CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1247833

Available online at: http://www.iajps.com Research Article

DEVELOPMENT AND EVALUATION OF ANTI-MICROBIAL HERBAL GEL FORMULATION CONTAINING *MAJORANA HORTENSIS* AND *CURCUMA LONGA* EXTRACTS

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Abstract:

In developing countries the gradual revival of use of medicinal plants is attributed to the safety of herbal medicines with minimal adverse effects as comparable to the synthetic drugs. The present study was aimed to evaluate the anti-microbial effect of formulated gel containing chloroform extract of M.hortensis and ethanolic extract of C. longa. An optimized topical gel (carbopol- 934, as gelling agent) formulation in combination with the two herbal drugs was prepared in the 2:3 ratio and was evaluated. The extracts were prepared using standard procedures and physico-chemical parameters of the formulated gel were determined. The anti-bacterial and anti-fungal activity was done using agar well diffusion method for determination of zone of inhibition. The studies indicated that with the increase in the concentration of the extract there is increase in the zone of inhibition with both the test extracts. The present study investigated the anti-bacterial activity of chloroform extract of M.hortensis and ethanolic extract of C. longa against various bacteria such as Staphylococcus aureus, Bacillus subtilis, Escherichia coli, P.aeroginosa and Candida albicans at different concentrations. In the anti-bacterial activity, the zone of inhibition (ZOI) was highest for S.aureus and P.aeroginosa at 150 mg/ml with the chloroform extract of M.hortensis and similarly ethanolic extract of C. longa produced highest zone of inhibition for E. Coli and B.subtilis. In the anti-fungal activity, the two test extracts chloroform extract of M.hortensis and ethanolic extract of C. longa showed 19 mm and 20 mm at 150 mg/ml as comparable to the standard. From the results it can be concluded that the formulated gel exhibited a potent anti-microbial activity.

Key Words: *Anti-microbial, anti-fungal, zone of inhibition, herbal extracts, formulated gel.*

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Please cite this article in press K. Lalitha and P.Sailaja Rao., ***Development and Evaluation of Anti-Microbial Herbal Gel Formulation Containing Majorana Hortensis and Curcuma Longa Extracts,*** *Indo Am. J. P. Sci, 2018; 05(05).*

INTRODUCTION:

Herbal plants are the major sources of drugs, with secondary metabolites. Natural products have greater importance and possess greater structural diversity as compared to compounds from standard combinatorial chemistry, as plant materials and herbal remedies derived from them represent a substantial portion of global drug market [1]. The use of medicinal plants for treatment of microbial diseases is well known and well documented since ancient times [2]. The focus is mainly on those herbal medicines that are easily available, cheaper, time tested and considered safer than most of the synthetic drugs [3]. In 80% of the world population the usage of antimicrobial agents derived from plants are considered as traditional health remedies. As there is an increase in the failure of synthetic drugs, moreover their side effects and development of antibiotic resistance by pathogenic microorganisms have lead to the identification and screening of several medicinal plants for their potential antimicrobial activity [4].

Majorana hortensis, commonly called as sweet marjoram, *belongs to the* family Lamiaceae,is a small perennial aromatic herb which can be grown in containers and moved indoors for winter. The plant is popularly used as flavouring agent. Various parts of the plant were commonly used in folklore medicine for wide variety of remedies. The aerial parts of the plant are used for isolation of essential oil, which has lot of uses in flavour, perfumery and pharmaceutical industry. The essential oil is employed for external application in bruises, sprain, stiffs and paralytic limbs and toothache and for hot fermentation, in acute diarrhoea. The plant was also reported to possess anticancer, antioxidant and antifungal properties. The main constituent-essential oil component is bicyclic monoterpene alcohol, cis-sabinene hydrate. Along with these, terpinenes, terpineols and cineol are also found in significant amounts. Polyphenols, flavanoids, terpenoids are some important phytoconstituents [5].

Curcuma longa or turmeric belongs to the family Zingiberaceae. The root part has traditionally been used as an insect repellent, anti-diabetic, anti-rheumatic, to treat skin diseases, intestinal worms, diarrhoea, intermittent fever, hepatic disorders, urinary discharges, inflammation and constipation. The phytochemical constituents include diarylheptanoids, diarylpentanoids, monoterpenes, sesquiterpenes, diterpenes, triterpenoids, alkaloids and sterols. Herbal drugs are extremely safe because they are basically the foods that are ground up and combined in formulas designed to maintain the herbal effectiveness. It is important to know the effect of combined therapy whether it gives numerous benefits that include treatment of infections caused by specific causative organisms, and to increase anti-microbial activity [6]. Hence, this study was taken up to evaluate the anti-microbial effect of formulated gel prepared from two herbs – *Majorana hortensis* and *Curcuma longa*.

MATERIALS AND METHODS:

Collection of Plant Material: Whole herbal plant *M.hortensis* and Rhizomes of *C.longa* were procured from the local market of Hyderabad and Botanical authentification of both the herbs was done at the Department of Botany, Yogi Vemana University Kadapa and sample species were deposited in the herbarium of Department of Pharmacology, Sri Venkateshwara College of Pharmacy, Hyderabad, India for future reference.

Monographic analysis of Herbs: The individual herbs were evaluated with regard to their standard specifications according to the Herbal Pharmacopeia of India. The tests carried out were loss on drying, extractive values and foreign organic matter, ash values.

Extraction: Shade dried aerial parts of *M.hortensis* and air dried rhizomes of *C.longa* were coarsely powered and were subjected to extraction with different solvents - chloroform and ethanol by maceration for 72 hrs and the collected extracts were concentrated on rotary evaporator and concentrated extract were kept in desiccator until used [7].

Preliminary Phytochemical Screening: The extracts obtained were evaluated for phytoconstituents using standard procedures. Chloroform extract of *M. hortensis* and methanolic extract of *C.longa* were combined in the ration of 2:3 for anti-microbial evaluation.

Preparation of Formulation: [8]

Extracts of the selected herbal drugs *M.hortensis* and *C. longa* were formulated into a topical gel using carbopol 934 as a gelling agent. The gelling agent was dispersed in distilled water (methyl paraben, propyl paraben) overnight with glycerine. The extracts and propylene glycol were added to the above mixture under continuous mixing using a magnetic stirrer. The mixture was then neutralised by drop wise addition of tri ethanol amine. Mixing was continued until a transparent gel was formed.

Physical evaluation: [9]

The colour, appearance and the feel on application of the formulated gel was noted. The properties such as consistency, texture and skin irritation tests were done. The pH was measured using digital pH meter.

Spreadability: Spreadability was determined by the apparatus which consists of a wooden block, which was provided by a pulley at one end. By this method spreadability was measured on the basis of slip and drag characteristics of gels. An excess of gel (about 2gms) under study was placed on the ground slide. The gel was then sandwiched between this slide and another glass slide having the dimensions of the fixed ground slide and provided with the hook. A 1kg weight was placed on the top of the two slides for 5 minutes to expel air and to provide a uniform film of the gel between the slides. Excess of gel was scrapped off from the edges. The top plate was subjected to pull of 80 gms with the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5cms be noted. A shorter interval indicates better spreadability. It was calculated using the formula: [10].

S = M x L /T

S = Spreadability

M = weight in the pan (tied to the upper slide)

L = length moved by the glass slide

T = time (in sec.) taken to separate the slides completely from each other

Viscosity of the prepared formulation was estimated using Brookfield viscometer. [11]

Stability studies: Accelerated stability testing was performed using standard procedure and recorded.

Good manufacturing practices need to be followed which are designed to take care of all aspects in preserving the integrity and properties of formulations throughout the shelf life. Stability studies were performed as per ICH guidelines. The formulated gel was filled in the collapsible tubes and stored at different temperatures and humidity conditions, viz, 25°C± 2°C /60% ± 5% RH (Relative humidity), 30°C ± 2°C /65%±5% RH, 40°C ± 2°C/75%±5% RH, for a period of 3months and studied for appearance, pH, viscosity and spreadability [12].

Acute skin irritation study for topical formulations:

Skin irritation test was performed following OECD guidelines 404. In skin irritation test, total 9 rats were taken of either sex weighing between 150-180 gms. Animals were divided into three groups of 3 each. Hairs were depleted from the back of the rats with the help of depilatories and area 4 cm^2 was marked on both the sides. One side served as control while the other as test. Test substance was applied and the substance should be attached to the skin. The animals were observed for 14 days for signs of oedema and erythema [13].

Anti-microbial evaluation:

Determination of MIC (Minimum inhibitory concentration): [14]

The minimum amount of drug that is required to inhibit the growth of microorganisms is called as minimum inhibitory concentration of that compound. Dilutions of the drugs were prepared and inoculated with test organisms; this test was employed when therapeutic dose has to be regulated. It can be determined by serial dilution technique, a series of dilutions were prepared and incubated overnight, and then MIC was determined. Fresh nutrient agar medium, soya bean casein digestive medium and sabouroud dextrose agar medium were prepared and sterilized. After sterilization, first nutrient agar medium was added to 10 assay tubes, inoculated with microorganisms, staplylococcus aureus, before addition of extracts of *Majoram* and *Curcuma* species. The tubes were agitated to maintain the uniformity of the broth mixture. Ten assay tubes were taken and labelled as 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 16.5 mg/ml, 3.125 mg/ml, 1.56 mg/ml, positive control and negative control for both extracts. The tubes were incubated at 37^0 C for a period of 24 hrs and the results were deciphered by noting the turbidity in the tubes. The smallest amount of extract that is capable of restricting the growth of bacteria (MIC) was determined. The same procedure was followed for organisms such as *Bacillus subtilis*, *Escherichia coli and Candida albicans* separately.

Anti-bacterial activity: [15]

The anti-bacterial activities of the extracts were evaluated by means of the agar well evaluated by means of the agar well diffusion assay. Weighed Muller Hinton Agar as per requirement and dissolved in distilled water as per the guidelines given by manufacturer. Autoclaved the media at 121°C for 15 minutes. After autoclaving, poured the media in sterile petriplates and kept for solidification. Cork borer was sterilized by autoclaving or disinfected it by rinsing in alcohol followed by sterile water.On a Muller Hinton Agar plate aseptically punched 5 holes using a cork borer. Using a marker, marked the underside of the petri to label the wells. Aseptically 20µl of the indicator organism was spread onto the MH agar plate. Allowed the plate to stand for 5 minutes. Placed 50, 75, 100 125 and 150 mg/ml of the extract in the appropriate wells. The plates were incubated at 37°C for 24 hrs. The zones of inhibition were measured in millimeters, using a ruler on the

underside of the plate. Same Procedure was repeated with standard with concentration of 50 µg.

Gram Positive Strain: *Bacillus subtilis* and *Staphylococcus aureus*

Gram Negative Strain: *Escheraria coli* and *Pseudomonas aeroginosa*

Standard Drug for Gram Positive: Ampicillin

Standard Drug for Gram Negative: Ampicillin

Anti-fungal activity: [16]

The in vitro anti-fungal activity was determined by using standard method – Agar well diffusion method. Standard cultures of *Candida albicans* were used for the study. Fluconazole was used as a standard anti-fungal agent. The inoculums were prepared as per Mac – Farland Nephlometer Standard. It was prepared by suspending a single isolated colony in about 5 ml of 0.9% w/v of normal saline. This was slowly mixed to achieve a smooth suspension. Later one drop of Tween-20 was added for filamentous fungi and the mould was broken by shaking. A sterile cotton swab was moistened in the inoculums suspension and the excess was removed by rolling the cotton swab inside of the tube. Above the fluid level 30 ml of sterile sabouraud's agar medium was poured in each plate and allowed to harden on a level surface. The surface was streaked in 4 different directions (at 90^0 C angle), so as to cover the entire surfaces. Using a flamed sterile borer the medium was bored and 0.1 ml of each prepared extract was added from the above in each bore. Same procedure was followed for *C.albicans* and was dried at 35^0C. Other bores were also prepared for test extracts taking fluconazole as a standard. A control having only DMSO was maintained in each plate, the plates were incubated at 35^0C for a period of 48 hrs. The values of Zone of inhibition were recorded.

The same methodology was followed for formulated gel with chloroform extract of *M.hortensis* and ethanolic extract of *C.longa*.

RESULTS AND DISCUSSION:

The preliminary phytochemical screening of chloroform extract of *M.hortensis* showed the presence of flavanoids, terpenoids and phenols whereas the ethanolic extract of *C.longa* revealed the presence of flavanoids, terpenoids, phenols, steroids and tannins respectively. The monographic analysis of the herbs was found to be within the pharmacopoeial limits and was depicted in table 1. The incorporated herbs into gel form were evaluated individually and in combination for the physical properties like appearance, spreadability, pH and colour. From the results of stability studies it was inferred that there was no change in pH, colour and spreadability of the formulated gel over time. The viscosity of the combined herbal formulated gel was determined and changes in the viscosity parameters were insignificant.

Table 1: Monographic analysis of the herbs

Parameters	Obtained values (w/w)		Pharmacopoeial limit for *C.longa*
	***M. hortensis* (%)**	***C.longa* (%)**	
Foreign organic matter	0.26±0.12	1.70±0.60	NMT 2%
Total ash	5.80±0.06	7.30±0.30	NMT 9%
Acid insoluble ash	0.89±0.31	0.90±0.01	NMT 1%
Alcohol soluble extractive	3.20±0.20	10.6±0.40	NLT 8%
Water soluble extractive	5.10±0.15	14.5±0.16	NLT 9%

(n=3; Mean± S.D)

Table 2: Physical evaluation of formulated herbal gels

Name of the formulation	Appearance / consistency	Spread ability	Washability	Colour and Odour	Clarity
Combined formulated gel (*M.hortnesis* and *C.longa*)	Smooth	Easy	Washable	Greenish	Clear and transparent

Table 3: Stability studies of topical combined herbal gel formulation for a period- 3 months

Parameters	1st month	2nd month	3rd month
Colour	Greenish	Greenish	Greenish
Odour	No change	No change	No change
Appearance	Smooth and clear	Smooth and clear	Smooth and clear
pH	6.8	6.8	6.8
Spreadibility	Easy	Easy	Easy
Viscosity (cps)	3984	3981	3979

Storage condition: 40°c ± 2°c / 75%RH ± 5%

Table 4: Effect of individual test extracts on Minimum inhibitory concentration (MIC)

Name of the bacteria	Growth in nutrient agar medium, soyabeancasein digestive medium, sabourouds dextrose agar medium containging at different concentration of extracts in mg/ml
Chloroform extract of *M.hortensis* of *Staphylococcus aureus* and *Candida albicans*	400 200 100 50 25 12.5 6.25 3.125 1.56 - - - - - - - - +
Ethanolic extract of *C.longa*, *Staphylococcus aureus* and *Candida albicans*	400 200 100 50 25 12.5 6.25 3.125 1.56 - - - - - - - + +

Keys: '-' ..... No growth indicates MIC;
'+' ..... Growth

Table 5: Effect of chloroform extract *M. Hortensis* on Zone of inhibition in mm (Anti – bacterial and Anti-fungal effect).

Test Organism	A 50mg/ml	B 75mg/ml	C 100mg/ml	D 125mg/ml	E 150mg/ml	Ampicillin 100ug/ml	Fluconazole 50ug/ml
S. aureus	11	15.8	20.4	26.4	26	20.1	-
B. subtilis	15.1	20.4	21.8	26	17.5	20	-
E. coli	9	10	13	15	15	19.3	-
P. aeurogenosa	20.6	3.7	24	30	31	19.1	-
C. albicans	10.4	14.3	18	19.6	19	-	18.2

Table 6 Effect of ethanolic extract of *C. longa* on Zone of inhibition in mm (Anti – bacterial and Anti-fungal effect).

Test Organism	A (50 mg/ml)	B 75mg/ml	C 100mg/ml	D 125mg/ml	E 150mg/ml	Ampicillin 100ug/ml	Fluconazole 50ug/ml
S. aureus	17.4	18.5	20.9	22.5	27	20.1	-
B. subtilis	13.1	17.7	20.4	22	29.1	20	-
E. coli	11	16.8	18.6	23.3	28	19.3	-
P. aeurogenosa	18.8	20.7	23.6	23.7	24	19.1	-
C. albicans	10.4	13.5	15.5	18.7	20.1	-	18.2

Table 7: Effect of anti-microbial activity of formulated gel of *M. Hortensis* and *C. longa*

Test Organism	ZOI of Formulated gel (mm)	ZOI of Standard (mm)
S. aureus	21.8	21.5
B. subtilis	24.4	20

E. coli	23.9	21.6
P. aeurogenosa	34.1	27.6
C. *albicans*	19.8	18.7

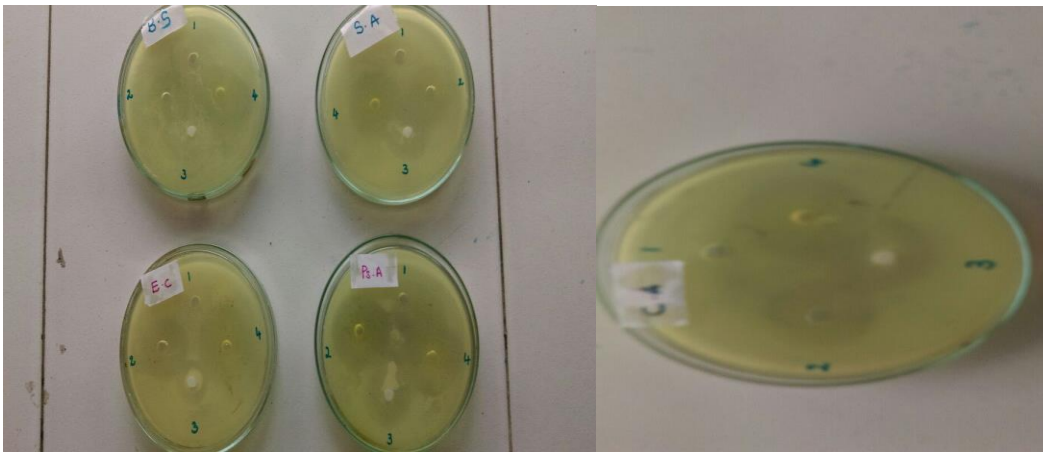

Fig 1: Representing the ZOIs of different test extracts of *M.horetensis* and *C.longa* in combination

The MIC study was performed on *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, P.aeroginosa* and *Candida albicans.* These pathogenic organisms were selected to explore the potentiality of the selected different concentrations of test extracts. In the first series of assays, the minimum inhibitory concentration (MIC) of extract against the tested microorganisms was screened by means of total solid diffusion test. The MIC of chloroform extract of *M.hortensis* and ethanolic extract of *C. longa* was observed to be 3.125 mg/ml and 6.25 mg/ml respectively (only two organisms were depicted in Table 4). The present study investigated the anti-bacterial activity of chloroform extract of *M.hortensis* and ethanolic extract of *C. longa* against various bacteria such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, P.aeroginosa* and *Candida albicans* at different concentrations. The effect of chloroform extract of *M.hortensis* and ethanolic extract of *C. longa* as anti-microbial agents were efficient on all investigated microorganisms.

The studies indicated that with the increase in the concentration of the extract there is increase in the zone of inhibition with both the test extracts. From the Table 6, in the anti-bacterial activity, the zone of inhibition (ZOI) was highest for *S.aureus* and *P.aeroginosa* at 150 mg/ml with the chloroform extract of *M.hortensis* and similarly ethanolic extract of *C. longa* produced highest zone of inhibition for *E. Coli* and *B.subtilis*. In the anti-fungal activity, the two test extracts chloroform extract of *M.hortensis* and ethanolic extract of *C. longa* showed 19 mm and 20 mm at 150 mg/ml as comparable to the standard. The studies indicated that with the increase in the concentration of the extract there is increase in the zone of inhibition with both the test extracts. The formulated gel with both the extracts was evaluated for anti-microbial activity using same methodology and was found that there is increase in ZOI for all organisms, highest being noted for *P.aeroginosa*.

CONCLUSION:

There are number of fungal and bacterial infections which are being treated by many traditional medicines. A formulation gel in combination with two herbal extracts chloroform extract of *M.hortensis* and similarly ethanolic extract of *C. longa* was proved to possess anti-bacterial and anti-fungal agents showing good anti-microbial effect.

CONFLICT OF INTEREST:

The authors have no conflict of interest with anyone.

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