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COMPARITIVE STUDY OF DRUGS EXTRACT AND ITS POLYHERBAL FORMULATION FOR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Foeniculum vulgare and *Ocimum tenuiflorum* are traditional medicinal plants that have antimicrobial activity against *S.aureus* and *E.coli*, a combination of these two plants have not been known for its activity against these bacteria. The purpose of this research was to formulate mouthwash, combination of *F.vulgare* and *O.tenuiflorum* which has antimicrobial activity against *S.aureus* and *E.coli*. The antimicrobial activity test of *F.vulgare* and *O. tenuiflorum* using agar well diffusion method was carried out. Test parameters for mouthwash includes organoleptic, pH, shelf life and stability studies. The results of this study showed that *F.vulgare* and *O.tenuiflorum* extracts had antimicrobial activity. Antimicrobial activity combination of *F.vulgare* and *O.tenuiflorum* extracts is synergistic. For the formulation materials, the concentration chosen is 1:1 (*F.vulgare : O.tenuiflorum*) because in that combination the zone of inhibition found in a good manner. And the physical examination states that the formulation was stable even after 60days.

Key words*:* *Foeniculum vulgare, Ocimum tenuiflorum*, Staphylococcus aureus, Escherichiacoli, Mouth wash.

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INTRODUCTION

In India, as in other developing countries, a very significant proportion of dental problems is due to microbial infections. Bacteria existing in the dental plaque or biofilm play an important role in the development of both dental caries and periodontal disease. Dental caries, also known as tooth decay or a cavity, is an infection, generally bacterial in origin, localized and transmissible, that results in the destruction of hard dental tissue. The occurrence of dental caries is approximately 60-65% among the Indian population.Mouth rinses have been used for centuries for medicinal and cosmetic purposes, but it is only in recent years that the rationale behind the use of chemical ingredients has been subject to scientific research and clinical trials. Despite several agents being commercially available, these chemicals can alter oral micro biota and have undesirable side effects such as vomiting, diarrhea, and tooth staining. Poly herbal are the formulations containing two or more than two herbs are called polyherbal formulations (PHF).Popularity of poly herbal formulation is due to

its high effectiveness towards a number of diseases. Poly herbal formulations are concentrated pharmaceutical preparation of plants obtained by removing active constituents with a suitable solvent, which is evaporated away and adjusting the residue to the prescribed standard. In the traditional system of Indian medicine , plant formulations and combined extracts of plants are choosen rather than individual ones. It is known that Ayurvedic herbals are prepared in a number of dosage forms, in which mostly all of them are PHF. Eventhough the active phytochemical constituents of individual plants have been well established, they usually present in minute amount and always, they are insufficient to achieve the desirable therapeutic effects. Due to synergism, poly herbalism confers some benefits not available in single herbal formulation. Mouthwashes are liquid preparations meant for cleansing, deodorising and rinsing of oral cavity. And it also provides protection for teeth and gums. Commonly mouthwashes are prescribed for oral infections like plaque, gingivitis etc. since brushing only covers 25% of the mouth, germs left behind the mouth may cause bad breath, plaque and gum problems. While mouthwash can reach almost all parts of the mouth and is able to kill 99% of the germs. It can be able to penetrate and is helpful in conditions like plaque. Mouthwash can be prepared by using traditional drugs. It is also due to the much stronger belief that the alternative therapy is with less side effects. That makes the demand of herbal products in the market. People are willing to follow the alternative therapy since now a day they are well bother about side effects of organic medicine like anti fungal, antibacterial activities (1-3)

MATERIALS AND METHODS

Plant Collection and Drying (4-10)

The plant *Ocimum tenuiflorum*& seeds of *Foeniculum vulgare* were collected from Kasaragod. The plant material was taxonomically identified by the botanist, Dr. VS Anil Kumar, Assistant professor, Department of Botany, Government College, Kasaragod. The plant dried under shade for about 7 days and then powdered with mechanical grinder and stored in an air tight container.

Pharmacognostic Studies of Plant

Macroscopic evaluation

Macroscopic evaluation can be done by means of organs of sense. This refers to the evaluation of drug by colour, odour, size, shape, taste and special features including touch, texture etc. For this purpose authentic specimen of the material under study and sample of Pharmacopoeial quality should be available to serve as a reference. However the judgment based on the sensory characteristics like odour, taste etc. may vary from person to person and time to time based on individual's nature. No preliminary treatment is necessary for evaluating the sample in this manner.

Colour-The untreated samples were examined under diffused sunlight or an artificial light source with wave length similar to day light. **Size-**Size was measured using graduated ruler in millimetres.

Odour and taste:Samples were crushed by gentle pressure and examined by repeated inhalation of air over the material. **Texture and fracture-**the texture was examined by taking small quantity of material and rubbed in between the thumb and fore finger. Bent and rupture caused to the sample provided information of the brittleness and appearance of the fractured plane as fibrous, smooth, rough, granular etc.

Microscopic evaluation

The sample was subjected to powder microscopy. Small quantity of powder sample was taken in watch glass. To this a few drops of chloral hydrate was added and heated for 2-3 minutes. Small amount of powdered drug was mounted on a slide with a drop of glycerin, covered with cover slip and observed under microscope for presence of calcium oxalate crystals. To visualize lignified tissues, 1:1 ratio of phloroglucinol: concentrated Hydrochloric acid was added to the powdered drug. After 2minutes, small amount of drug powder was mounted on a slide with a drop of glycerin, covered with a cover slip and observed under microscope.

Physico-Chemical evaluation

Determination of moisture content

2.5g drug powders were placed in a tared evaporating dish. Drying was carried out at 105^{0} C for 5 hours. The drying was continued at 1 hour intervals until difference between two successive weighing corresponded to not more than 0.25%. Constant weight was reached when two consecutive weighing, after drying for 30 minutes and cooling for 30 minutes in desiccators, showed not more than 0.01gm

difference.

Determination of ash value

The ash value is an important parameter for the evaluation of crude drugs, due to the variation of values within fairly wide limits. The ash value of any organic material is composed of inorganic materials like metallic salts and silica. The following three different methods were adopted for the determination of ash values (Total ash, Acid insoluble ash and Water soluble ash). Ashing involves an oxidation of the components of the products and a high ash value involves the contamination, substitution, adulteration or carelessness in the preparation of crude drugs for marketing.

Determination of Extractive Values

This method determines the amount of active constituents in a given amount of plant material when extracted with solvent. The extractive value is used as a means of evaluating crude drug and not readily estimated by other means. For example, lowering from the prescribed values indicate the addition of exhausted or unwanted material with original drug or incorrect processing of the drug.

Alcohol soluble extractive value-Macerated 2.5 grams of coarsely powdered air dried drugs with 50ml ethanol in a stoppered flask for 24 hours, with occasional shaking during the first 6 hours and then allowed to stand undisturbed for another 18 hours. Filtered rapidly, by taking precaution against loss of alcohol. Then 12.5 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish, dried at 105 ^{0} C and weighed. Calculated the percentage w/w ethanol soluble extractive with reference to the air dried material. **Water soluble extractive value-** Macerated 2.5 grams of coarsely powdered air dried drug with 50ml chloroform water in a stoppered flask for 24 hours, with occasional shaking during the first 6 hours and then allowed to stand for another 18 hours. Filtered rapidly, then 12.5 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish and dried at 105 ^{0} C and weighed. Calculated the percentage w/w of water soluble extractive with reference to air dried material.

Preparation of Extract

Soxhlet extraction

Dried leaves of *Ocimum tenuiflorum* and seeds of *Foeniculum vulgare* were grounded using mixer grinder into coarse powder. The powdered drugs were then extracted by using solvent methanol. Around 35g of powdered drugs were weighed, moistened with methanol and packed in the soxhlet extractor and was then extracted with 500ml methanol. The extract was then collected and the solvent distilled off and finally the dried extract was obtained.

Preliminary phytochemical screening of plant extracts

Preliminary phytochemical screening like chemical tests for alkaloids, glycosides, phenolic compounds and tannins, flavanones and flavanoids, carbohydrate,proteins and amino acids, terpenoids, sterols, saponins,gums and mucilages, volatile oil was carried out.

Formulation of Polyherbal Mouthwash of *O.tenuiflorum* and *F.vulgare*

The polyherbal mouthwash formulation were prepared based on following ingredients with little modifications was adopted. Three batches of mouthwashes were formulated with plant extracts (Table-1). A study on formulation was carried out to determine the best formula. The best formulation was selected on analysing the stability on storage under room temperature and refrigeration.

Table-1Formulation of mouth wash

SL.NO	INGREDIENTS	BATCH 1	BATCH 2	BATCH 3
1	*O.tenuiflorum*	0.5g	0.5g	0.5g
2	*F.vulgare*	0.5g	0.5g	0.5g
3	Chloroform water	6ml	6ml	-
4	Ethanol	-	-	6ml
5	Peppermint oil	1.25ml	1.25ml	1.25ml
6	Tween 80	2.5ml	3.5ml	3.5ml
7	Distilled water	50ml	50ml	50ml

Procedure

Sufficient quantity of drug extracts were weighed and dissolved in little of distilled water. And these solutions were mixed and kept aside. Ingredients like chloroform water, peppermint oil, tween 80 were mixed in 3/4 th of water with vigorous shaking. The drug solution was then incorporated with other ingredients. The volume was made up to 50ml with distilled water.

Physico-Chemical evaluation of mouthwash

The physical parameters of mouthwash like colour, appearance, odour, pH, surface tensions were evaluated. **Visual appearance**-The preparation was visually inspected for its clarity, colour and transparency. It was also evaluated for the presence of particles. **Stability Study**-Placebo and medicated mouthwash were evaluated for their thermo stability. 20ml of mouth wash was taken in two 50ml beakers. One was kept at room temperature and other was kept at 40℃ for 4 weeks. It was also examined for pH, appearance and homogeneity. **Determination of Stability of the Formulation Using Surface Tension-**The study was carried out using drop volume method. A pipette was used to measure the volume of a drop of preparation by taking the average of 5 drops. The weight of the drop was measured using an analytical weighing balance. The surface tension of the batches were determined over a period of 4 weeks using the equation below.

γ (surface tension) = 2πrf Where, m = weight, g = acceleration due to gravity, f = correction factor 2π = constant. pH Studies-Well calibrated pH meter was used to determine the pH of different batches of mouth wash preparations were kept on shelf and in refrigeration (1 2 ^{0} C) respectively. The results were documented and compared over 4 weeks.

Screening of Antibacterial Activity

Combined methanolic extract of *O.tenuiflorum* and *F.vulgare* and formulated mouthwash are studied for its antimicrobial properties. However there are no reports regarding the evaluation of antimicrobial activity of these herbs in combination. Hence the drug was evaluated for antimicrobial activity in *Staphylococcus aureus* and *E.coli.*

Preparation of Mueller Hinton Agar

Suspend 38.0g in 1000ml distilled water, heat to boiling to dissolve the medium completely. Sterilized by autoclaving at 15lbs pressure(121^{0} C) for 15 minutes. Mix well before pouring.

Screening by Agar well diffusion method

Mueller Hinton agar was prepared. Then it was poured on sterilized petric plate and allowed to solidify. The bacterial culture (*S.aureus* and *E.coli*) was swabbed over the plates. Then , the wells are prepared by using sterilized cork borer. To that well separate drug extracts of *O.tenuiflorum* and *F.vulgare*, combined drug extracts (*O.tenuiflorum and F.vulgare*) and formulated mouthwash as well as standard (Ciprofloxacin) was added. Then the plates were incubated at 37^{0}C for 24-48hrs. The zone of inhibition was measured in mm.

RESULTS AND DISCUSSION

Plant Collection and Drying

Dried leaves of *O.tenuiflorum* and seeds *F.vulgare* used for the study was collected from Kasaragod district, Kerala. The same drugs were identified and authenticated by Dr. VS Anil kumar, Assistant professor, Govt. College Kasaragod.

Pharmacognostic Studies of Plant

Macroscopic evaluation

The samples were subjected to macroscopic identification based on colour, shape, size, odour, taste and features of drug. The morphological details of drugs are tabulated in Table-2.

Table-2 Morphological features of leaves of *O.tenuiflorum* and seeds *F.vulgare*

Characteristics	***Ocimum tenuiflorum* leaves**	***Foeniculum vulgare* fruits**
Colour	Green or purple	Green to yellowish brown
Shape	Ovate and have petioles, toothed	Straight or slightly curved
Size	Leaves up to 5cm long,Plants are 30-60cm tall	5-10mm long and 2-4mmwider
Taste	Spicy, Pungent	Strongly aromatic
Other features	Upright bushy shrub Stem is hairy	Dried ripe seeds Fruits are five-sided form of cremocarps
Odour	Aromatic	Sweet aromatic

Microscopic Evaluation

The sample was subjected to powder microscopy. Microscopic features were observed and compared with standard description in Indian Herbal Pharmacopoeia. The microscopic characters for *F.Vulgare* fruits are; parquetry arrangement of endocarp, thick walled endosperm cells containing aleurone grains, oil globules and minute calcium oxalate crystals. *O.Tenuiflorum* leaf shows covering or nongranular trichomes with thick and lignified wall, broken xylem elements, epidermal cells with paracytic stomata and sphaero crystals of calcium oxalate.

Physico-Chemical evaluation

The moisture content, total ash, water soluble extractive value and acid soluble extractive values of the drugs are tabulated in table-3 analysis was carried out for two samples.

Table-3 values for total ash, acid insoluble ash, water soluble extractive value and alcohol soluble extractive values for drugs

Parameters	**Sample(%)**		**Standard in IP(%)**	
	O.tenuiflorum	*F.vulgare*	*O.tenuiflorum*	*F.vulgare*
Total ash	13.8%	11.0%	NMT 16.0%	NMT 12.0%
Acid insoluble ash	5.8%	13.0%	NMT 6.0%	NMT 15.0%
Water soluble extractive value	1.0%	1.0%	NLT 1.0%	NLT 1.0%
Alcohol soluble extractive value	2.0%	4.3%	NLT 2.0%	NLT 4.0%

Preparation of Extract

The extracts of *O.tenuiflorum and F.vulgare* were prepared by soxhlet extraction. Then the extract was evaporated. The percentage yield of extracts was calculated and results are shown in table-4.

Table-4 Percentage yield of extraction of *O.tenuiflorum* and *F.vulgare* by soxhlet extraction

SL No.	**DRUGS**	**%YIELD OF EXTRACTS**	
		Methanolic extract	**Chloroform extract**
1	***O.tenuiflorum***	11%	3.8%
2	***F.vulgare***	9.7%	4%

Preliminary Phytochemical Screening of Plant Extracts

Preliminary Phytochemical Screening of Plant Extracts was carried out and results were shown in table-5.

Table-5 Phytochemical screening of plant extracts of *O.tenuiflorum* and *F.vulgare*

PHYTOCHEMICALS	**TESTS**	**METHANOLIC EXTRACT**	
		O.tenuiflorum	***F.vulgare***
Alkaloid	Mayer's test	+	+
	Wagner's test	+	+
	Hager's test	+	+
	Dragondroff's test	+	+
Glycoside	Legal's test	+	+
	Balget's test	+	+
	Liebermann-Burchard's test	+	+
	Borntrager' test	+	+
	Modified Bontrager's test	+	+
Phenol	Ferric chloride test	+	+
	Gelatin test	+	-
	Lead acetate test	+	+
	Decolourisation test	-	-
Flavanoid	Aq.sodium hydroxide test	+	+
Carbohydrate	Molisch's test	+	+
	Fehling's test	+	+
	Benedict's test	-	-
Protein and Amino acids	Million's test	-	+
	Biuret's test	-	-
	Ninhydrin's test	+	+
Terpenoids	Salkowlski'test	+	+
Sterols	Liebermann-Burchard's test	+	+
	Salkowski'test	-	-
Saponin	Foam test	+	+
	Hemolysis test	+	+
Gum and Mucilage		-	+
Volatile Oil		+	+

Formulation of Polyherbal Mouthwash

The polyherbal mouthwash formulations were prepared based on the formulas mentioned in the table-3 among the formulas the batch 1 was found stable on storage. Thus it was chosen for final preparation.

Visual appearance

When the optimized formulation was evaluated for 4 weeks, the visual appearance of the mouth wash was found to be unchanged, stable and acceptable.

Stability study

The formulation was stored and observed under room temperature and elevate temperature (40^0 C) for a period of 4 weeks. On observation the preparation was found stable, unchanged and good in appearance. Hence it was found to be thermo stable. The results were shown in table-6.

Table-6 Results of stability studies

Formulation	Appearance	Homogeneity	pH
Batch 1	Stable and unchanged	Maintained	6.8±0.05

Determination of Stability of Formulation by Surface Tension

On analysing the documented data, the batch 1 was found stable (Table-7). Since the other two batches possess randomly changing surface tensions under two different storage conditions.

Table-7 Surface tension by drop weight method

Batches	Surface tension (dyn/cm)							
	Shelf				Refrigeration			
week	1	2	3	4	1	2	3	4
Batch 1	31.5	31.5	32	32.4	35	33	35.2	35.6
Batch 2	37.2	29	31	36.8	42	28	28.3	31
Batch 3	34	41.2	36	43	27			

pH STUDY

From the table-8 it can be seen that the range of pH for shelf storage and refrigerator storage over 4 weeks corresponds. And from the data it shows, formulation was stable on storage and it maintains the pH on storage. The pH of the preparation was also found compatible with oral cavity.

Table-8 pH under storage conditions

pH							
Shelf				Refrigeration			
Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
6.78	6.8	6.82	6.82	6.81	6.82	6.81	6.8

Evaluation of formulated mouthwash combination of *O. tenuiflorum* and *F. vulgare* for antibacterial activity

Antibacterial activity of the extract of tulsi and fennel were studied in different concentration of such as 10% and 15% extract against *S.aureus* and *E.coli* (Table-9).

Table-9 Evaluation of antibacterial activity by agar well diffusion method

SAMPLE	CONCENTRATION	ZONE OF INHIBITION 100µL/well	BACTERIAL CULTURE
Tulsi leaf extract	10%	16mm	*S.aureus*
	15%	20mm	
	10%	12mm	*E.coli*
	15%	14mm	
Fennel extract	10%	12mm	*S.aureus*
	15%	15mm	
	10%	10 mm	*E.coli*
	15%	12 mm	
Combined extract of tulsi and fennel	1:1	22 mm	*S.aureus*
	1:2	16mm	
	2:1	17 mm	
	1:1	30 mm	*E.coli*
	1:2	11 mm	
	2:1	11mm	
Formulation	1:1	35mm	*S.aureus*
	1:1	22 mm	*E.coli*
Standard (ciprofloxacin)		40mm	*S.aureus*
		30mm	*E.coli*

Antibacterial potential of extracts were assessed in terms of zone of inhibition of bacterial growth. The antibacterial activity was observed to be increased with increase in concentration of extracts. The zone of inhibition was measured as 22mm for *S.aureus* and 30mm for *E.coli* in the 1:1 combination of *O.tenuiflorum* and *F.vulgare*. Thus the 1:1 combination of the extracts was chosen for the preparation of mouth wash. While performing antibacterial activity using the prepared mouth wash the zone of inhibition against the two bacterial cultures was found and results of antibacterial activities are presented in table-10

Table-10 Antibacterial activity of mouthwash

SAMPLE	COMPOSITION	ZONE OF INHIBITION	BACTERIAL CULTURE
Formulation	1:1	35mm	*E.coli*
	1:1	22 mm	*S.aureus*
Standard (ciprofloxacin)		40 mm	*E.coli*
		30 mm	*S.aureus*

The results shows that the mouth wash of *O.tenuiflorum* and *F.vulgare* extracts was found to be more effective against *S.aureus* and *E.coli*. Among these bacteria it was found to be moreeffective against *E.coli.*

CONCLUSION

The present study aimed at formulation of *O.tenuiflorum* and *F.vulgare* and evaluation of the same for antibacterial activity. Extraction of *O.tenuiflorum* and *F.vulgare* were done by soxhlet extraction technique. Methanolic extract of tulsi leaf yielded and fennel fruit yielded Phytochemical studies on methanolic extract of *O.tenuiflorum* showed the presence of alkaloid, glycoside, phenol, protein and amino acids, flavanoid, carbohydrates, terpenoid, sterols and volatile oil. The phytochemical analysis on the methanolic extract of *F.vulgare* showed the presence of alkaloid, glycoside, phenol, protein and amino acids, flavanoid, carbohydrates, terpenoid, sterols and volatile oil. Herbal mouthwash preparation of methanolic extract of *O.tenuiflorum* and *F.vulgare* combination (1:1) were prepared using chloroform water, tween 80 and distilled water, it yielded a formulation of satisfactory physicochemical appearance. The stability studies of the formulation were carried out satisfactory. Study for the effect of the extracts and the finished herbal mouthwash on individually, in combination and as formulated producing antibacterial activity. As per our research work, it reveals that drug extracts of *O.tenuiflorum* and *F.vulgare* found to be good antibacterial. And their methanolic extract was formulated with good physicochemical parameters, however further studies to be done to improve the stability of the formulation.

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