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IN VITRO ANTIMICROBIAL ACTIVITY OF POLYHERBAL OINTMENT AGAINST HUMAN PATHOGENIC BACTERIA

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Abstract
The aim of the study was assess the antimicrobial effect of some medicinal plants and its formulation against *Escherichia coli, Candida albicans, Aspergillus niger*. Medicinal plants are the important source of potentially useful structures for the development of novel chemotherapeutic agents. Historically, plants have provided a source of the development for novel drugs and plant derived drugs which have made large contributions to human health and wellbeing. The hydro-alcoholic extract of selected medicinal plant show presence of major phyto-constituent such as alkaloids, glycosides, steroids, flavonoids, tannins etc. The antimicrobial activities of selected plants extracts were evaluated using the disk diffusion method .In the present study, polyherbal formulation was screened against microorganism at the concentration of 100 /ml with reference standard DERMIKEM OC Cream. At above concentration there is antibacterial activity found in PHF A and B They not show antifungal activity.

Keywords: Medicinal plants, Phytochemical Investigation, Polyherbal Ointment, antimicrobial activity

INTRODUCTION
Many plant based medicine are being used in the different kind of microbial infection but modern system of medicine that is allopathy mostly to treat microbial infection synthetic drugs are used which are very expensive, and having severe adverse effect and we focus on herbal drugs which are natural, less expensive, negligible side effect and not only eliminate the disease from the body but also enhance body vigour and immunity.
Over the years scientific research is expanded and they provide basic study of medicinal plant but these are still large number of medicinal plant in which all active constituent have not yet been even there medicinal effect is established by traditional and modern system of medicine .The research work is essential for modern era in which we used different herbal drugs for management and treatment of different kind of microbial infection [1].

MATERIAL AND METHOD
Selection of Plant Material
Pongamia pinnata (Family-*fabaceae*): Commonly known as Karanj.It grows to about 15-25 meters in hight it has a straight or crooked trunk [20-30 inch], the leaves are soft shiny and deep green. The important chemical constituent are karanjin, ovalitenone, kanugin, pongapin, 3-metho-oxypongapin,

pongaglabrone, kqnjone, pongol, gamatin, glabrin etc. Traditionally it is used to treat wounds, abscesses, scabies, herpes and rheumatism. Its oil shows antifungal, antibacterial activity [2].

Lanata camara (Family-*Verbenaceae*): Commonly known as Tantani. It is hardy, evergreen shrub with characteristic odour, height about 3 m with or without prickels on branches. It have majority of their activity is due to bioactive compounds viz. flavones, isoflvones, flavonoids, anthocynins, coumarins, saponins and triterpenoids camarilic acid and camaricinic acid [3]. Medicinal properties of *L.camara* are anticancer, antiproliferative, antibacterial, antioxidant, haemolytic, antifungal, antihyperglycemic, anti-inflammatory, wound healing, mosquito contolling, antimutagenic, antiulcerogenic, effect on R.B.C.s [4].

Vitex Nugundo (Family-*Verbenaceae*): It is also known as Nirgundi, Nila. It is irect shrub growing from 2 to 8 m. Its leaves are digitate, with five lanceolate leaflets, sometime three. It contain hydroxy-3,6,7,3′,4′-pentamethoxyflavone,6′-p-hydroxybenzoyl mussaenosidic acid; 2′-p-hydroxybenzoyl mussaenosidic acid, 5, 3′-dihydroxy-7,8,4′-trimethoxyflavanone; 5,3′-dihydroxy-6,7,4′-trimethoxyflavanone,viridiflorol; β caryophyllene; sabinene;4-terpineol; gamma-terpinene; caryophylleneoxide; 1-oceten-3-ol; globulo. It show anti-feedant, anti-bacteria, anti-filarial, anti-fungal, anti-larval, antiviral, insecticidal, larvicidal, Mosquito repellent [5, 6].

Annona Squamosa **(**Family-Annonaceae): It is also known as sugar apples, shitaphal. It is small, well-branched tree that bears edible fruit called sugar apples. Chemical constituent of *A sqaumosa* include the alkaloids oxophoebine, reticuline, isocorydine, and methyl corydaldine and flavonoid Traditionally it is used to dysentery ,urinary tract infection The leaves are rubbed on floors and put in hens to repel lice and also used to wound healing [7].

Calotropis procera (Family-Apocynaceae*):* It is also known as rui, ruchkin. It is flowering plant .The leaves are gray green are 15-30 cm long and 2.5-10 cm broad. The milky sap contain complex mix of chemicals (cardiac aglycones) calotropis glycosides there name are calotropin, calotoxin ,calactin, It is used for G.I. tract disorder including diarrhoea, constipation, stomach ulcer, for painful condition such as toothache, cramps ,joint pain and also useful for parasitic infection and skin disease [8, 9].

Thevetia peruviana (Family-Apocynaceae)*:* It is commonly known as kanher, yellow oleander. It is small tree, leaves are green flower colour is yellow leaves are present waxy coating to reduce the water loss of the plant .The chemical constituents in *Thevetia peruviana* are cardio active glycoside such as thevetin A, thevetin B, cerebroside, peruvoside, nerrifolin, thevenerin, peruvosidic acid. It show antimicrobial, antifungal, piscicidal, antitermite ,antispermatogenic, rodenticidal, anti-inflammatory pharmacological activity [10].

Carica papaya (Family-Caricaceae): This is also known as papaya. It is small, single steam growing from 5-10 m tall with spirally arrange leaves fresh leaves are green and fruit is edible. It contain various phytochemicals including carotenoids, polyphenols, benzyl isothiocynate, benzyl glucosinate, seeds also contain cynogenic substance prunasin[11]. It is used for diabetes, as birth control, as an antiseptic, antimicrobial, diuretic, control parasites, reduse inflammation, lower blood pressure, it also used for bed sores and wounds and treat intestinal worm [12].

Ricinus communis (Family: Euphorbeaceae): It is also known as castor (English) errand (Hindi). It is fast growing small tree around 12m. The glossy leaves are 15-45 cm green and some dark reddish purple colour. It contain alkaloids ricinine, N-dimethylricinine , and flovanol glycosides kaempferol-3-0-beta-D-xylopyranoside, kaempferol-3-0-beta-D-gluco pyranoside, kaempferl-3-0-beta-D-rutinoside and xyloside, glycosides. The alcoholic extract of leaves show liver protective action, and it also show antimicrobial activity against pathogenic bacteria [13].

Azadirachta indica (Family-Meliaceae): It is commonly known as Neem. It is fast growing tree, hight about 15-20 m, evergreen, leaves are green and when dry [ripe] becomes yellow and bitter in taste. It contain nimbidin, nimbinin, azadirachtin, isomeldenin, nimbandiol, immobile, nimocinol, quercetin, and beta –sitosterol. It show anthelmintic, antifungal, anti-bacterial, antiviral, contraceptive, sedative activity. Neem oil is used for healthy hairs, improve liver function, detoxify blood balance blood sugar level [14,15].

METHODOLOGY

Air dried leaves of *Pongamia pinnata, Lanata camara, Vitex nugundo, Annona squamosa ,Calotropis procera, Thevetia peruviana, Carica papaya, Ricinus comunis and Azadirachta indica* were collected from hills boradi region in satpuda hills.All the plant material were authenticated by Dr D.A. Patil

Table no. 01: Composition of plant material for herbal formulation

Sr. No	Name of plant	Part of plant
1	*Azadriachta indica*	Leaves
2	*Annona squamosa*	Leaves
3	*Calotropis procera*	Leaves
4	*Pongamia pinnato*	Leaves
5	*Virex negundo*	Leaves
6	*Ricinus communis*	Leaves
7	*Carica papaya*	Leaves
8	*Lanata camara*	Leaves
9	*Nerium indicum*	Leaves

Determination of Physical Constant [16,17]

Total ash value:

Weigh accurately 1 gm. of air–dried leaves of all selected plant material in a tared platinum or silica dish and incinerated at a temperature not exceeding 450^0C until it will be free from carbon, cool and weigh. Percentage yield of ash was calculated for each plant material respectively.

Loss on Drying:

Accurately weighed quantity of sample was taken in a tared glass bottle and initial weight was taken. The sample was heated at 105^0C in an oven and weighed. This procedure was repeated until a constant weight was obtained. The moisture content of the sample was calculated with reference to air-dried drug.

Loss on drying (%) = loss in weight x 100/ w

Where w = weight of the leaf powder

Table no.02: Physical Constant of selected Plants

Sr. No	Name of plant	Ash Value (%)	Loss on Drying (%)
1	*Azadriachta indica*	11 %	4 %
2	*Annona squamosa*	7 %	8 %
3	*Calotropis procera*	11 %	12 %
4	*Pongamia pinnato*	15 %	19 %
5	*Virex negundo*	15 %	13 %
6	*Ricinus communis*	14 %	4 %
7	*Carica papaya*	10 %	10 %
8	*Lanata camara*	13 %	9 %
9	*Nerium indicum*	5 %	5 *%*
10	*Mixture*	10 %	8 *%*

Preparation of Extracts

The plant material first washed with water thoroughly to remove dirt and soil deposits and dried under shade until complete removal of moisture content, such dried plants were powdered by mechanically and passed through sieve no 80. About 100 grams(each plants) of leaves of *Pongamia pinnata, lanata camara, Vitex nugundo, Annona squamosa, Calotropis procera, Thevetia peruviana, Carica papaya ,Ricinus comunis and Azadirachta indica* were powdered and subjected to extraction by two methods maceration and soxhlet extractor by using hydro alcoholic solvent after defatation with petrolium ether. In maceration individual plant material is used and separate

extraction were performed for each plant material and for soxhlet 1:1 composition which contains each plant material used up to 100 gm. The extracts were filtered and concentrate at reduced pressure. The dried lyophilized extracts were stored carefully for further investigation.

Phytochemical investigation

All the Preliminary qualitative phytochemical analysis of all the extracts were carried out by employing standard conventional protocols for preliminary phytochemical screening. The result of phytochemical investigation was shown in Table no.4

Table no. 03: Preliminary phytochemical investigation of hydroalcoholic extracts of selected medicinal plant

Sr. No	Name of plants	Carbo-hydrates	Alka-loid	Glyco-sides	Sapo-nins	Ster-Oides	Terp-enoides	Flavo-nides	Tanni-ns
1	*Azadriachta indica*	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
2	*Annona squamosa*	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve
3	*Calotropis procera*	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
4	*Pongamia pinnato*	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve
5	*Virex negundo*	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
6	*Ricinus communis*	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve
7	*Carica papaya*	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve
8	*Lanata camara*	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve
9	*Nerium indicum*	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
10	Mixture(1:1)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Preparation of Polyherbal Ointment [18]

The evaporated lyophilized residue hydro alcoholic extracts of all selected medicinal plants were prepared by using two topical ointment bases of varying degree of aqueous/anhydrous character namely; simple B.P. and emulsifying ointment B.P. by fusion method (Table no.2). The constituents of the base were placed together in a melting pan and allow melting at 70^0C. After melting, the ingredients were stirred gently at 70^0 C for five min. and then cooled with continuous stirring. Incorporation of 10 gm of the extract into the various bases was achieved by triturating in a ceramic mortal with a pestle to obtain 100 g of herbal ointment. The prepared herbal ointments were put in wide mouth containers, labeled were stored at 25^0 C. The two formulated ointments PHF A and PHF B and the Standard, Dermikem OC cream were evaluated.

Table no. 04: Polyherbal formulation A (For preparation of 100 g)

Sr. no.	Ingredients	Quantity
1	Extract	10g
2	Cetostearyl alcohol	4.5 ml
3	Hard paraffin	4.5 gm
4	White soft paraffin	76.5 gm
5	Wool fat	4.5 gm

Table no. 05: Polyherbal formulation B (For preparation of 100 g) :

Sr. No.	Ingredients	Quantity
1	Extract	10g
2	Liquid paraffin	18g
3	Emulsifying wax	27g
4	White soft paraffin	45g

PHF A: This Herbal preparation contain extract of mixture of nine drugs and this extract is extracted from mixture herbal drugs by previous defatted and soxhlet extraction method.

PHF B: This herbal preparation contain extract of mixture of nine drugs but these extract are separately extracted by simple maceration process

Preparation of Stock solution:
A stock extract solution (100 ug/ml) was prepared by dissolving the extract in pure dimethylsulfoxide (DMSO) and diluting it two times. This stock extract was sterilised and filtered using filter paper (0.2 µm) and stored in Eppendorf tubes at 4°C.

Evaluation of Anti-microbial activity of the Polyherbal Ointment [19,20].
*E.Coli, Candida albicans, Asperagillusniger*are the commonly occurring bacteria, fungi causing skin infection, hence, were selected for the study.
All the extracts prepared by using maceration andsoxhlet extraction were evaluated by using Agar diffusion method as per I.P .

Method Used :
Agar diffusion assay (Disk diffusion method, Disk size 6 mm)

Concentrations of compounds:
Stock solution [1000 microgram per ml] of each compound was prepared in Water. Assay carried out by taking concentration 100 microrgram per disk.
Hi-media antibiotics disk:

Media Used
Microbiological media used for bacteria (*Escherichia coli)* is Nutrient agar (Hi media) Composition (gL-1**)::** Sodium chloride, 5.0; Beef extract 10.0; Peptone 10.0 (pH 7.2)
Microbiological media for yeast (*Candida albicans*) is MGYP (all ingredients of Hi-media) Composition(gL-1) : : malt extract ,3.0 ; Glucose,10.0;Yeast extract ,3.0;Peptone,5.

Microbiological media For fungi *(Aspergillus niger*): Potato Dextose both is used (all ingredient Hi-media) composition (gL-1) :potatoes infusion ,200 ; Dextrose, 20 ; Final pH(at 25^{o} C) 5.6 +- 0.2
Standard: This marketed formulation (Dermikem OC) contain Terbinafine (1%w/w), Clobetasol (0.05% w/ w) , Ofloxacin (0.75%w/w), Ornidazole(2%) .

Agar well diffusion method
Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) , MYGP for Yeast and Potato Dextrose Agar (PDA) plates were swabbed by using cotton sterile swab with 8 hour old - broth culture of respective bacteria, fungi. Wells (6 mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extracts was prepared at a concentration of 1 mg/ml in DMSO. About 100 µl of concentrations of PHF A , PHF B and Standard Dermikem OC were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured.

Concentrations of compounds:
Stock solution [1000 microgram per ml] of each compound was prepared in DMSO. Assay carried out by taking concentration 100 microrgram per disk.

Table no. 06 Effect of polyherbal ointment on pathogenic bacteria prepared from selected plants.

Sr. No.	Sample code 100 ug/ml	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
1.	PHF A (Mixture)	17.65 mm	-	-
2.	PHF B	9.73mm	-	-
3.	Standard (DERMIKEM OINTMENT)	38.21mm	-	30.36mm

Fig.01: standard formulation Dermikem OC

Fig no. 02 Polyherbal formulation A and B

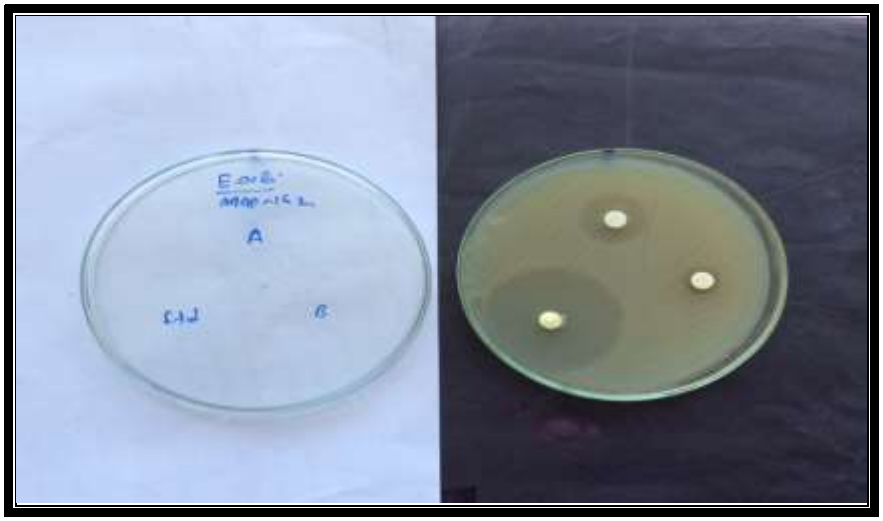

Fig no. 03: Antimicrobial activity of formulation A and B against *E.Coli*

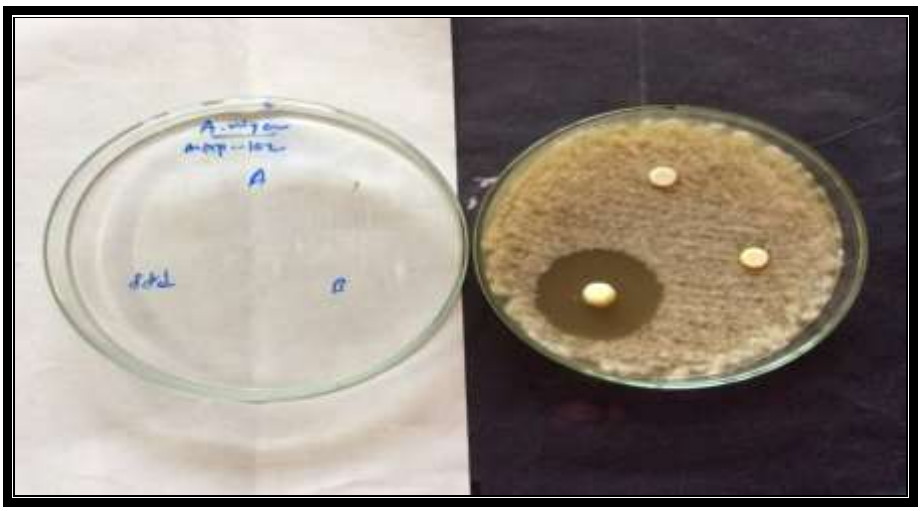

Fig no. 04: Antimicrobial activity of formulation A and B against *Aspergillusniger*

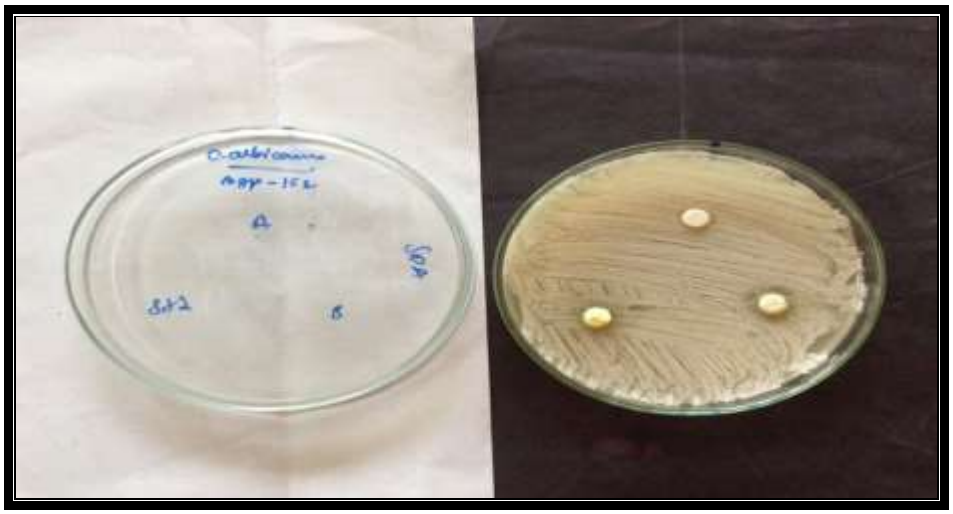

Fig no. 05: Antimicrobial activity of formulation A and B against *C. albicans*

RESULTS AND DISCUSSION

In the present study, selected medicinal plant was collected from hills satpuda near boradi and authenticated from ayurvedic college boradi. The medicinal plant was collected, dried and subjected for determination of physical constant after that subjected simple maceration extraction method. Physical constant are mention in Table [2]

In this present study physical constant such ash value and LODof following drugs observed and it found to be as per prescribed standards respectively; as follows *Azadriachta indica* (11%,4%), *Annona squamosa*(7%,8%), *Calotropis procera*(11%12%), *Pongamia pinnato* (15%19%), *Virex negundo* (15%13%), *Ricinus communis* (4%,4%), *Carica papaya* (8%,10%), *Lanata camara* (7%,9%), *Nerium indicum* (5%,5%), 1:1 composition (Mixture) (1%8%).

Preliminary phytochemical investigation of the leaf extract of 1:1 Mixture shows carbohydrate , alkaloid , glycosides , saponins , steroids , terpenoids , favonoids tannins and macerated commonly gives the presence of carbohydrate, alkaloides, glycosides, flavonoides etc respectively[Table no.3].

Antimicrobial activity of selected medicinal plant was carried out by using Agar diffusion method. There is antimicrobial activity was found in given concentration of polyherbal formulation A (17.65mm) as compare to DERMIKEM OINTMENT (38.21 mm) and standard and polyherbal formulation B(9.73 mm) B against *E.coli.* PHF A and B not show any significant activity against *C. albicans*, *Aspergillus niger*.

In the present study it was found that PHF A ointment consist of shows antimicrobial activity as compare to PHF B contains individual extracts by using maceration. Both the formulation doesn't show any antifungal activity at same concentration (100 ug/ml).

The *in-vitro* antimicrobial activity showed that the formulated ointment of 1:1 proportion of selected medicinal plant previously defatted with pet ether and extracted with Hydro-alcoholic solvent (PHF A) showed comparatively more against the bacterial rather than PHF B contains individual extracts by using maceration.

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