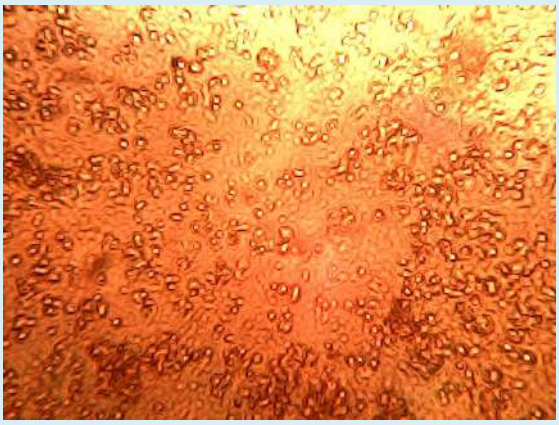
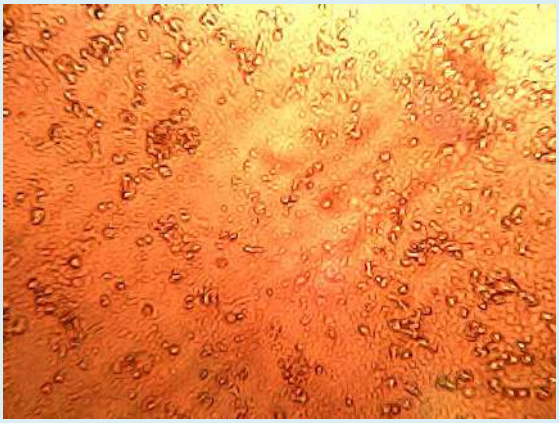


VN A



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EVIDENCE BASED HERBAL MEDICINE - PART - VI

Pharmacological evaluation of Rasagenthi Mezhugu

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Abstract *–Rasagenthi Mezhugu (RGM) is a polyherbal Ayurvedic formulation prepared with ten different herbal ingredients, each one containing chief phytoconstituents. It is a good therapeutic and dietary medicine for women, which may be easily prepared at home.It is used in the treatment of cancer.In the current study, the ability of RGM in Determination of DPPH radical scavenging activity, Anti-inflammatory activity and Antibacterial activity were investigated.*

Keywords *–RGM, DPPH assay, anti-oxidant activity*

1. INTRODUCTION

Rasagenthi Mezhugu (RGM) is a herbomineral formulation in the Siddha system of traditional medicine and is prescribed in the southern parts of India as a remedy for all kinds of cancers. Rasagenthi Mezhugu, a Siddha medicine, is a formulation containing 6 different botanicals and 8 inorganic substances, some of which are heavy metals. Siddha practitioners prescribe RGM as a therapy for different cancers. However, scientific evidence for the antioxidant, antibacterial and antiinflammatory activities are yet to be proved. Hence this study focused to identify the various pharmacological properties of Rasagenthi Mezhugu.

Composition

ChukkuZingber officinalls-114.240mgs
MilaguPiper nigrum-11.240mgs
ThippliPiper longum-11.240mgs
VallaraiCentalla asiatica-5.183 mgs
Rasaganthi MezhuguRasaganthi mezhugu-31.096mgs
Pal SarkkaraiLactose IP-30.000mgs

2. MATERIALS & METHODS

Determination of DPPH radical scavenging activity

Antioxidant activity in the sample RGM were estimated for their free radical scavengingactivity by using DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals (George et al., 1996) [6]. 100µL of RGM extract was taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively and read the plate on Elisa plate reader at 490nm Standard ascorbic acid was used as reference. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

In vitro anti-inflammatory activity-inhibition of albumin denaturation

The reaction mixture of 0.5ml aqueous extract contains drug 0.05ml in the concentration (1mg/ml) with 0.45 ml aqueous solution of bovine albumin fraction (5%). The pH (6.3) of the solution was adjusted using a small amount of 0.1N HCl at 37oC for 20 min, then heat to 57 0C

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for 30 min. Cool the solution and transfer it to the 96-well plate and measure the absorbance at 660nm. Standard was used as Diclofenac sodium (1000µg/ml) and the control contain 0.05ml distilled water [14].

The percentage of inhibition of albumin denaturation was calculated by the following formula,

Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.

A sample - absorbance reaction mixture with Sample)

Antibacterial activity

The antibacterial activity of evaluation of RGM extracts against staphylococcus aureus, Klebsiella pneumonia, E. coli, and Pseudomonas aeruginous was performed by well diffusion methods [15]. The inoculum of themicroorganism was prepared from the bacterial cultures. 15 ml of nutrient agar (Hi media) medium was poured into clean sterilized Petri plates and allowed to cool and solidify. 100 µl of broth of bacterial strain was pipette out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 6 mm in diameter were bored using a sterile cork borer. Solutions of all the extracts (1mg/ml) in DMSO were prepared. 100µl of extracts solutions was added to the wells. The Petri plates were incubated at 37°C for 24 h. streptomycin (1mg/ml) was prepared as a positive control DMSO was taken as the negative control. Antibacterial activity was evaluated by measuring the diameters of the zone of inhibitions (ZI) all the determinations were performed in triplicates. RGM extract was also tested for antibacterial activity against human pathogens like staphylococcus aureus, Klebsiella pneumonia, E. coli, and Pseudomonas aeruginous.

3. RESULTS& DISCUSSION

RGM is polyherbal formulation which had demonstrated significant antioxidant activity of 60% when compared with standard ascorbic acid (Vitamin C) of 85.18 % under similar conditions. It also exhibited dose dependent activity.RGM exhibited moderately good anti-inflammatory

activity of 95% inhibition of protein denaturation when compared to the standard diclofenac sodium of 93% at 100 mg/mL concentration under similar conditions.RGM documented for significant antioxidant and anti-inflammatory due to their versatile poly herbal combinations and their enriched phytochemicals content.

Table 1:*In vitro* anti-oxidant activity by DPPH assay

sample	Concentration	COD	SOD	% Inhibition	Average
STD VIT C	1 mg/ml	0.36	0.05	86.11	85.18%
		0.36	0.06	83.33	
		0.36	0.05	86.11	
RASAGANDHIMELLUGU	1 mg/ml	0.36	0.15	58.33	60.0%
		0.36	0.14	61.11	
		0.36	0.12	66.67	
		0.36	0.15	58.33	
		0.36	0.16	55.55	
		0.36	0.15	58.33	

sample	Concentration	COD	SOD	% Inhibition	Average
STD VIT C	1 mg/ml	0.36	0.05	86.11	86.11%
		0.36	0.05	86.11	
		0.36	0.05	86.11	
RASAGANDHIMELLUGU	1 mg/ml	1.00	0.47	55.0	50.0%
		1.00	0.50	50.0	
		1.00	0.53	47.0	
	2 mg/ml	1.0	0.59	41.0	42.6%
		1.0	0.59	41.0	
		1.0	0.54	46.0	
	3mg/ml	1.0	0.59	41.0	41.3%
		1.0	0.58	42.0	
		1.0	0.59	41.0	
	4 mg/ml	1.0	0.48	42.0	52.6%
		1.0	0.47	43.0	
		1.0	0.47	43.0	
	5mg/ml	0.36	0.49	51.0	52.3 %
		0.36	0.47	53.0	

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		0.36	0.47	53.0	

Rasakanthi mezhugu (0.1)

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.47	55.0%
2	520	1.00	0.50	50.0%
3	520	1.00	0.53	47.0%
				50.0%

Rasakanthi mezhugu (0.2)

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.59	41.0%
2	520	1.00	0.59	41.0%
3	520	1.00	0.54	46.0%
				42.6%

Rasakanthi mezhugu (0.3)

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.59	41.0%
2	520	1.00	0.58	42.0%
3	520	1.00	0.59	41.0%
				41.3%

Rasakanthi mezhugu (0.4)

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.48	52.0%
2	520	1.00	0.47	53.0%
3	520	1.00	0.47	53.0%
				52.6%

Rasakanthi mezhugu (0.5)

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	1.00	0.49	51.0%
2	520	1.00	0.47	53.0%
3	520	1.00	0.47	53.0%
				52.3%

Table 2: Anti-inflammatory action for inhibition of herbal medicine DICLOFENAC INJECTION

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s.no	nm	C.O.D	S.O.D	Inhibition
1	520	0.30	0.02	93.3%
2	520	0.29	0.01	96.6%
3	520	0.30	0.01	96.7%
				95.5%

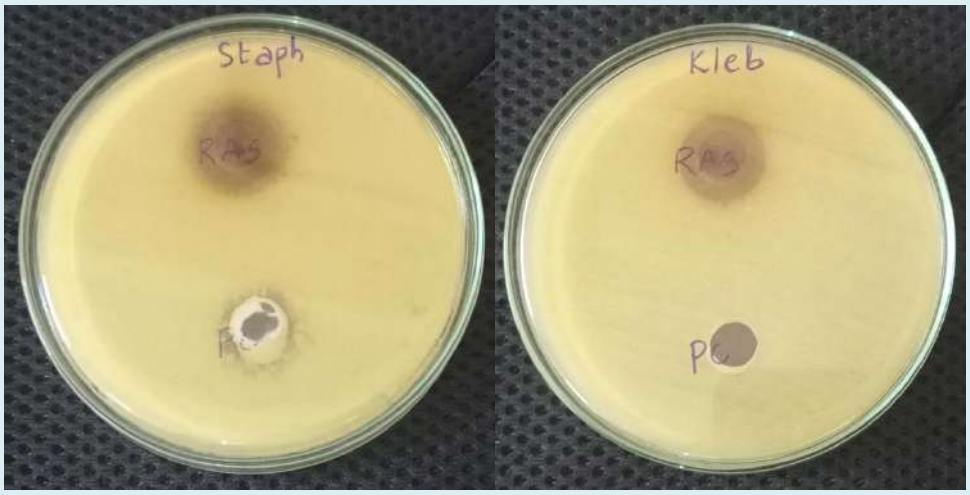
Rasakanthi mezhugu capsule

s.no	nm	C.O.D	S.O.D	Inhibition
1	520	0.30	0.03	90.0%
2	520	0.29	0.03	89.7%
3	520	0.30	0.01	96.7%
				92.1%

Table 3:*Invitro* antibacterialactivity and anti-oxidantactivity of Rasagandhimelluku

Antimicrobial activity of Rasagandhimelugu againstMDR bacteria at 250 ul

S. No.	Organism	Zone of Inhibition (mm) ZOI mm80 ul	PC
1	*Staphylococcus aureus*MRSA	20	0
2	*Klebsiella pneumoniae* MDR	19	0
3	*E. coli* MDR	19	0



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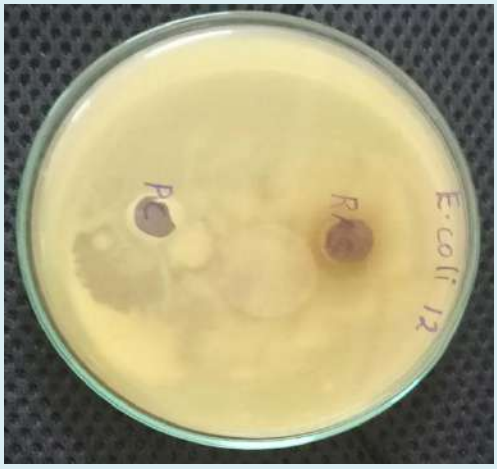

The RGM extract is against bacterial strains such as Staphylococcus aureus MRSA, Klebsiella pneumoniae MDR and E. coli MDR.

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In vitro antioxidant activity, antimicrobial and anti-inflammatory activity of PIMPLE GEL (PG)

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Abstract *–Pimple Gel is a polyherbal Ayurvedic cosmetic formulation contains ingredients Azadirachta Indica (NEEM) , Tulsi Ocimum santum (Tulsi), Santalum album (sandal), Aloe vera L (aloes), among them aloe vera is one of its chief constituents and base for gel. It is a good therapeutic medicine for pimple skin disease, which may be easily prepared at home and used in the treatment of complications that arise during skin problems. In the current study, the ability of PG in scavenging the free radicals was assessed by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, and the in vitro anti-inflammatory studies tested by protein denaturation assay MTT assay. The results proved PG with marked antioxidant activity. PG investigated for invitro antioxidant activity at the concentration of 1 mg/mL by DPPH method. PG had significant antioxidant activity, as compared to standard Vitamin C (ascorbic acid) under similar conditions PG had significant anti-inflammatory activity when compared with standard diclofenac sodium under similar conditions.Invitro antimicrobial activity of PAG tested against staphylococcus aureus, streptococcus epidermis and Candida albicans by well plate method and compared with standard streptomycin under similar condition. PAG had significant activity against staphylococcus aureus and mild activity against other organism. The study suggests that, this herbal composition possess good source of natural antioxidants, and is considered useful in combating the advances of oxidative stress, thereby preventing from the diseases that are caused by the free radicals.*

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Keywords *–Aloe vera gel, antioxidant activity, DPPH assay, anti-inflammatory assay, Azadirachta indica (neem), Smilax china, Oleaeuropaea (olive oil), Aloe vera L (aloes)*

1. INTRODUCTION

PAG is herbal formulation, composed of versatile medicinal plants such as*Azadirachta Indica* (NEEM) [1] ,Ocimum santum **(Tulsi**) [2], *Santalum album* (sandal) [3], *Aloe vera* L (aloes) [4], with excellent pharmacological and medicinal value due to the presence of enriched bioactive molecules and nutraceuticals. The phytochemical contents of herbal formulation of PG is responsible for antioxidant activity, which leads to many therapeutic and medicinal values.Antioxidant potential of bioactive molecules of herbs is responsible for the recommendation of treatment many diseases including Skin problem. Present work is envisaged to study the *in vitro* antioxidant activity by using DPPH assay method to explore the level of antioxidant potential. invitro anti-inflammatory activity also investigated by protein denaturation assay using diclofenac sodium.*Invitro* antimicrobialactivity of PAG tested against*staphylococcus aureus*, *streptococcus epidermis* and *candida albicans*bywell plate method and compared with standard streptomycinunder similar condition . Herbal formulation with antioxidant activity essential for the treatment of many human diseases. Free radicals are mainly responsible for various disordersand containing the excellent herbal combinations is used as skin.

2. MATERIALS & METHODS

2.1. In vitro antioxidant activity

PG is investigated for *in vitro* antioxidant activity by DPPH, ABTS, FRAP and NO for the estimation of anti-oxidant potential of pimple gel [5].

2.2. Determination of DPPH Radical scavenging activity

Antioxidant activity in the sample PG were estimated for their free radical scavengingactivity by using DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals (George *et al.*, 1996) [6].

100µL ofPG extract was taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively and read the plate on Elisa plate reader at 490nm Standard ascorbic acid was used as reference. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

2.3. In vitro anti-inflammatory activity-Inhibition of albumin denaturation

Reaction mixture 0.5ml aqueous extract of PG contains drug 0.05ml in the concentration (1mg/ml) with 0.45 ml aqueous solution of bovine albumin fraction (5%). The pH (6.3) of solution was adjusted using small amount of 0.1N HCl at 37 ^{0}C for 20 min, then heat to 57 ^{0}C for 30 min. Cool the solution and transfer to the 96 well plate and measure the absorbance at 660nm. Standard was used as Diclofenac sodium (1000µg/ml) and control contain 0.05ml distilled water [7].The percentage of inhibition of albumin denaturation was calculated by the following formula, Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.

A sample - absorbance reaction mixture with Sample)

3. RESULTS& DISCUSSION

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Pimple Gel(PG)were investigated for *invitro* antioxidant activity at the concentration of 1 mg/mL by DPPH method to ensure the antioxidant activity. The results obtained from the antioxidant study indicated that PG exhibit significant free radical scavenging potential. The study suggests that PG has a good origin of natural antioxidants and might be beneficial in impeding the oxidative stress.The results suggested that PG possessmarked antioxidant activity (Table 1, and 2).They had exhibited significant antioxidant activity compared to standard ascorbic acid under similar conditions (Table 3).In the *invitro* anti-inflammatoryactivityPG hadpotent anti-inflammatoryactivity when compared with standard diclofenac sodium(Table 5). The antioxidant potential and the anti-inflammatory efficiency of PG formulation could aid for both prophylactic and therapeutic tool against skin diseases including inflammatory. *Invitro* antimicrobialactivity of PAG tested against*staphylococcus aureus, streptococcus epidermis* and *candida albicans*bywell plate method and compared with standard streptomycinunder similar condition . PAGhad significantactivity againstskin pathogenic bacteria *staphylococcus aureus*and mild activityagainst other organism like *candida albicans* and *S.epidermis.*

Table 1:*In vitro* anti-oxidant activity of PIMPLE ALOE GEL by DPPH assay

S.NO	COD	SOD	% inhibition	Average
1	0.36	0.16	55.56%	51.85 %
2	0.36	0.17	52.78%	
3	0.36	0.15	58.33%	
4	0.36	0.17	52.78%	
5	0.36	0.20	44.44%	
6	0.36	0.19	47.22%	

Table 2:*In vitro* anti-oxidant activity of PIMPLE ALOE GEL by DPPH assay (After 12 Hours)

S.NO	COD	SOD	% inhibition	Average
1	0.38	0.19	50.00%	50.88%
2	0.38	0.18	52.63%	
3	0.38	0.17	55.26%	
4	0.38	0.19	50.00%	
5	0.38	0.20	47.37%	
6	0.38	0.19	50%	

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Table 3:*In vitro* anti-oxidant activity of Vit C (STD) by DPPH assay

S.NO	COD	SOD	% inhibition	Average
1	0.36	0.06	83.30	81.45 %
2	0.36	0.07	80.50	
3	0.36	0.06	80.50	

Table 4:*In vitro* anti-inflammatoty activity of PAG PIMPLE ALOE VERA GEL

S.NO	COD	SOD	% inhibition	Average
1	0.30	0.08	73.33 %	74.44%
2	0.30	0.08	73.33 %	
3	0.30	0.07	76.67 %	

Table 5:*In vitro* anti-inflammatoty activity of PAG PIMPLE ALOE VERA GEL

S.NO	COD	SOD	% inhibition	Average
1	0.30	0.10	66.67%	61.67 % 62%
2	0.30	0.14	53.33%	
3	0.30	0.15	50.00%	
4	0.30	0.10	66.67%	
5	0.30	0.10	66.67%	
6	0.30	0.10	66.67%	

Table 6:*In vitro* anti-inflammatoty activity of Diclofenac Sodium

S.NO	COD	SOD	% inhibition	Average
1	0.34	0.05	82.29	81.37%
2	0.34	0.07	79.41	
3	0.34	0.07	79.41	

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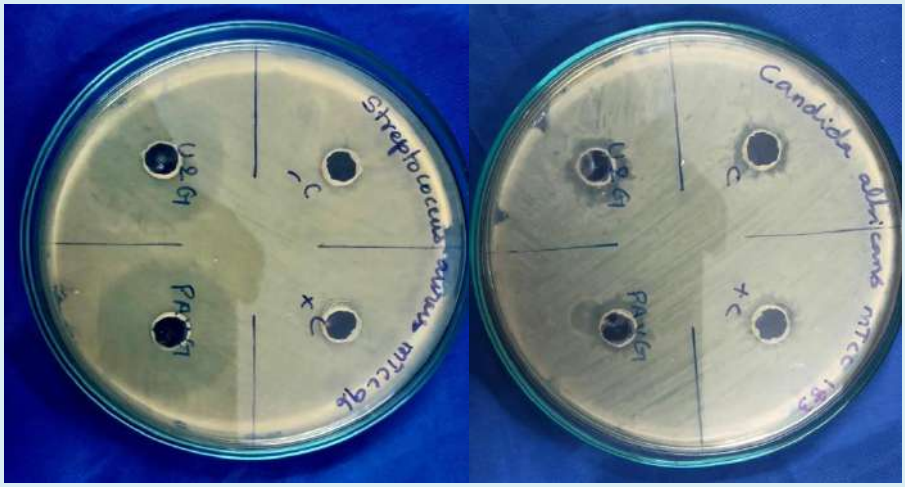

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Assessment on in vitro antibacterial activity of *Vitex negundo* L. leaves extracts

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Abstract – *Phytochemicals are ecologically derived secondary metabolites that plants release to protect themselves against environmental stress and pathogenic microbial invasion. These phytochemicals have been shown to have both positive and pharmacological effects in the treatment of human illnesses. In this study, the antibacterial activity of aqueous and ethanol extract of Vitex negundo L. (Verbenaceae) leaves was assessed against certain pathogenic bacteria using disc diffusion method. Both extracts of V. negundo leaves exhibited satisfactory antibacterial activities against gram positive and gram negative bacterial pathogens. Ethanolic extract showed maximum zone of inhibition of 28 mm against Escherichia coli and Pseudomonas aeruginosa. On the other hand, aqueous extract revealed maximum zone of inhibition of 32 mm against Staphylococcus aureus. Findings of this investigation suggested promising role of V. negundo leaves as antibacterial agent.*

Keywords – *Vitex negundo, Antibacterial activity, Disc diffusion, Aqueous and ethanol extract*

1. INTRODUCTION

In developing countries, infectious diseases are one of the major causes of mortality (Khusro *et al*., 2016). Antibiotics are being used as prime therapeutic approach against varied bacterial

infections. Unfortunately, the over-exploitation of antibiotics led to the emergence of drug-resistant bacteria. A constant increase in the drug-resistant bacteria is a major threat globally (Aarti *et al*., 2021). Therefore, the identification of new therapeutic agents from natural sources is in demand now.

Plants used in traditional medicine represent a priceless tank of new bioactive molecules (Eftekhari *et al*., 2021). *Vitex negundo* L. (Verbenaceae) is one of the important plants from traditional system of medicine found all over the world (Rastogi *et al*., 2017). *V. negundo* is large and an erect aromatic shrub which grows to a height of 3-6 m or slender tree with quadrangular branchlets distributed throughout India. The leaves have five leaflets in a palmately arrangement, which are lanceolate, 5–11 cm long, hairy beneath and pointed at both ends. The bluish purple flowers are numerous. The fruit is succulent, black when ripe, rounded and about 4 mm in diameter (Suva *et al*., 2014).

The various chemical constituents present in leaves of *V. negundo* leaves are friedelin, vitamin-C, carotene, casticin, artemetin, terpinen-4-ol, α-terpineol, sabenine, globulol, spathulenol, β- farnesene, farnesol, bis (1,1dimethyl) methylphenol, α-pinene, β-pinene, linalool, terpinyl acetate, caryophyllene epoxide, caryophyllenol, vitexicarpin, viridiflorol, 4,4″- dimethoxy-trans-stilbene, 5,6,7,8,3′4′5- heptamethoxy, 5-hydroxy-6,7,8,3′4′pentamethoxy (5-Odesmethylnobiletin), 5-hydroxy-6,7,8,3′,4′,5-hexamethoxy(gardeninA), 5-hydroxy-6,7,8,4′tetramethoxy (gardeninB), 5- hydroxy-7,3′,4′,5′-tetramethoxyflavone (corymbosin), terpinen-4-ol, αcopaene, β-caryophyllene, β-elemene, camphene, α-thujene, α-pinene, sebinene, linalool, stearic acid and behenic acid (Rana and Rana, 2014; Sachin *et al*., 2017). The seeds of *V. negundo* contain chemical constituents such as n-tritriacontane, n-hentriacontanol, n-hentricontane, n-pentatricontane, n-nonacosane, β-sitosterol, phydroxybenzoic acid, 5-oxyisophthalic acid, 3, 4-dihydroxybenzoic acid, artemetin, 3-β-acetoxyolean-12-en-27-oic acid, 5-β-hydro-8,11,13-abietatrien6α-ol, 2α,3α-dihydroxyoleana-5,12-dien-28-oic acid, 2-β,3α-diacetoxyoleana-5,12-dien-28-oic acid and 2α,3β diacetoxy-18-hydroxyoleana-5 (Ahmed and Fatima, 2018). The various chemical constituents present in the stem and bark are 3,6,7,3′,4′-pentamethoxy-5-oglucopyranosylrhamnoside, vitexin cafeate, 4′-o-methyl myricetin- 3-o-[4′-o-β-D-

galactosyl]-β-D-galactopyranoside, β-amyrin, epifriedelinol, oleanolic acid, hepta methyl-phenyl-cyclotetra siloxane, cyclo heptasiloxane, tetra decamethyl nona methyl, phenyl-cyclopenta siloxane, cyclo octa siloxane, hexadeca methyl, borazine, 2,4,6-tripheny-l1, 3, 5-tryophl, nonamethyl, phenyl-cyclopenta siloxane. vitexoside, agnuside, R-dalbergiphenol, negundin A, negundin B, 6-hydroxy-4-(4-hydroxy-3-methoxy)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaledehyde, vitrofolal E, (+)-lyoniresinol, (+)-lyoniresinol-3α-o-βd-glucoside, (+)-(−)-pinoresinol and (+)-diasyringaresinol (Venkateswarlu, 2012; Fauziya *et al*., 2014).

In view of the presence of diversified bioactive compounds in *V. negundo*, the present study was investigated to determine the antibacterial activity of *V. negundo* leaves extracts against certain bacterial pathogens.

2. MATERIALS & METHODS

Collection of plant

V. negundo was collected from Aravindh herbal laboratory in Rajapalayam, Tamilnadu (Figure 1).

Figure 1: *V. negundo* plant

Extracts preparation

Ten grams of powdered leaves of *V. negundo* was mixed into 100 ml of distilled water and ethanol separately. The mixture heated for 5 h in a water bath with a reflux condenser, then cooled and filtered. Aqueous and ethanolic extract of *V. negundo* was obtained by vacuum evaporation of the filtrate.

Antibacterial activity

The disc diffusion method was used to test the antibacterial activity of plant extracts against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aureginosa*. The bacterial cultures were used to make the microorganism's inoculum. In clean sterilized petri dishes, 15 ml of nutrient agar (Hi Media) medium was poured. Hundred µl of bacterial culture was pipette out and evenly dispersed over the medium with a spreading rod until it dried. A sterile cork borer was used to make wells of 6 mm in diameter. All the extracts were dissolved in DMSO at a concentration of 1 mg/ml. The wells were filled with 100 µl of extracts. The petri plates were incubated for 24 h at 37°C. Streptomycin (1 mg/ml) was used as positive control, while DMSO was used as negative control. The diameters of the zone of inhibitions were measured to determine antibacterial activity, and all measurements were done in triplicate.

3. RESULTS & DISCUSSION

Aqueous and ethanolic extract of *V. negundo* leaves showed potential antibacterial activity against the tested bacterial pathogens. Ethanolic extract exhibited maximum zone of inhibition of 28 mm against *E. coli* and *P. aeruginosa* (Fig. 2). On the other hand, aqueous extract revealed maximum zone of inhibition of 32 mm against *S. aureus* (Fig. 3). Both extracts showed comparatively lower antibacterial activity against *K. pneumoniae*.

Figure 2: Antibacterial activity of ethanolic extract of *V. negundo*

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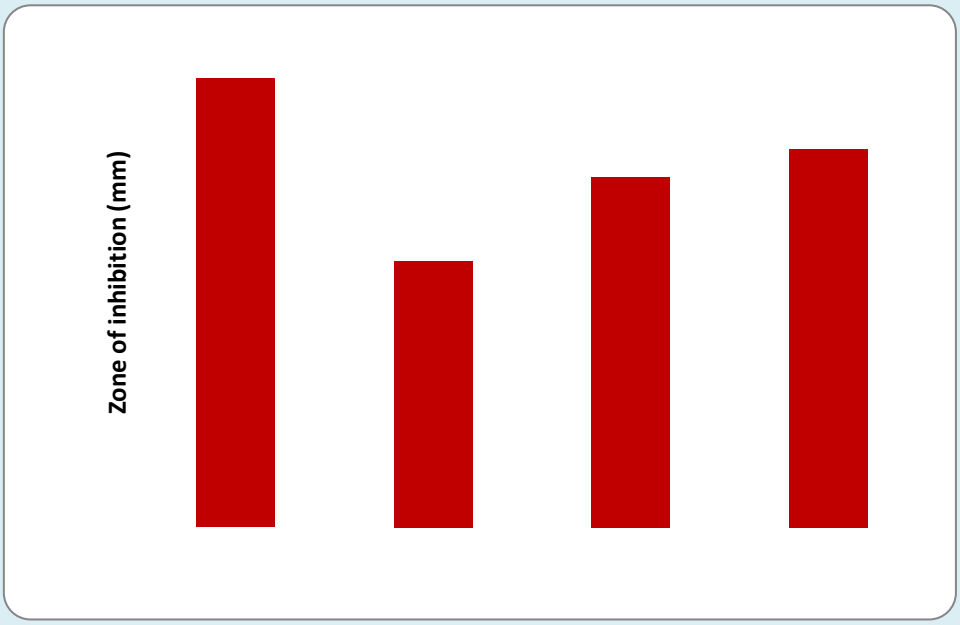

Figure 3: Antibacterial activity of aqueous extract of *V. negundo*

Medicinal plants are being used for therapeutic agents since ancient periods (Khusro *et al*., 2013). Traditional methods of therapy utilize diverse medicinal plants for the treatment of infectious diseases. In fact, modern medicines are originated from ancient herbal tradition. At present, there are several plants which show antibacterial properties (Khusro *et al*., 2014). The antibacterial nature of those medicinal plants relies on the presence of plethora of bioactive phytoconstituents in it. In this study, the aqueous and ethanolic extract of *V. negundo* leaves showed potential antibacterial activity against certain gram positive and gram negative bacteria. Kamruzzaman *et al*. (2013) demonstrated antibacterial activity of *V. negundo* leaves against enteric pathogens, particularly *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *E. coli*, *Shigella* spp., and *Aeromonas* spp. In a different study, Kurapatti *et al*. (2017) revealed antibacterial activity of methanolic and ethanolic extract of *V. negundo* leaves against *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *Salmonella typhi*. In a recent investigation, Koirala *et al*. (2020) reported antibacterial activity of methanolic extract of *V. negundo* leaves against *B. subtilis* and *S. aureus*.

CONCLUSIONS

In conclusion, the aqueous and ethanolic extract of *V. negundo* leaves exhibited promising antibacterial activity against gram positive and gram negative bacterial pathogens. Ethanolic extract showed maximum zone of inhibition of 28 mm against *E. coli* and *P. aeruginosa*. On the other hand, aqueous extract revealed maximum zone of inhibition of 32 mm against *S. aureus*. Findings of this investigation suggested promising role of *V. negundo* leaves as antibacterial agent.

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