RESEARCH ARTICLE

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Enhancement of Antimicrobial Activity of *Curcuma longa* with the Combination of *Ocimum sanctum* and *Gingiber officinale* and Development and Evaluation of Topical Formulations

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Abstract: The present investigation was designed to check antimicrobial activity of combination of three extracts of *Curcuma longa* (CL), *Ocimum santum* (OS) and *Gingiber officinal*e (GO) together and the development of suitable dosage form. Crude samples of CL, OS and GO were collected, air dried, powdered and extracted with 50% ethanol by maceration method and subjected for phytochemical analysis. Antimicrobial activity of individual extracts of CL, OS and GO CL, OS, GO and combined extract was carried out by the agar well diffusion method and tube dilution method. The extracts were tested on clinical isolates include aerobic, facultative bacteria namely *Stapyhlococcus aureus* ATCC 25921, *Escherichia coli* ATCC 25922, *Bacillus subtilis* and fungus *Aspergillus niger.* The combined extract showed greater antimicrobial activity than individual hydroalcoholic extracts. The minimum inhibitory concentration (MIC) of extracts varied from species of microorganisms, it was found to be 1 to 1.2 mg/ml. Further combined extract was used for preparation of topical formulations such as gel (1%) and ointment (1%). The further antimicrobial study of gel and ointment was compared which showed no loss of antimicrobial activity of extracts after six months. Then both the formulations were evaluated for physical characteristics and stability study.

INTRODUCTION

Since ancient times, plants have been an exemplary source of medicines. India has about 45000 plants species and among them, several thousands have been claimed to possess medicinal properties. [1] The demand for plant remedies results from several factors like effectiveness of plant medicine and the side effects of synthetic drugs. Several important drugs used in modern medicine have come from plant. [2]

We had selected three herbs tulsi, turmeric and ginger to check synergistic activity of extracts when mixed altogether.

Aqueous extract of OS showed growth inhibition for *Klesbiella, E. coli, Proteus* and *Staphylococcus aureus*; while alcoholic extract of OS showed growth inhibition for *Vibrio cholera*. The alcoholic extract of OS was also found to be active against multidrug resistant strains of *S. aureus* that are also resistant to common beta lactam antibiotics. Similarly, OS was found to be active against resistant *Neisseria gonorrhea* strains. [3] Curcumin (diferuloylmethane) is a principal yellow pigment present in the rhizome of turmeric (*Curcuma longa* L.) and related species. *C. longa* of family Zingiberaceae, a perennial herb. A wide array of pharmacological and biological activities of curcumin has been reported in literature. [4] Antioxidant and anti-inflammatory antibacterial effects of this compound have been assessed in various *in-vitro* systems and in experimental animal models. Only few studies have shown the stimulatory effect of curcumin on antibody production from splenocytes and natural killer (NK) cell activity *in-vivo*. Curcumin increases total white blood cells counts and circulating antibody titer against sheep red blood cells (SRBC). [5]

Ginger is a medicinal plant that has been widely used in Chinese, Ayurvedic and Tibb-Unani herbal medicines all over the world and has a long history of use in traditional systems of medicine. The primary pungent agents are due to the presence of phenylalkylketones or vanillyl ketones. Gingerol and shogaol are two most active constituents of ginger based preparations. They are reported to demonstrate antiemetic, antipyretic, analgesic, antiarthritic and anti-inflammatory activities. Ingenol and (6)–shogaol, isolated from gingerrhizome, demonstrated antiviral activity. (10)-gingerol has been reported as active inhibitor of *M. avium* and *M. tuberculosis in-vitro.* Gingerol and related compounds have been investigated for antimicrobial activities. (6)-gingerol and (12)-gingerol, isolated from ginger rhizome, demonstrated antibacterial activity against periodontal bacteria. [6]

India has a rich resource base of medicinal plants, plush with approximately 8,000 different species according to the Government of India. [7] Evidently the bioactive component of plants has been used for a long time against a wide variety of microorganisms and curing various infectious diseases. Due to failure of chemically synthesized antibiotic to protect the emergence of multi drug resistant bacteria, so there is need to evaluate the antibacterial activity of these herbs for the purpose of searching out newer and safer antibacterial drug. Considering all views, the present study evaluates the efficiency of antibacterial activity and can be used new tools for novel drug development.

MATERIALS AND METHODS

Collection of Crude Drugs

Rhizomes of *Gingiber officinale* and roots of *Curcuma longa* were procured from local market, Mumbai and authenticated at G. N. Khalsa College, Mumbai, India. Then samples were air-dried at 45°C in a hot air oven. Fresh leaves of *Ocimum sanctum* were collected and dried in shed. Dried samples were ground to powder using a mechanical grinder and stored in a sealed plastic container.

Extraction

The dried powder of herbs *gingiber officinale, Curcuma longa* and *Ocimum sanctum* was extracted by maceration

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using solvents was 50% ethanol (Herb to menstrum ratio 1:7). [5] It was kept for maceration in stoppered container with frequent agitation for 10 hr. The filtrate was separated and solvent was removed by distillation. Further extracts were subjected to phytochemical investigation.

Collection of Microorganisms

Bacterial and fungal cultures of *Escherichia coli* and Gram-positive bacteria Staphylococcus aureus, *Bacillus subtilis* and *Aspergillus niger* were collected from the microbiology laboratory, at Bombay college of Pharmacy, Santacruz, Mumbai.

Cultivation of Microorganism [8]

5 days old cultures of *E-coli* ATCC 25922*, Bacillus subtilis, Staphylococcus aureus* (ATCC 25921) cultivated on Nutrient Agar medium were transferred to nutrient broth and allowed to grow for 2 days at 37±2°C. In case of *A. niger*, 2 days old culture were transferred to Sabraud dextrose broth and allowed to grow for 2 days at 25°C.

Preparation of Culture Medium

HiMedia broth contains peptone, NaCl and yeast extract was prepared as below:

Nutrient broth (NB): To 1000 ml of distilled water, 13 gm of nutrient broth was suspended. [8]

Nutrient agar: To 1000 ml of distilled water, 13 gm of nutrient broth was suspended. To this nutrient broth 20 gm agar was added. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Sabraud dextrose broth (SD): To 1000 ml distilled water 30 g of sabraud dextrose broth was suspended and heated to dissolve in the medium completely. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Sabraud dextrose agar: To 1000 ml distilled water 65 g of sabraud dextrose agar was suspended and heated to dissolve in the medium completely. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Preparation of 0.5 McFarland Standard: 9.95 ml of 1% H_2SO_4 was added to 0.05 ml of 1% $BaCl_2$ to obtain McFarland's No. 0.5 solution. A 0.5 Mc Farland standard is comparable to a bacterial suspension of 10^8 to 10^6 cfu/ml.

Preparation of Inoculum

The stock cultures of microorganisms used in this study were maintained on Agar slants at +4°C. The bacterial culture medium was prepared as following; nutrient broth and soft agar medium adjusted to pH 6.6 and autoclaved at 121°C for 20 min. Cell suspensions were prepared by inoculation of loop full bacteria into 10 ml of Nutrient Broth (NB Hi media). Incubation was performed at 37°C for 24 h. After optimum growth was observed, the Cultures of *E. coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) agar medium were transferred to nutrient broth and allowed to grow for 2 days, at 37±2°C. In the same way cultures of fungus *Aspergillus niger* from Sabraud dextrose (SD) agar medium were transferred to Sabraud dextrose broth and allowed to grow for 2 days, at 25±2°C. After growth, the culture solutions of bacteria and fungus adjusted to a turbidity equivalent to 0.5 McFarland with sterile water. [9]

Screening of Antimicrobial Activity of Extracts

50% ethanolic extracts of turmeric, tulsi and ginger were evaluated for antimicrobial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and fungus *Aspergillus niger* by agar well diffusion assay and broth dilution method.

1. Agar Well Diffusion Assay

The *in-vitro* inhibition assay of extracts and standard were carried out by agar well diffusion assay**.** NB agar and SD agar were used as a medium and bore well method was used for studies. Sterile NB agar and SD agar 500 ml was seeded with 25 µl of microbial culture and poured in petriplates. Well was made with the help of borer after cooling NB and SD agar. Solution of extracts (4000 µg/100 µl, 3000 µg/100 µl, 2000 µg/100 µl, 1000 µg/100 µl) were prepared and amoxicillin (500 µg/100 µl) used as standard (positive control). Each set of microorganism contained standard, negative control, drug extract and sterility control and incubated bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2ºC. After incubation period, the zone of inhibition was measured as a diameter of well for extracts and standard. The results of individual extract of herb and combined extract of herbs were compared. [9]

2. Broth Dilution Method

Cultures of *E. coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) were diluted with sterile water to 10 µl/ml and transferred to test tubes containing NB. In the same way culture of a fungus *Aspergillus niger* from Sabraud dextrose (SD) broth was diluted with sterile water to 10 µl/ml and transferred to test tubes containing SD broth. The extracts of concentrations 4000 µg/1000 µl, 3000 µg/1000 µl, 2000 µg/1000 µl and 1000 µg/1000 µl were added in test tubes. Along with this, standard (500 µg/1000 µl), negative control and sterility broth were prepared as shown in Table 1. Bacteria allowed growing for 24 h at 37±2°C and fungus for 24 h at 25±2°C. [8] Estimation of growth and inhibition of microorganism and fungus were performed by viable count method. [8]

a. Viable Count Method

5 µl of above NB and SD broth in each test tube were diluted to 100 µl with sterile water and added in petri plates containing NB and SD agar. It was kept for incubation for 24 h at 37±2°C and 25±2°C respectively. Numbers of colonies of bacteria and fungus were calculated and results of different concentrations of extracts were compared. [8]

Formulation Topical Gel

The attempt was made to formulate equal amount of extract CL, GO and OS into gel with different polymers. The extracts of CL (Turmeric), OS (Tulsi) and GO (Ginger) (2.5%) was formulated into gel with polymers (Sodium CMC, Carbopol 974) using various formulae. The

Table 1: Test Tubes Dilutions

S. No.	Test tubes	Broth (ml)	Culture (ml)	Standard (ml)	Extracts (ml)	Water (ml)
1	Blank (-ve control)	9	___	___	___	1
2	Blank (Standard)	9	___	0.1	___	0.9
3	Blank (Extract)	9	___	___	0.1	0.9
4	Negative control	9	0.9	___	___	0.1
5	Standard	9	0.9	0.1	___	___
6	Extracts	9	0.9	___	0.1	___

Table 2: Composition of Gel (1%w/w)

S. No.	Ingredients	Quantity
1	Combined extract of CL, OS and GO	1gm
2	Carbopol 974	1 gm
3	Benzalkonium chloride	(0.01% w/v) 0.01 ml
4	Triethanolamine (q. s)	1.2 ml
5	Distilled water	Up to 100 ml

Table 3: Evaluation of Topical Gel of Combined Extract (0 Month)

S. No.	Parameters	Observation
1	Color	Greenish
2	Appearance	Homogeneous
3	pH	6.98
4	Spreadibility	20 (dyn.s/cm^2)
5	Viscosity (at 20 RPM, Spindle#7)	40000 to 41000 cps

Table 4: Stability Testing at 0°C±2°C (3 Month) of Topical Gel of Combined Extract

Color	Appearance	Spreadability (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	20.38	6.9	40500 to 44450

Table 5: Stability Testing at 25°C±2°C (3 Month) of Topical Gel of Combined Extract

Color	Appearance	Spreadability (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	21.65	6.98	42000 to 43500

Table 6: Stability Testing at 40°C±2°C (3 Month) of Topical Gel of Combined Extract

Color	Appearance	Spreadibility (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	20.22	7.2	43200 to 43900

combination with Carbopol 974 resulted in the best gel formulation, which was smooth and stable. [10]

1 g of Carbopol 934 was dispersed in 50 ml of distilled water with continuous stirring. 5 ml of distilled water was taken and required quantity of benzalkonium chloride was dissolved in water. The solution was cooled and further required quantity of extract was mixed to the above mixture and volume was made up to 100 ml with distilled water. Finally ingredients were mixed with carbopol 934 gel by continuous stirring and triethanolamine was added drop wise to the formulation for adjustment of required skin pH (6.8-7). The composition of gel is given in Table 2, to obtain the gel at required consistency. The same method was followed for preparation of control sample without adding extract. [10]

Evaluation of Topical Gel [11, 12]

Gel was evaluated for physical characteristics, pH, spreadibility, viscosity and stability. Results are given in Table 3-6.

Formulation of Ointment [13]

The ointment from 50% ethanolic extract of herbs (2.5% w/w) was prepared by fusion method and composition given in Table 7. The weighed quantity of cetosteryl alcohol, hard paraffin, lanolin and white soft paraffin were melted together at 45-50°C. Then the extract was accurately weighed and added to the melted base and stirred properly to get uniform ointment. [13]

Evaluation of Physicochemical Properties

The ointment was evaluated for physicochemical properties and stability. Observations were recorded in Table 8-9. [12]

Evaluation of Antimicrobial Activity of Formulations

1. Agar Well Diffusion Assay

The *in-vitro* inhibition assay of gel, ointment and standards were carried out agar well diffusion assay. NB agar and SD agar were used as a culture growth medium and bore well method was used for studies. Sterile NB agar and SD agar

Table 7: Composition of Ointment (1% w/w)

S. No.	Ingredients	Quantity
1	Combined extract	100 mg
2	Cetosteryl alcohol	500 mg
3	Hard paraffin	500 mg
4	Lanolin	250 mg
5	White soft paraffin	8.5 g
6	Lemon oil (fragrance)	Q.S

Table 8: Evaluation of Physicochemical Parameters of Formulation

S. No.	Formulation Properties	Observations
1	Appearance	Smooth
2	Color	Green
3	Odor	Lemon oil
4	Appearance	Homogeneous

Table 9: Stability Testing after 3 Month of Topical Ointment Combined Extract

Temperature	Appearance	Color	Appearance
0°C±2°C	Smooth	Green	Homogeneous
25°C±2°C	Smooth	Green	Homogeneous
40°C±2°C	Smooth	Green	Homogeneous

Table 10: Zone of Inhibition of Gel, Ointment and Standard against Microorganism

S. No.	Formulation	*B. subtilis*	*E. coli*	*S. aureus*	*A. niger*
1	Ointment (100 mg)	25± 3 mm	19.5±1 mm	22±1 mm	15±1 mm
2	Gel (100 mg)	24± 0.5 mm	22.±2 mm	24.±1 mm	18±1 mm
3	Standard (500 ug)	25.25±1 mm	26±1 mm	31±1 mm	36.4±1.2 mm

Table 11: Viable Count for 5 µl of Broth Containing Microbes with Ointment, Standard and Control Sample

S. No.	Formulation	*B. subtilis*	*E. coli*	*S. aureus*	*A. niger*
1	Ointment (100 mg)	150	80	156	5
2	Gel (100 mg)	65	11	112	2
3	Standard (500 ug)	24	09	78	0
4	Control	362	415	374	14

500 ml was seeded with 25 µl of culture and poured on petriplates. Well was made after cooling NB and SD agar. In agar well 100 mg of gel and ointment were added (100 mg gel contain 2500 µg of extracts) and amoxicillin capsule (500 µg/100 µl) used as a standard. In case of fungus fluconazole (500 µg/100 µl) used as a standard. Each set of microorganism contained standard, negative control, drug extract and sterility control and incubated bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2°C. [9] After incubation period, the zone of inhibition was measured as a diameter of well for formulations and standards. The results of gel, ointment and standard are given in Table 10.

2. Broth Dilution Method

Cultures of *E. coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) were diluted with sterile water and transferred to test tubes containing NB. In the same way culture of a fungus *Aspergillus niger* from Sabraud dextrose (SyD) broth was diluted with sterile water and transferred to test tubes containing SD broth. In each test tube 100 mg of gel and ointment were added (100 mg gel contain 2500 µg of extract) and amoxicillin capsule (500 µg/100 µl) used as a standard. In case of fungus fluconazole (500 µg/100 µl) used as a standard. Along with this, negative control and sterility broth were maintained and allowed to grow bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2°C (ointment mixed with small amount of methanol before addition in to test tubes). [8]

a. Viable Count Method

5 µl of above NB and SD broth in each test tube were diluted to 100 µl with sterile water and added in petriplates containing NB and SD agar. It was kept for incubation for 24 h at 37±2°C and 25±2°C respectively. Numbers of colonies of bacteria and fungus were calculated and results of different extract concentrations were compared as given in Table 11.

RESULTS AND DISCUSSION

The yield of hydroalcoholic extracts of CL, GO and OC was around 14%, 9% and 16 % w/w respectively. The preliminary phytochemical analysis of extracts has showed the presence of resins, flavonoid, tannins and phenolic compounds; in addition to this they showed the presence of carbohydrate and mucilage.

Table 12: Zone of Inhibition by CL, OS, G0 and Combined Extract by Well Diffusion Method

S. No.	Ethanolic Extract of Leaves	*B. subtilis*	*E. coli*	*S. aureus*	*A. niger*
1	1000 µg (CL)	Not found	12±1 mm	Not found	15±1 mm
2	1500 µg (CL)	14±2 mm	14±1.5 mm	14±3 mm	23±2 mm
3	1000 µg (OS)	16±3 mm	14±1 mm	22±3 mm	34±2 mm
4	1500 µg (OS)	16±1 mm	16±3.5 mm	22±1 mm	34±3 mm
5	1000 µg (GO)	18±1 mm	19±1 mm	29±1 mm	35±2 mm
6	1500µg (GO)	18±2 mm	20±2 mm	31±2 mm	35±2 mm
7	Combined extract, 1000 µg (CL, OS and G0)	26.25±1.2 mm	25± 0.9 mm	35.25± 1.4	36.4±0.9 mm

Table 13: Comparison of % Growth Inhibition of Combined Extract by Broth Dilution Method

S. No.	Concentration of Extract/Standard	*E. coli*	*B. subtilis*	*S. aureus*	*A. niger*
1	1000 µg (CL)	26.58	28.32	3.505	63.77
2	1500 µg(CL)	45.39	41.39	15.02	68.59
3	1000 µg (OS)	78.52	46.18	29.71	75.75
4	1500 µg (OS)	89.36	69.71	49.24	78.51
5	1000 µg (GO)	92.84	79.73	70.95	80.85
	1500µg (GO)	78.52	46.18	29.71	75.75
6	Combined extract, 1000 µg (CL, OS and G0)	96.11	86.49	91.81	95.45

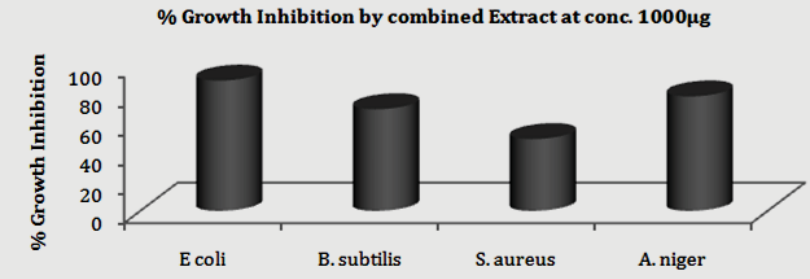
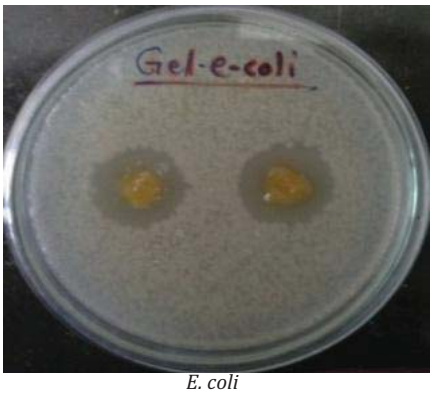
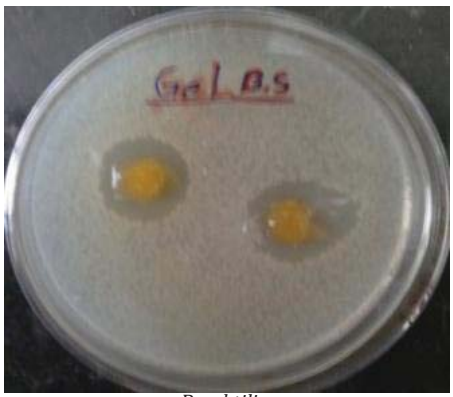


Figure 1: % Growth inhibition by combined extract at concentration 1000 µg (broth dilution method)

S. aureus



E. coli



B. subtilis

A. niger

Figure 2: Petri Plates photographs for zone of inhibition by 1% gel (100 mg) against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Aspergillus niger*

The individual extracts of CL, GO, OC and combined extract of all three were subjected to *in-vitro* antimicrobial studies by pour plate method and broth dilution method. The combined extract showed more promising antimicrobial activity as compared to individual extracts of CL, GO and OC. In pour plate method, the combined extract had showed significant bactericidal activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and fungistatic against *Aspergillus niger* at concentration 1 mg/ml as given in Table 12. The further comparison of antimicrobial studies of hydroalcoholic extract of CL, GO, OC and combined extract of all three were done by broth dilution method. In broth dilution method also combined extract showed better activity against microbes as compared to individual herbal extract. The minimum inhibitory concentration of combined extract was found to be 1 to 1.2 mg/ml. So, the combined hydroalcoholic extract was selected for topical formulations. The ethanolic extract of leaf was formulated into 1% w/w carbopol gel and 1% w/w simple ointment IP. The excipients compatibility with extracts was checked by repeating the antimicrobial studies after one, three and six months. The study showed that the combined extract was compatible with excipients of gel and ointment because antimicrobial activity of extracts retained in *in-vitro* tests. The gel and ointment was characterized for physicochemical stability at different temperature conditions. The results showed that the both formulations retained antimicrobial activity of the extract but gel has shown more promising activity than ointment.

CONCLUSION

The enhancement of antimicrobial activity of combined extracts of CL, GO and OS was probably due to the synergism of some important secondary metabolites. Therefore, this property of synergism of chemical component in these herbs can be used to discover natural bioactive products that potentially enhance the therapeutic value in the development of novel pharmaceutical research activities and suggesting for using potential antibacterial agents to cure bacterial infection.

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Abbreviations

Curcuma longa (CL*), Ocimum santum* (OS) and *Gingiber officinal*e (GO), Nutrient broth-NB, Sabraud Dextrose broth-SD and Minimum Inhibitory Concentration (MIC).

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