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RESEARCH ARTICLE
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EFFECT OF STABILITY, BIO-BURDEN, KILLING TIME, PRESERVATIVE ASSESSMENT, AND GC-ANALYSIS ON NOVEL DEVELOPED HERBISOL - A NEW MOUTH WASH

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ABSTRACT

In Pakistan, individuals with oral issues are consistently increasing with an alarming rate and cases are routinely reported to dental practioners. Oral cleanliness is vital to get freed from plaque and gum disease which is possible by utilization of various mouthwashes in which certain fundamental oil having antimicrobial and sedative properties are incorporated. A few organic compounds are known to present in various oils of plants. This research was conducted to evaluate the stability, bioburden, Antimicrobial potentials, time measurement, and preservative tests to determine antimicrobial impacts and assessment of the movement of mouthwashes against pathogens included in dental caries and oral diseases. GC analysis was also observed to quantify herbal constituents present in the newly formulated mouth wash. Mouth wash Herbisol was separately extracted with N-hexane. Our results showed that herbal mouth wash were effective against different oral pathogens as compared to herbal dental rinse solution. Sodium benzoate is used as a preservative effective against *E.coli*, *S. aureus* & *A. brazilensis*. The recovery was 99% - 96.6% for menthol, 96.8% for eugenol, and 91.4% for eucalyptol which are ingredients of herbisol. GC analysis affirmed that herbisol are a naturally dynamic mouth wash showing that a specific quantity of herbal actives attained in the mouth wash. our results indicated that using herbisol mouth wash twice a day, will maintain oral hygiene from microorganisms. Herbisol maintains stability level in all parameters. Any bio-burden and specified pathogens were not seen.

Keywords: Peridontal infection, Dental solution, Bioburden, Preservative efficacy, Essential oils, Gas Chromatography

INTRODUCTION

Dental biofilm formation in the oral cavity is one the leading cause of the most prevalent oral infections including dental caries, gingivitis, and periodontal inflammations (Aro *et al.,* 2019). Dental plaque is consequences of formation of multifaceted biofilm on the surface of teeth involving more than 500 bacterial species. These microorganisms are reported to cause several other long-lasting infectious diseases in humans (Attoub *et al.,* 2014). Supra-gingival plaque is commonly prevented by using various tools including mechanical or electrical tooth brushing, dental floss, or interdental brushing (Bhavna & vidya, 2012; Bozorgi *et al.,*2013). Similarly, some other agents involved in plaque control are chemical & therapeutic agents like sprays, chewing gums, and oral mouthwash solutions (Brown & Wright, 2016). However, the use of mouthwash has been accepted as the simple, safe, and easiest mode of oral hygiene (Cortelli *et al.,*2014). Mouthwashes are considered as the main mode of oral care in medically compromised and aged people where maintenance of adequate oral hygiene could be a major challenge (Costa *et al.,*2016).

Oral and teeth hygiene remained a significant practice since ancient times and across various civilizations of the world. The first documented oral mouthwashes solution was prepared by a Greek physician, named Dioscorides, consists of milk, plants extract, oil, and or vinegar (Dua *et al.,* 2015). Mouthwash preparations are specialized liquid solutions that are deemed as a potent and safe tool used to remove oral microorganisms, act as an astringent and prevent dental caries by rinsing the mouth. These findings also figured out a reduces survival risk of debilitated strains produced by fecal water and food from legal rustling mouthwash(Eslami *et al.,* 2015).

Natural prescriptions, gotten from organic sources, have been applied in dentistry for a long history to hamper microorganisms, lessen aggravation, mitigate troubles, and relax irritations (Filiphi *et al.,* 2013). It has been reported that a significant number of homemade mouth washes have formulated that brings about plaque and gum disease control (Janta *et al.,* 2018).

Natural mouthwashes are planned and incorporated with different concentrates of fundamental oils. Novel herbal mouth wash has been introduced and tested however, the results of existing literature are consistent regarding the in-vitro effects of herbisol on both dental plaque and gingival inflammation control confirmed after stability studies, preservative activities, and bio-burden and kill time measurements (Janta *et al.,* 2021). Different evidences highlighted the overall effects of herbal mouthwashes as adjuncts to the daily self-performed oral hygiene of patients with gingivitis (Jayanti *et al.,* 2018). Therefore, this study aimed to develop a novel mouth wash and perform physical, chemical & microbiological analysis, and GC to quantify menthol, eugenol & eucalyptol. Overall effects of herbalmouthwashes as supplements to the daily oral hygiene on both plaque and inflammation control (Khan *et al.,*2018).

MATERIALS AND METHODS

Development of mouthwashes

Mouthwashes with novel and advanced formulations have been prepared under an aseptic condition in association with the Research & Development department of Herbion Pakistan Pvt. Ltd. Herbal mouthwashwas developed using herbs& herbal compositions including antimicrobial reagents ,different essential oils & preservatives. The composition and ingredients of mouthwash are shown in Table 1.

Table 1. Herbisol composition with percentages/100ml

S. No.	Name of Ingredients	
Oil Phase A		
1	Common essential oils With modified concentrations	Clove oil
2		Eucalyptus oil
3		Tea tree oil
4		Peppermint oil
5		Menthol
6	Novel & additional essential oils	Basil oil
7	Emulsifiers	Croduret LD 40
8		Polysorbate-20
Water Phase B (Part 1)		
9	Water	De-ionized water (Hot & sterilized)75°C for 10 minutes
10	Preservatives	Sodium benzoate
11	Humectant	Glycerin
12	Sweetener	Sodium Saccharin
Water Phase B (Part 2)		
13	Water	De-ionized water (Hot & sterilized) 75°C for 10 minutes
14	Preservatives	Citric acid
15		Sodium citrate
Water Phase B (Part 3)		
16	Water	De-ionized water (Hot & sterilized) 75°C for 10 minutes
17	Anti-tarter	Sodium fluoride
18	Foaming agent	Sodium lauryl sarcosinate
Water Phase B (Part 4)		
19	Water	De-ionized water (Hot & sterilized- cool) 75°C for 10 minutes
20	Coloring agent	Green color
Water Phase F (Part 5)		
23	Flavor	Tooty Fruity
Filter & Makeup		

Stability profile in terms of physical and microbiological Parameters

The stability profile of the oral solution was evaluated using parameters including,different characteristic i.e color, odor, and foaming appearance on shaking.The pH of the oral samples was determined using a standard glass electrode while the density was determined using water as standard according to the protocol of (Kirkin *et al.,* 2014).

Bioburden count and Pathogens Detection

A. Total Aerobic Microbial count (TAMC) and Total Yeast and Mold Count (TYMC)

Herbisol were diluted by adding 10ml of oral solution in 90ml of peptone.From this diluted oral solution 1ml was transferred to sterilized trypticase soya agar (TSA) and sterilized sabouraud dextrose agar medium (SDA) plates previously prepared. TSA plates were incubated for 3 days at 32.5 ± 2.5°C while SDA plates for 5 days at 22.5±2.5°C. Following incubation, the plates were examined for growth and colony count (Lebanov *et al.,* 2019).

B. Detection method for specific microorganisms

Specific detection tests were performed for the detection of specific microorganisms possibly grow in mouth,. Initially, oral solutions samples were spread on soybean casein digest medium (TSB) to

observe the possible growth of microorganisms. Following this, mannitol salt agar (MSA) medium and coagulase test was used for growth and detection of *Staphylococcus aureus* while cetrimide agar (CA) for *Pseudomonas aeruginosa*. MacConkey's agar medium for the detection of *E.coli*. Moreover, xylose lysine deoxycholate agar medium for the presence of *Salmonella typhimurium.* The presence/absence of *Enterobactericeae* was confirmed by sub-culturing on violet red bile glucose agar. Test for*Bacillus spizizenii*was performed by spreading on *Bacillus cereus* using a sterile glass spreader (Nasim *et al.,* 2017).

Efficiency of Bactericidal rate with different time interval by Herbisol

A modified plating technique was used for the determination of the rate of killing of bacteria by mouth wash preparations. Three different dilutions (1:10, 1:100, and 1:1000) of mouthwashes were prepared in tryptone soya broth in McCartney bottles and inoculated with test organisms (10^5 CFU/mL). The bottles were incubated at 37°C on an orbital shaker at 120 rpm and 100 µL sampling was done at 0, 4, 8, 12, 24, 48 & 72 hrs. for the determination of CFU/mL by the plate count technique. Positive control and negative controls were prepared accordingly (Costa *et al.,* 2016).

Preservative efficacy Test (PET)

Preservative efficacy test (PET)/antimicrobial efficacy test was performed to confirm the proper neutralization and minimal accepted level of contamination (<10 CFU/gm) in the designed product. The mouth wash sample were tested for their potential to reduce/rebound the high inoculum (10^6-10^7 cfu/g) of five ATCC established strains including; *E. coli* (8739), *S. aureus* (9027), *P. aeruginosa* (6358), *C. albicans* (10231), and *A. brasiliensis* (16405) in a sample for up to 28 days. Samples were collected from each culture preparation, positive and negative controls at the appropriate intervals of 0, 14th, and 28th days for the bacterial and fungal count. The log reduction was calculated for day 14^{th} using the following formula and results were interpreted as explained by (Aro *et al.,* 2019).

Log Reduction = (Log Initial Inoculum) – (Log Day 14 count cfu/ml))

Gas Chromatography (GC)

Gas chromatography(GC) addresses an insightful method reasonable for the subjective and quantitative investigation of essential oils (EO) since it offers high affectability, extraordinary solidness, and an uncommonly high straight unique reach that permits the examination of unpredictable segments of the EO at exceptionally low fixations or following levels (Sukkarwall *et al.,* 2013; Sullivan *et al.,* 2011). Most the fundamental oils acquired from vascular plants are potentin treating parasitic and bacterial diseases (Silva *et al.,* 2016).

Quantitative estimation of menthol, eugenol & eucalyptol by GC

25 g (accurate weight) of the Sample were taken in a container and moved into isolating channel, 25 ml of N-hexane were added and continuously shaken for 25 minutes at 25°C and make up volume according to detachment prerequisite with a similar dissolvable and shaken further for 60 minutes. The solution which dissolved the layers showed up on the upper meniscus and gradually mixes partially in watery layer without disturbing the layer and follows the same procedure 3 times (3x) with the layer for 1hour.A clear dissolvable layer has been isolated (Sciarrone *et al.,* 2012; Seeley, 2012). Thereafter shifted on Whatmann filterpaper containing sodium sulfate. However, to guarantee humidity control, silica gel were added, and further shifted by 0.2µm membrane filter 3 times,and then moved into 200ml volumetric cup. Readmade vial with the assistance of 5ml needle installed 0.2µm plate filterpaper. Test chromatograms with their noticed peak contrasted with standard chromatograms (Tranchida *et al.,* 2012).

Preparation of Standard solutions

0.1 g of (accurate weight) of menthol in a discrete 10 ml volumetric cup were mixed & diluted in N-hexane and make volume to 10ml

Preparation of diluent

Flushed all GC vials with the chosen dissolvable (N-hexane) and filled one vial by the utilization of needle with N-hexane to wash and get the obstruction-free if already someone has used it. Diluent showed a clean chromatogram with no pinnacles that demonstrate the smooth progression of the instrument with no past items cooperation (Tong *et al.*, 2018).

Chromatography conditions

Column	Teknokroma; TRB-5, 30m x 0.25 x 0.25um, capillary column or equivalent.
Column thermostat temperature	120°C for 4 minutes
Flow speed	10°/min;
Detector	200 nm;
Introduction volume	1 ml
Stop time	15 min
Hold periods	Menthol – around 15 min; Eugenol – around 15 min; Eucalyptol – around 15 min.

The introduced sample volume is 1 mcl of tested solution (measure conduct not less than 3 times).
Stop time: 15 min.
Contents X (%) of menthol calculated by formula:

$$X = \frac{S \times m_o \times 25 \times P \times D \times 100}{S_o \times m \times 10 \times 100}$$

Sample Area	□	**Weight of Standard**	□		**Dilution of sample**	□	**Potency of standard**	□	**Density □□100**
Standard Area		**10 ml (amount of standard makeup)**			**weight of sample**	**100**			

Where:
S– Average value of respective content peak on tested solution chromatograms;
S_0 – Average value of respective standard peaks on standard solution chromatogram;
m – Sample weight; g
m_0 – Respective standard weight; g
P – Potency of standard

Statistical Analysis

In this study, the data obtained were presented as mean ± standard deviation. To determine the coefficient of variation, the relative standard deviation was performed. Menthol, eugenol & eucalyptol (RSD-limit) should be less than 2%.

RESULTS

Physical and microbiological stability profiling in oral washes

The Physical and microbiological tests of the oral sample were performed from the initial date when the oral solution were developed and tested after 3 months, 6 months, 9 months, 12 months, 18 months & 24 months at 30ºC and 40°C ± 2 which confirmed the stability of the product retained till 2 years. Temperature is inversely proportional to density and pH. Mouth wash was found to be satisfactory in terms of physical and microbiological parameters when compared with international standards as shown in Table 2 and Table 3 in which different parameters are compared at 30ºC and 40°C respectively.

Table 2. Stability profiling of herbisol oral wash at 30ºC (Mean in triplicates n=3 samples shown)

Product Name: **Herbisol oral Wash**		Packaging: **Plastic Bottle**	Shelf Life: **2 years**
Batch No.: **TR-001**	Batch Objective: **Commercial batch**	Batch Size: **300ml**	Mfg. date**: October 2019**
Active Content: **clove oil, Eucalyptus oil, Tea tree oil, peppermint oil, basil oil**			Kept on Stability: **October 2019**
Active Manufacturer: **In-house**		Mfg. Lot. No.: **TR-001**	Report No.: **121/19**
Storage Condition: (On labeled)	**Store Below 30ºC.**	Stability Condition: **Room temperature**	**On-going (30ºC ± 2, RH 65% ± 5)**

Sample density and RD bottle 43.9-44.3 g/ml

S #	TEST	SPECIFICATION	RESULTS						
			Initial	3 months	6 months	9 months	12 months	18 months	24 months
01	Appearance	Light green, clear, transparent solution	Comply	Comply	Comply	Comply	Comply	Comply	Comply
02	Odor	Characteristic odor of Peppermint & basil	Comply	Comply	Comply	Comply	Comply	Comply	Comply
03	Foaming	Foaming appears on shaking	Comply	Comply	Comply	Comply	Comply	Comply	Comply
04	pH	4 – 8	7.23	7.48	7.23	7.41	7.40	7.46	7.39
05	Density	0.970 – 1.095 g/ml	1.026g/ml	1.038g/ml	1.037g/ml	1.037g/ml	1.039g/ml	1.038g/ml	1.040g/ml
06	Average volume of bottle content	Not less than 300ml	300ml	305ml	301ml	300ml	302ml	301ml	302ml
07	Microbiological Test	Category B (Ref: BP 2017 Vol-V Appendix 2.6.31-Page XVIF VA511-A515	Comply	Comply	Comply	Comply	Comply	Comply	Comply

Table 3. Stability profiling of herbisol oral wash at 40ºC (Mean in triplicates n=3 samples shown)

Product Name: **Herbisol oral Wash**			Packaging: **Plastic Bottle**					Shelf Life: **2 years**
Batch No.: **TR-001**	Batch Objective: **Commercial batch**		Batch Size: **300ml**					Mfg. date**: October 2019**
Active Content: **clove oil,Eucalyptus oil , Tea tree oil, peppermint oil & basil oil**								Kept on Stability: **October 2019**
Active Manufacturer: **In-house**			Mfg. Lot. No.: **TR-001**					Report No.: **122/19**
Storage Condition: (On labeled)	**Store Below 40ºC.**		Stability Condition: **Accelerated temperature**			**On-going (40°C ± 2°C RH=75%)**		
Sample density and RD bottle 43.5-43.8 g/ml								
S #	**TEST**	**SPECIFICATION**	**RESULTS**					
			Initial	3 months	6 months	9 months	12 months	18 months	24 months
01	Appearance	Light green color, clear, transparent solution	Comply	Comply	Comply	Comply	Comply	Comply	Comply
02	Odor	Characteristic odor of Peppermint & basil oil	Comply	Comply	Comply	Comply	Comply	Comply	Comply
03	Foaming	Foaming appears on shaking	Comply	Comply	Comply	Comply	Comply	Comply	Comply
04	pH	4 – 8	6.91	6.96	6.98	6.94	7.01	6.98	6.97
05	Density	0.970 – 1.095 g/ml	1.012g/ml	1.011g/ml	1.012g/ml	1.014g/ml	1.013g/ml	1.012g/ml	1.000g/ml
06	Average volume of bottle content	Not less than 300ml	306ml	307ml	304ml	301ml	305ml	303ml	305ml
07	Microbiological Test	Category B (Ref: BP 2017 Vol-V Appendix 2.6.31-Page XVIF VA511-A515	Comply	Comply	Comply	Comply	Comply	Comply	Comply

Bio-burden by microbial limit test method and specified micro-organisms detection

Herbisol showed no bacterial count & fungal count in 1:10 dilution. Total yeast and mold count and specified pathogens were absent in mouth wash and every ATCC strain was used as a positive control in TSA, SDA & their specified media, strengthening the efficacy of this mouthwash (peptone water) was used as a negative control.This experiment demonstrate that how effective is Herbisol against the pathogenic bacteria which are harmful for oral infections.

Table 4. Bioburden count and pathogens detection in newly developed mouthwashes by microbial limit test method (cfu/ml) and streak plate method (absent/ml) (Mean in triplicates n=3 samples shown)

Tests Performed	**Standard Limits of Herbal Oral wash**	**Herbisol (1:10 dilution) CFU/ml**
	Herbal	
Bio-burden by microbial limit test method (CFU/ml)		
Total Aerobic Count	$<10^4$	<10
Total Combined Yeast and Mold Count	$<10^2$	<10
Specified pathogens detection by streak plate method (Absent/ml)		
Staphylococcus aureus	Absent	
Pseudomonas aeruginosa	Absent	
Salmonella typhi	Absent	
Enterobacteriaceae	Absent	
Escherichia coli	Absent	
Candida albicans	Absent	
Aspergillus brazilensis	Absent	
Bacillus spizizenii	Absent	

Bacteriocidal assessment of Herbisol.

The Bacteriocidal activities of newly synthesized Herbisol mouthwash were checked against microorganisms mentioned in table 5. It was noticed that herbisolreduced the growth rate upto 48hrs and 72 hrs against many micro-organisms.However it was observed fewer bacteria i.e*Escherichia coli (ATCC-8739 & 14169), Salmonella typhi (ATCC-14028), Salmonella enterica (ATCC-6017) & Candida albicans (ATCC- 10231) has started growth after 48hrs and prominently after 72hrsPseudomonas aeruginosa (ATCC-9027)*showed growth after 12hrs. *No* growth was observed against in the rest of microorganisms mentioned in Table 5.

Table 5. In vitro time-kill assessment of the mouthwashes against Pathogens

Test isolates (1×10^5cfu/ml)	**Herbisol**						
	1:10 dilution (0.1ml organisms into 10 ml of mouth wash)						
	0h	**4h**	**8h**	**12h**	**24h**	**48h**	**72h**
P. gingivalis ATCC-33277	-	-	-	-	-	-	-
V. parvula ATCC-10790	-	-	-	-	-	-	-
P.aeruginosa ATCC-9027	-	-	-	+	++	++	+++
E.coli ATCC-8739	-	-	-	-	-	+	++
E.coli ATCC-14169	-	-	-	-	-	+	++
S.aureus	-	-	-	-	-	-	-

ATCC-6538							
S.typhi ATCC-14028	-	-	-	-	-	+	++
S.enterica ATCC-6017	-	-	-	-	-	+	++
B.spizizenii ATCC-6633	-	-	-	-	-	-	-
C.albicans ATCC-10231	-	-	-	-	-	+	++
A.brazilensis ATCC-6017	-	-	-	-	-	-	-

Keys: + indicate less growth, ++ indicate moderate growth, +++ indicate maximum growth, - No growth

Preservative efficacy Test

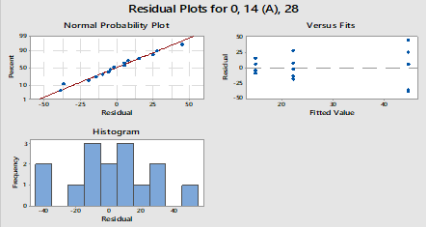
Efficacy and log data of Herbisol (oral wash)

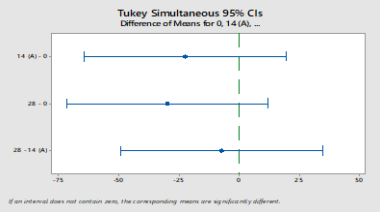
Microbial counts of herbisol rinse solution showed good results, i.e., microbiological counts were decreasing when compared with an initial inoculum. These results showed that oral wash constituents and their preservatives have good microbicidal activity against *S.aureus, E.coli & A.brazilensis* but no killing activity/increase recovery was observed against *P.aeruginosa*&*C.albicans*. as shown in Table-6.

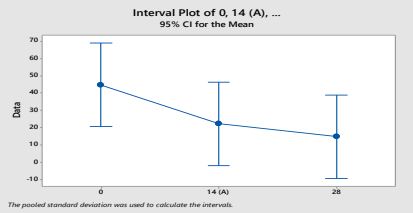
Table 6. Log reduction of Herbisol oral wash (Mean in triplicates n=3 samples shown)

S. No	**Microorganisms**	**ATCC**	**Initial Count (CFU/ml)**	**After Inoculum Addition (No of Days)**			**Log Red**	**Interpretation**
			X	**0**	**14 (A)**	**28**	**Log X – Log A**	
1	***E. coli***	8739	$4x10^6$	70	30	20	**6.602-1.477 = 5.125**	**Decrease Recovery**
2	***P. aeruginosa***	9027	$6x10^6$	$6x10^6$	$8x10^6$	$9x10^6$	**6.778-6.903=-0.125**	**Increase Recovery**
3	***S. aureus***	6538	$8x10^6$	50	20	10	**6.903-1.301 = 5.602**	**Decrease Recovery**
4	***C. albicans***	10231	$5x10^6$	$8x10^6$	$3x10^7$	$5x10^7$	**6.698-7.477 = -0.779**	**Increase Recovery**
5	***A. brazilensis***	16405	$2x10^6$	90	50	30	**6.301-1.698 = 4.603**	**Decrease Recovery**

Keys:
Increase Recovery: Microorganisms increased their growth.
Decrease Recovery: Microorganisms starts decreasing or killed.







Standards chromatogram

In our review, the comparative percent recuperation of 90-close to 100% of menthol, eugenol and eucalyptol have been accomplished in natural mouth wash when contrasted and the name guarantee, we added for assembling. Our results showed that the made dental plan consolidates herbisol recovered >96.68078% menthol,> 96.78605% eugenol, and> 91.4251% eucalyptol. The normal utilization of herbisol mouth wash noticed a notable reduce in their dynamic parts when assessed by gas chromatography Mark guarantee and the real amount got after gas chromatography were corresponded with one another. The percent relative standard deviation in menthol, eugenol and eucalyptol was noticed under 2%.The menthol mouth wash showed best result up to 90% compared to others which reflected similar results presented in previousstudies that menthol related mouth washes were more effective than other mouth washes used in market.

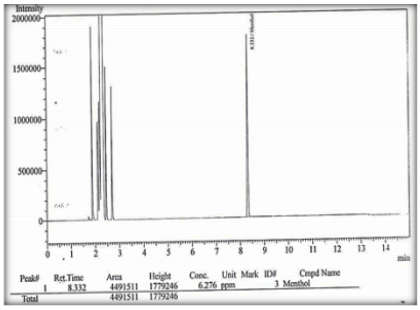


Fig.1. Menthol chromatogram

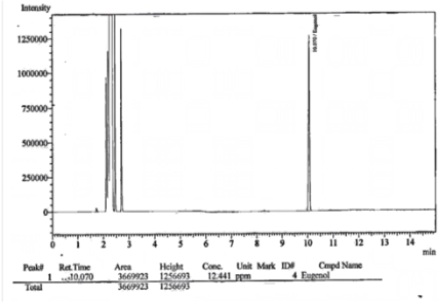


Fig. 2. Eugenol chromatogram

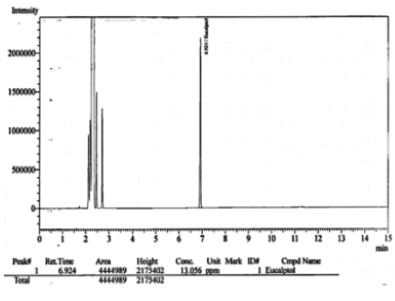


Fig. 3. Eucalyptol chromatogram

Diluent chromatogram (N-hexane)

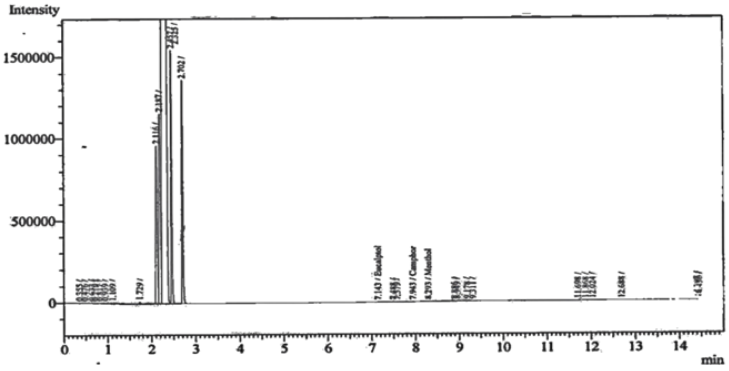


Fig. 4. N-Hexane chromatogram

Herbisol standards chromatograms

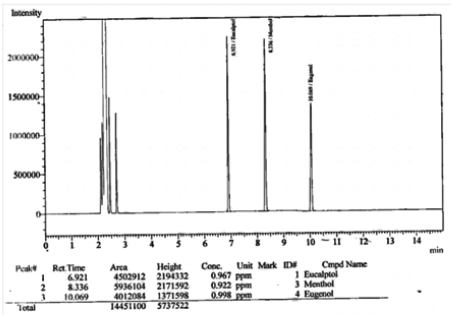


Fig. 5. Standard 1-herbisol chromatogram

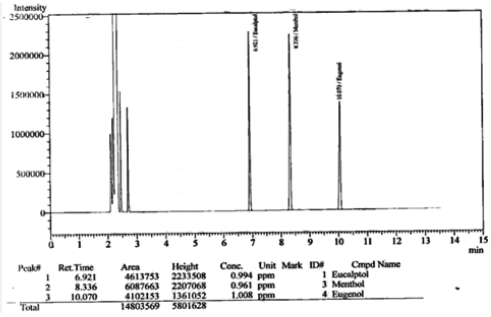


Fig. 6. Standard 2-herbisol chromatogram

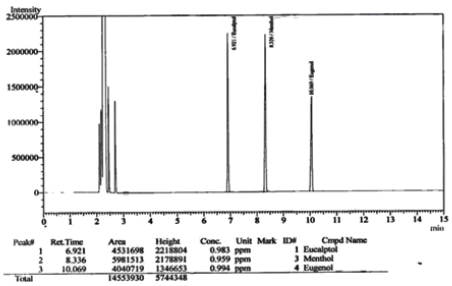


Fig. 7. Standard 3-herbisol chromatogram

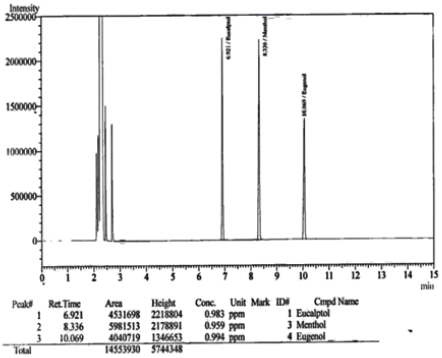


Fig. 8. Standard 4-herbisol chromatogram

Herbisol dental solution Chromatograms

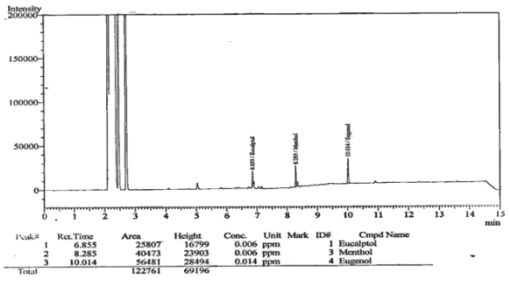


Fig. 9. Sample A1-herbisol chromatogram

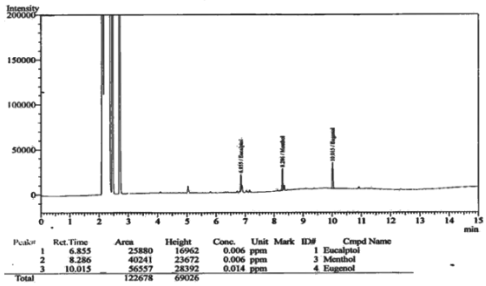


Fig. 10. Sample A2-herbisol chromatogram

Table 7. Areas of standards (Mean ± SD4times (n=4)

Standards	**Menthol**	**Eugenol**	**Eucalyptol**
	Area		
STD-1	5936104	4012084	4502912
STD-2	6087663	4102153	4613753
STD-3	5981513	4040719	4531698
STD-4	5981513	4040719	4531698
Average	**6001760**	**4051652**	**4549454**
SD	**77781.67**	**46019.06**	**57514.32**
% RSD	**1.30**	**1.14**	**1.26**

Table 8. Areas of herbisol (Mean ± SD each sample run in triplicates n=3)

Samples	**Menthol**	**Eugenol**	**Eucalyptol**
	Area		
Sample-1 (Herbisol)	40473	56489	25807
Sample-2 (Herbisol)	40241	56557	25880
Average	**40357**	**56523**	**25843.5**
SD	**164.0488**	**48.08326**	**51.6188**
% RSD	**0.406494**	**0.085068**	**0.199736**

DISCUSSION

Mouthwash -preparations are done with the aim of adequate oral cleanliness. Its usability notwithstanding the huge capacity to diminish dental plaque development made mouthwashes a sensible technique to restrict gum disease and periodontitis (Cortelli *et al.,* 2014; Costa *et al.,* 2016). The current investigation indicated that the oral sample was developed under aseptic conditions. All physical (appearance, odor, foaming, pH, density, and average volume of bottle content) and microbiological (bio-burden of aerobic, yeast and mold count and specified pathogens) stability profiling tests have been performed from the initial date and tested against different monthly intervals; include 3, 69 months,12 months, 18 and24 months at room temperature and at elevated temperatures and confirmed the stability of the product lasted till 2 years. Similar test has been also reported by (Dua *et al.,* 2015). The temperature was found to be inversely proportional to density and pH. Therefore, it was observed that pH and density decreased with the higher temperature at 40ºC as compared to 30°C. Similar findings were also reported by ….et al in …mouthwash ….et al in …mouthwash etc. The menthol mouth wash shows the best result as 90% studied earlier that menthol related mouth washes were more effective to other mouth washes used in market. It gives usually sweet taste with mint coolness which make it effective as compared other mouth washes. The softness make it usable for people of every age. Oral sample shave been identified for the evaluation of stability data. Herbisol is prepared from advanced &different formulations. Stability performed at 40°C±2°C RH=75% &30ºC ± 2, RH 65%±5 at different intervals. Mouth wash was tested according to the approved test method of (Kirkin *et al.,* 2014).During the stability studies, physical characteristics, average weight, microbiological purity of the product was observed. Similar tests has also been conducted by(Attoub *et al.,* 2014).Microbiological purities were also found to be in range when compared with specified limits throughout the shelf life. Our results suggested that the composition and concentration of essential oils along with other ingredients in all herbisol mouthwash was potent enough to control periodontitis, plague, peri-implants diseases, and oral infections as compared to the rest of the frequently used mouthwashes (Khan *et al.,* 2018).

No significant growth has been observed against majority of microorganisms from day 1 to 14 and from day 14 to 28 except *P. aeruginosa* & *C. albicans*. Similar tests were also performed by (Janta *et al.,* 2021) and their findings are consistent with our findings. It means that partial log reduction occurred by inoculum which were added in the wash initially at day zero. These results showed that mouth wash constituents and their preservatives have good microbicidal activity against USP recommended some microorganisms. Sodium benzoate, sodium citrate & citric acid are used as a preservative in the above oral wash (Bhavna & Vidhya, 2012).

Antimicrobial potentials of current mouthwash were checked against different microorganisms. Herbisol noticed development rate at 48 and 72 hrs *Escherichia coli* (ATCC-8739 and 14169), *Salmonella typhi* (ATCC-14028), *Salmonella enterica* (ATCC-6017) and *Candida albicans* (ATCC-10231). *Pseudomonas aeruginosa* (ATCC-9027) showed development after 12hrs due to high resistance behavior in presence of antimicrobial substances. -Our findings were done according to the reports of (Seeley, 2012).

In our study, the percent recovery of 90-99% of menthol, eugenol & eucalyptol have been achieved in herbal mouth wash when compared with the label claim, as the similar claim was about the manufacturer we added is (Vlachojannis *et al.,* 2015). Our outcomes showed that the created dental

arrangement incorporates herbisol recuperated 96.68078% menthol>, 96.78605% eugenol>, and 91.4251% eucalyptol>.The common use of herbisol (newly developed) mouth wash observed a remarkable decrease in their active components when estimated by gas chromatography (Nasim*et al.,* 2017; Sciarrone *et al.,* 2012) as reported by these mentioned research. Our researched mouth wash have the ability to recover throat infection as mint had the ability to recover. Herbisol mouth wash gives the highest percent of bactericidal potential to make our gums more fresh as compared to others mouthwash. similar findings has also been reported by (Mustafa et al., 2019). Which strengthen our data.

The previous studies could not detect the significant presence of herbal actives in herbal mouth rinse wash. As per past research of (Kirkin *et al.,* 2014; Seeley, 2012), Their mentioned quantity and the actual quantity obtained after gas chromatography in commercial dental solutions were not correlated with each other. Therefore, either the active ingredients provided on commercial brands just for distraction and increase the market value of the product, or inactivation of herbal ingredients would also be possible that may be due to improper/insufficient stabilities studies (Kirkin *et al.,* 2014).

Difference inin vitro and in vivo conditions parametrs(such as the presence of saliva in the oral cavity, natural buffering capacity of saliva, pH of the oral cavity, and presence of epithelial mucosa as a surface for bacteria to adhere) has also been brought into consideration. Herbal ingredients have long-term retention in products without degradation. Good recovery has been observed in samples. Menthol, eugenol & eucalyptol maintain nearby content in newly developed Herbisol mouthwash and our findings are consistent with the findings of (Lebanov *et al.,* 2019).

CONCLUSION

Results indicate that the quantification (GC) of herbisol mouth wash recovered 90-99 %, suggesting that under the observable situation, the mouth wash has antimicrobial potentials and is effective against germs and dental infections. The current research showed developed product is novel and stable in physical, chemical, and microbiological parameters. The herbisol composed of variety of essential oils and preservatives decreases the increasing growth of *S.aureus, E.coli &A. brazilensis*. The used essential oils can subsequently be a decent possibility to be utilized as elective oral treatment. Be that as it may, broad examination of the negligible parts of the concentrates should be completed to distinguish pharmacologically dynamic mixtures to explain the activity component and to ensure its wellbeing, and decide the appropriate portion.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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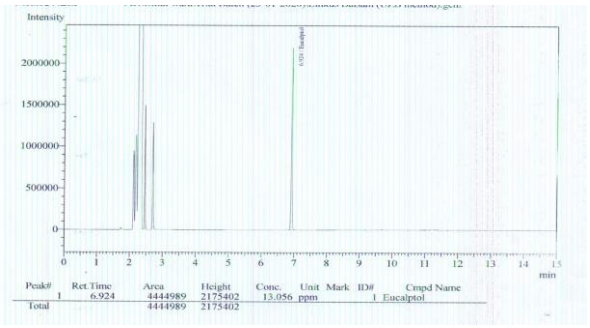
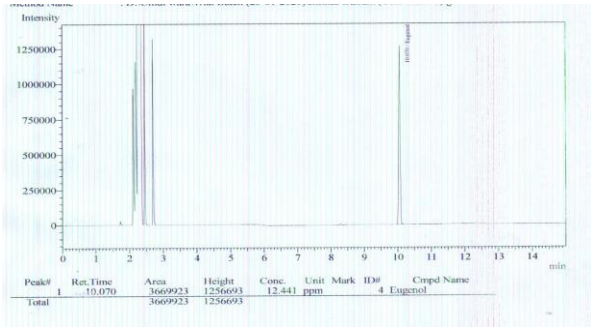
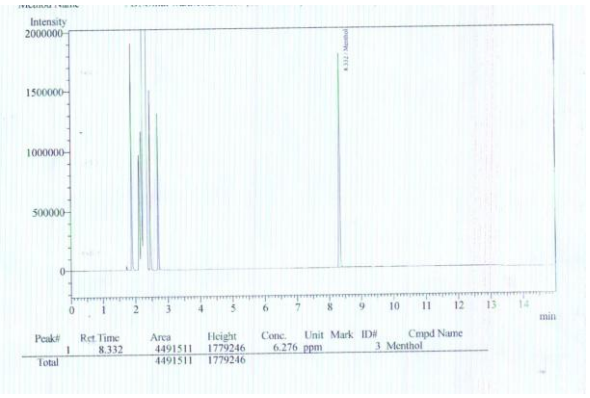
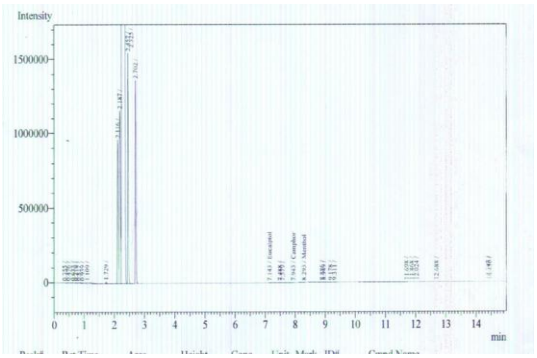
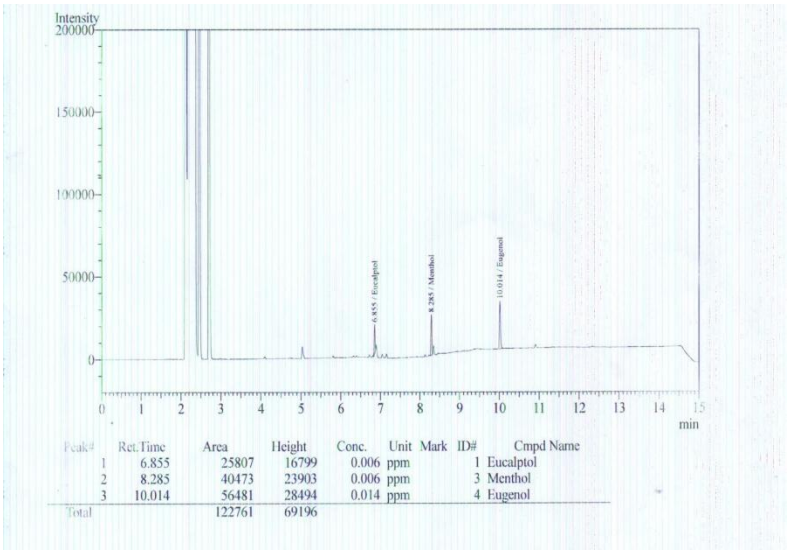


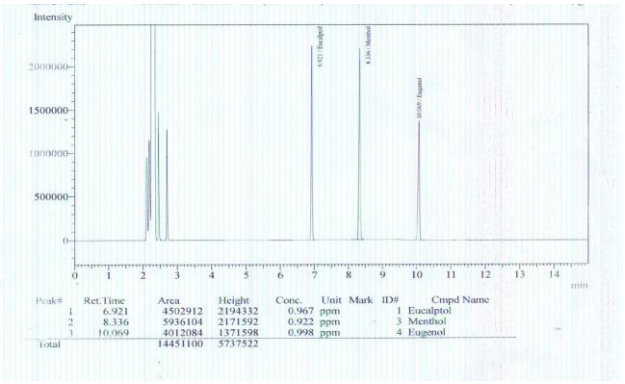




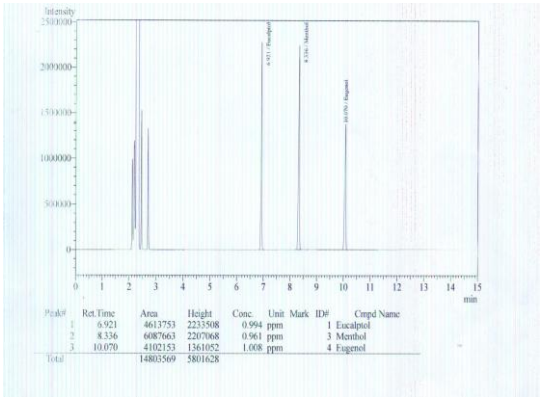




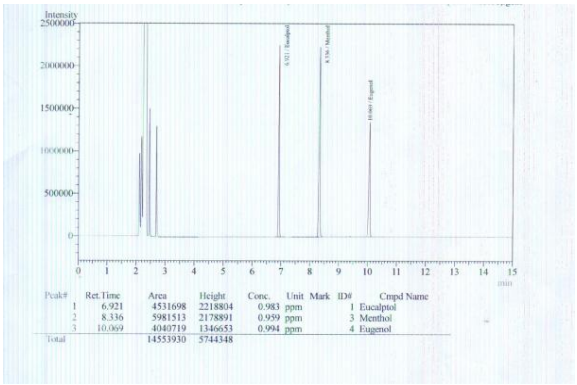
Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	6.855	25807	16799	0.006	ppm		1	Eucalptol
2	8.285	40473	23903	0.006	ppm		3	Menthol
3	10.014	56481	28494	0.014	ppm		4	Eugenol
Total		122761	69196					



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	6.921	4502912	2194332	0.967	ppm		1	Eucalptol
2	8.336	5936104	2171592	0.922	ppm		3	Menthol
3	10.069	4012084	1371598	0.998	ppm		4	Eugenol
Total		14451100	5737522					



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	6.921	4613753	2223508	0.994	ppm		1	Eucalptol
2	8.336	6087663	2207068	0.961	ppm		3	Menthol
3	10.070	4102153	1361052	1.008	ppm		4	Eugenol
Total		14803569	5801628					



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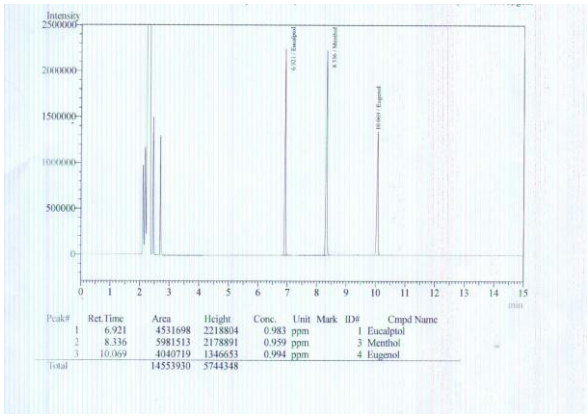
2	8.336	5981513	2178891	0.959	ppm		3	Menthol
3	10.069	4040719	1346653	0.994	ppm		4	Eugenol
Total		14553930	5744348					



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	6.921	4531698	2218804	0.983	ppm		1	Eucalptol
2	8.336	5981513	2178891	0.959	ppm		3	Menthol
3	10.069	4040719	1346653	0.994	ppm		4	Eugenol
Total		14553930	5744348					