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Original Research Article

Assessing the effectiveness of a herbal mouthwash against oral pathogens: *In vitro* analysis

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ABSTRACT

The most common infectious diseases caused by numerous viruses and bacteria include dental caries and periodontal diseases, which can develop at different stages of a person's life. Herbal mouthwash is highly promising because of its positive effects and works on oral infections and bacteria to quickly cure pain. In this study, a herbal mouthwash with a range of plant extracts was prepared. Medicinal plants like Azadirachta indica, Ocimum tenuiflorum, and Clincanthus nutans was used. Their usefulness in halting the growth of harmful bacterial species such Staphylococcus sp was evaluated. Clinacanthus nutans has strong antibacterial properties and non- toxic to cell lines. Phytochemical was carried out and characterization analysis such as FTIR and GC-MS analysis were performed to identify the function groups and important constituents. In vivo toxicity analysis was performed and the LC50 value for the three formulations A, B, C were found to be 16.0598 μg/ml,15.948 μg/ml,16.058 μg/ml respectively.The physical characteristics such as pH, colour, and stability, have been assessed. Antibacterial activity against an oral sample on blood agar showed good zone of inhibition for three different formulations. The formulation C showed maximum inhibition of 25mm for 100μl concentration of the sample.

1. Background

The definition of mouthwash is a liquid solution, typically antiseptic, used to clean the mouth, teeth, and breathe. For the prevention and treatment of a number of oral problems, mouthwash is frequently suggested in dentistry.[1] The usage of naturally occurring goods, also known as grandmother's remedy, has increased significantly in recent years. This has prompted the development of newer generations of mouthwashes, but this study examines whether they are on par with or even superior to the gold standard mouthwashes.[2] Mouthwashes are liquids with analgesic, anti-microbial, and anti-inflammatory properties. They come in two varieties: chemical mouthwash and herbal mouthwash.[3] Dental caries and periodontal disease are both significantly influenced by bacteria found in dental plaque, one of the primary opportunistic pathogens of dental caries is Streptococcus mutants.[4] The prevalence of dental caries is between 60-65% among Indians, its chemical makeup also makes it dangerous providing side effects. As a cure, herbal mouthwash was found.[5]

Natural plant extracts are the main component of herbal mouthwashes. Because they are non-irritating, non-staining, and free of alcohol, herbal mouthwash has gained popularity over chemical mouthwashes.[6] Nearly all chemical mouthwashes contain fluoride and alcohol, both of which are hazardous to the body in excess amounts. Therefore, the majority of herbal mouthwashes are a safe solution for diabetics, dry mouth sufferers, pregnant women, and kids.[7] This study was done to determine how efficient

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and safe the herbal mouthwash compared to the chemical one. In adddition to mouthwash type, this study also looked at mouthwash quantity to be used.[8] To develop a herbal mouthwash employing the herbs Azadirachta indica, Ocimum tenuiflorum and Clinacanthus nutans that would be more effective and environmentally friendly. Mechanical plaque control is less technically complex than chemical plaque reduction techniques, such as mouthwashes.[9] The leaves of four distinct plants, Azadirachta indica, Ocimum tenuiflorum and Clinacanthus nutans, were combined to create an antibacterial herbal mouthwash. The ancient dental hygiene methods in India have included chewing neem leaves after meals and brushing with neem twigs. Halitosis can also be successfully avoided with tulsi.

Using herbal mouthwash allows us to avoid potentially dangerous ingredients, which is a positive step toward better oral hygiene and general health. Hence Herbal mouthwashes are an alternative to chemical mouthwashes and offer additional advantages or benefits over commercial mouthwashes.[10] Due to its numerous medical virtues, Azadirachta indica also known as neem, has gained fame on a global scale recently. The Meliaceae family includes neem (Azadirachta indica), and its importance as a health-promoting agent is linked to its abundance in antioxidants.[11] In the treatment and prevention of many ailments, it has been utilized extensively worldwide in Chinese, Ayurvedic, and Unani treatments, especially in the Indian Subcontinent.[12] Neem and its contents are thought to have a part in the scavenging of free radical production and the prevention of disease pathogenesis, according to prior study.[13] Neem has become well-liked in modern medicine as a result of its vast use in Ayurveda, Unani, and homoeopathic treatments. The neem plant produces a wide range of physiologically active chemicals with a wide range of chemical make-up and complicated structural makeup. The various components of the neem plant contain more than 140 distinct chemicals.[14] Traditional medicine uses the leaves, blossoms, seeds, fruits, roots, and bark of the neem tree to treat inflammation, infections, fever, skin conditions, and dental problems.[15] Immunomodulatory, anti-inflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic, and anticarcinogenic properties. and anti-inflammatory properties of neem leaf and its components.[16]

The plant tulsi (Ocimum sanctum Linn.), which is a member of the genus Ocimum, is well-known for its many therapeutic benefits. Holy basil is the English translation of the Hindi word tulsi.[17] Tulsi is used as a plant in Indian homes for a variety of ailments and is regarded as sacred by the Hindu religion. The worlds tropical and semitropical regions are home to the bushy tulsi plant. It tastes different and has a distinctive aroma. It can reach heights of three to five feet. Ayurvedic medications are frequently made with tulsi leaves. The common cold, heart diseases, headaches, stomach issues, kidney stones, and many other conditions may benefit from the use of Tulsi extracts.[18] The Tulsi plant also offers defense against flies, insects, and mosquitoes. It could also aid in the battle against malarial fever. Also renowned for their potential medicinal value are tulsi leaves.[19]

The Clinacanthus nutans Lindau, a plant in the Acanthaceae family, is sometimes known as snake grass.[20] This plant is used in traditional herbal medicine in Malaysia, Indonesia, Thailand, and China to cure a number of diseases, including diabetes, gout, herpes simplex lesions, skin rashes, bug bites, and snake bites.[21] Studies on phytochemistry have shown that this plant contains a variety of bioactive substances, including flavonoids, glycosides, glycoglycerolipids, cerebrosides, and mono-acylmonogalatosyl glycerol.[22] The results of the pharmacological analysis revealed the presence of a wide variety of biological properties in several types of extracts and pure chemicals from this species, including anti-inflammatory, antiviral, antioxidant, and anti-diabetic actions.[23] The results of a toxicity research revealed that this plants extracts did not exhibit any toxicity, making them potent therapeutic agents for particular sick situations.[24] To fully understand the phytochemical profile and determine whether they are suitable for use in future medications, more research on chemical components and their modes of action that demonstrate biological activities is necessary.[25]

2. Materials and Methods

2.1. Collection and Extraction of Azadirachta indica, Ocimum tenuiflorum and Clinacanthus nutans

Azadirachta indica (Figure 1), Ocimum tenuiflorum (Figures 3 and 2), and Clinacanthus nutans's (Figure 3) dried leaves are gathered and roughly grinded into tiny fragments. To make these extracts, the dried coarse piece was extracted in hot aqueous[26] (Figures 4, 5 and 6). These extracts were purified using muslin cloth, and the filtrate was then run through filter paper before being sealed in a jar. After a day, filter paper were used to purify the aqueous extract.[27] To analyze the maximum zone of inhibition of this, used three distinct composition of the aqueous plant extract.[28]

2.2. Screening of oral pathogenic microbiota

The present study comprised of 5-6 students of age 20-22 and their oral samples were collected through sterile swab before brushing their teeth and streaked in Blood agar plates as it is the potential nutrient source for the growth of pathogenic microorganisms (Figure 7). Then the plate was incubated for 24 hours and screened for the microorganism present in it (Figure 8).[29]

Figure 1: Leaves of Azadirachta indica

Figure 2: Leaves of Ocimum tenuiflorum

Figure 3: Leaves of Clinacanthus nutans

Figure 4: Aqueous extraction of Azadirachta indica

Figure 5: Aqueous extraction of Ocimum tenuiflorum

Figure 6: Aqueous extraction of Clinacanthus nutans

Figure 7: Blood agar

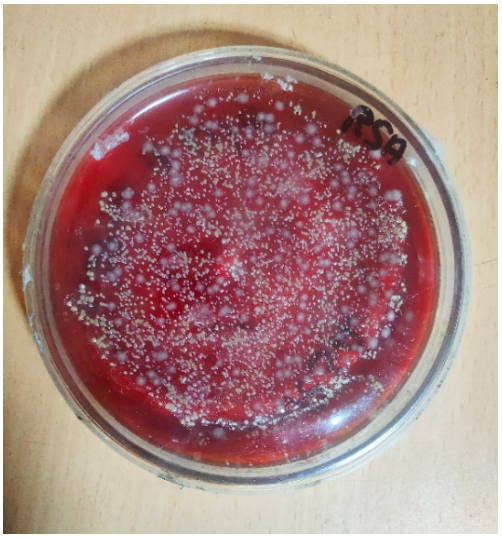

Figure 8: Cultured microorganisms in blood agar

Figure 9: Sub-cultured microorganism

2.3. Identification of microbiota and construction of phylogenetic tree:

After amplifying it in PCR, Gene sequencing studies were performed through which the phylogenetic tree has been constructed using maximum likelihood method (Figure 9).[30]

2.4. Characteristic analysis

2.4.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis for Azaridichta indica, Ocimum tenuiflorum, Clinacanthus nutans Fourier transform infrared spectroscopy is often referred to as FTIR analysis or FTIR spectroscopy. It is possible to detect organic, polymeric, and occasionally inorganic functional groups using the analytical method known as FTIR analysis.[31] Infrared light is used in this technique to scan test materials and examine chemical characteristics. The plant sample was fed into an FTIR spectroscope (Shimadzu, IRAffinity1, Japan), which has a scan range of 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1}.[32]

2.4.2. Gas chromatography - Mass Spectrometry (GC-MS)

The Plant extract was subjected to a GC-MS analysis utilising Thermo GCTrace Ultra Version: 5.0, Thermo MS DSQ II. The apparatus includes a non-polar DB 35 - MS Capillary Standard column with the film's 30 mm x 0.25 mm ID x 0.25 m measurements. Helium is employed as the carrier gas, with a modest flow rate of 1.0 ml/min.[7] The oven temperature was programmed as follows, with the injector running at 250 °C: 15 minutes at 60 °C, followed by

a 3-minute climb to 280 °C. Willey and NIST libraries, as well as a comparison of their retention indices, were used to identify the components. After comparison with those found in the computer library (NIST and Willey) connected to the GC-MS instrument, the constituents were determined.[33]

2.5. Formulation Of Herbal Mouthwash

The Figure 3 was used to formulate the herbal mouth with three different formulation such as Formulation A, Formulation B, Formulation C respectively.

2.6. Evaluation of Herbal-Mouthwash

2.6.1. Hue and smell

Physical features, such as colour and fragrance, were evaluated through visual inspection.[34]

2.6.2. PH test

With the aid of a digital pH meter, the pH of prepared herbal mouthwash was determined. A standard buffer was used to calibrate the pH meter. One milliliter of mouthwash was weighed, diluted in fifty milliliters of purified water, and its pH was measured.[35]

2.6.3. Check for microbial development in Mouthwash formulations

The prepared bio mouthwash was infected in the agar medium plates using the streak plate method, while a control was made. The incubator was filled with the plates, where they would remain for 24 hours at 37°C. Plates were taken out of the incubation time and compared to the control to look for signs of microbial growth.[17]

2.6.4. Test for stability

The stability of the final product is evaluated based on the formulation. In Accelerated stability tests, the product is subjected to heating at high degree in accordance with ICH requirements, are a standardized approach for estimating the stability of any product[36] For a period of three months, an accelerated short-term stability assessment of the produced formulation was conducted. The samples were kept at 3-50 C, 250 C with a 60% relative humidity, and 400 C with a 2% relative humidity. Finally, a monthly sample that had been maintained under rapid study was taken out and examined.[37]

2.6.5. In vitro Anti-bacterial activity

Anti-bacterial activity against the oral species has been studied using the herbal mouth wash prepared. Since, Blood agar is the potential nutrient source for various bacterial organisms hence, it is been prepared in our lab using the conventional protocol.[38] Live samples have been taken from the normal people, streaked and incubated for 24 hours. Up on studying gene sequence it was found out to be pathogenic species Staphylococcus aureus. Minimum zone of inhibition was studied for various formulations made were investigated[39] & Table.2.In millimetres, the zone of inhibition was measured[40] Refer Table 7.

2.7. Cytotoxicity Activity

2.7.1. Hatching of brine shrimp

A cylindrical container was filled with 1 liter of distilled water, 38g of Sodium Chloride, and was thoroughly swirled. To ensure proper aeration the air pump was inserted at base of the tank. A 60-100Watt light bulb was set a few inches away from the container. At the top of the containers water level, 10g of brine shrimp eggs were introduced and stirred. Nauplius hatched after a 20–24-hour incubation period. The hatchling nauplii were taken from the barren egg by turning off the lamp and the air supply. By doing this, the abandoned eggs are certain to be hovering on top and the brine shrimp are gathered at the water's surface. To improve visibility in the clear freshwater, the nauplii were taken out of the fish tank using a pipette and filtered.[41]

2.7.2. Microscopic evaluation of brine shrimp

On a clean slide, two brine shrimp eggs were inoculated with two drops of distilled water. 40X and 10X magnifications of a light microscope were used to observe them. Shrimps possessed a single, 22mm- long eye and an unsegmented body.[42]

2.7.3. Brine shrimp lethality assay

The shrimp larvae were used for the experimental bioassay 48 hours after hatching. 100ml of distilled water was placed in each of the four beakers. Pipetting 50 nauplii into each beaker. The beakers received 10ml of each of the three extract compositions, and the fourth beaker was held for control as in the previous hatchability. The dead shrimp larvae were counted using a stereomicroscope after five hours. By deducting the number of dead larvae after five hours from the total number of dead larvae in each beaker, the number of living larvae was calculated.[43]

2.7.4. Mortality rate

The difference between the mean survival of larvae in extract-treated tubes and control tubes was used to assess lethality. The logarithm of concentrations was plotted against the mean percentage mortality. The linear equation's antilogarithm was used to compute the concentration (LC50) that would kill 50% of the larvae. The positive control utilized was potassium dichromate.[44]

Mortality (%) is calculated as follows: (Number of dead nauplii /Total A. nauplii) * 100

3. Result and Discussion

3.1. Collection and extraction of leaves

The leaves of Azadirachta indica, Ocimum tenuiflorum and Clinacanthus nutans's were dried and the aqueous extract of each sample was subjected to further characterization study.

3.2. Screening and identification of oral microbes

The oral sample collected from students were screened for the presence of microbiota and the screened oral microbe was identified by 16S rRNA sequencing and the strain was identified as Staphylococcus aureus strain LN871053. The phylogenetic tree disclosed that the Staphylococcus aureus was similar to Staphylococcus family (Figure 10).

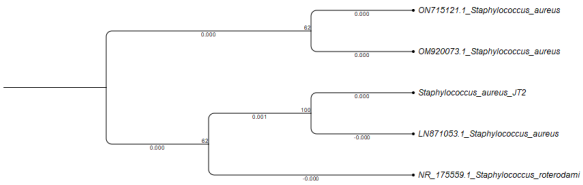

Figure 10: Phylogenetic tree

3.3. Characterization of plant extracts

3.3.1. FT-IR

Due to the fine structure of the Azadirachta indica sample used in the study (Figure 11), it was observed from all these vibration modes of frequency that the bands of the fine powders used in the current study were shifted to longer wavelength region or shorter energy region in comparison to the corresponding bands of leaf extract(Table 1).

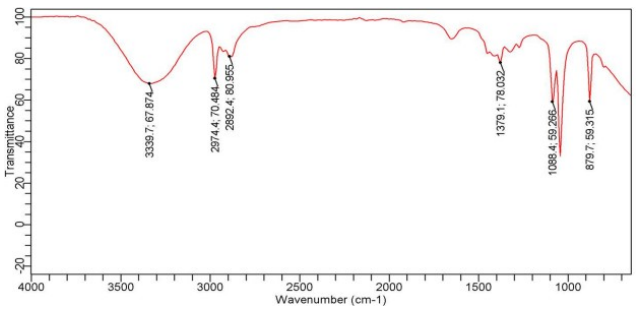

Figure 11: FTIR analysis of Azadirachta indica

In Ocimum tenuiflorum the broad natured band (Figure 12) may have resulted from the overlapping of the O-H and N-H stretching modes of vibration of alcohol/water present in the carotenoid and amide present in all plant leaves (confirmed later with other modes) (Table 2).

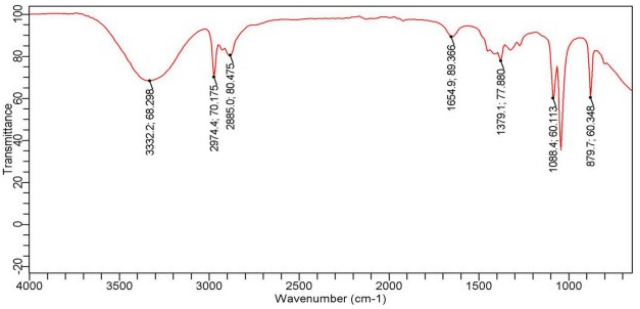

Figure 12: FTIR analysis of Ocimum tenuiflorum

By contrasting the bonds (functional groups) present within the sample, the Fourier Transform Infrared Spectrophotometer (FTIR) can identify flavonoid (Figure 13). Due to their ability to decrease NF-B production, flavonoids have a significant role in the healing of wounds. According to the literature review, Clinacanthus nutans possess antioxidant qualities that may help wounds heal more quickly(Table 3).

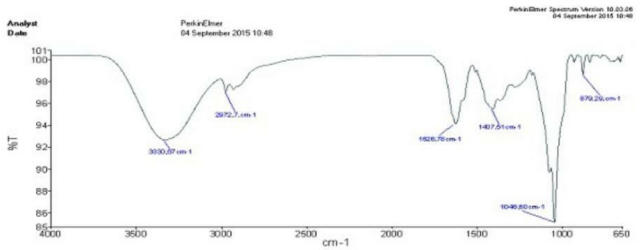

Figure 13: FTIR analysis of Clinacanthus nutans

3.3.2. GC-MS analysis

Five significant peaks were visible in the GC-MS chromatogram of the aqueous extract of Azadirachta indica (Figure 14) indicating the presence of five phytocomponents. These peaks were recognized after comparing the mass spectra with the NIST library (Table 4). According to the findings, the main constituents of the extract were N-Hexadecanoic acid (also known as palmitic acid), Tridecanoic acid (also known as tridecylic acid), 3, 7, 11, 15, tetramethyl-2-hexadecen-1-ol (also known as phytol), 9, 12, 15, octadecatrienoic acid (also known as linolenic acid or -Linolenic acid), lists the phytochemicals that contribute to the therapeutic properties of plant leaves. Phytol is said to have antioxidant, antiallergic, antinociceptive, and anti-inflammatory properties. Phytol is a superior immunostimulant, according to recent studies; In terms of inducing long-term memory and activating both innate and acquired immunity, is superior to a number of commercial adjuvants.[45] Phytol has also demonstrated antibacterial efficacy against Staphylococcus aureus and

Table 1: Functional groups inAzadirachta indica

Absorption	Appearance	Group	Compound class	Comments
3339.7;67.874	Strong, broad	O-H stretching	Alcohol	Intermolecular bonded
2974.4;70.484	Strong, broad	N-H stretching	Amine salt	Intermolecular bonded
2892.4;80.955	Strong, broad	N-H stretching	Amine salt	Intermolecular bonded
1379.1;78.032	Medium	O-H stretching	Carboxylic acid	Gem dimethyl
1088.4;59.266	Strong	C-O stretching	Secondary alcohol	Hydrate
879.7;59.315	Strong	C-H bending	1,3- disubstituted	

Table 2: Functional groups in Ocimum tenuiflorum

Absorption	Appearance	Group	Compound class	Comments
3332.2;68.298	Strong, broad	O-H stretching	Alcohol	Intermolecular bonded
2974.4;70.175	Strong, broad	N-H stretching	Alkyne	
2885.0;80.475	Medium	C-H stretching	Alkane	
1654.9;89.366	Strong	C=O stretching	Tertiary amide	Free
1379.1;77.880	Medium	O-H bending	Phenol	
879.7;60.348	Strong	C=C bending	Alkene	disubstituted

Table 3: Functional groups inClinacanthus nutans

Absorption	Appearance	Group	Compound class	Comments
3930.07	Medium, sharp	O-H stretching	Alcohol	Free
2072.7	Strong	N=C=S stretching	Isothiocyanate	
1628.78	Weak	C-H bending	Aromatic compound	Overtone
1407.51	Medium	C-H bending	Alkane	Methyl group
1045.50	Strong, broad	CO-O-CO stretching	Anhydride	
879.29	Strong	C=C bending	Alkene	vinylidene

Mycobacterium tuberculosis. It is well known that linolenic acid has potential antibacterial, antifungal, anti-arthritic, and anti-inflammatory properties. Due to its anti-inflammatory and anti-cancer properties, as well as its application in the treatment of rheumatoid arthritis, homo-linolenic acid has gained importance.[46]

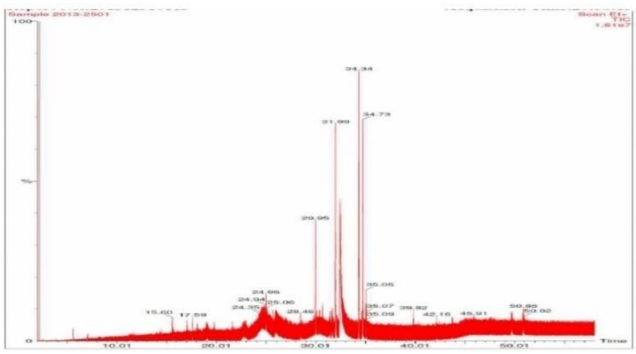

Figure 14: GC-MS analysis of Azadirachta indica

After comparing the mass spectra with the NIST library, the GC-MS chromatogram of the aqueous extract of Ocimum tenuiflorum revealed three main peaks (Figure 15) confirming the existence of three phytocomponents. According to the findings, Benzene, 1, 2-dimethoxy-4- (2- propenyl) - (also known as Methyl-Isoeugenol), Isocaryophyllene (also known as Caryophyllene), and Eugenol (also known as 2-Methoxy-4-(2-propenyl) phenol) were the extract's main constituents.(Table 5) contains a list of the phytochemicals that help the plant leaves' therapeutic qualities. The properties of methyl-isoeugenol include antifungal, nematicidal, and antifeedant action. Caryophyllene is widely known for its cytotoxic, antifungal, and anti-inflammatory properties 13–18. According to reports, eugenol has anti-insect, anti-cancer, anti-candida, anti-desinfection, anti-parasitic, and anti-mycotic properties.[47]

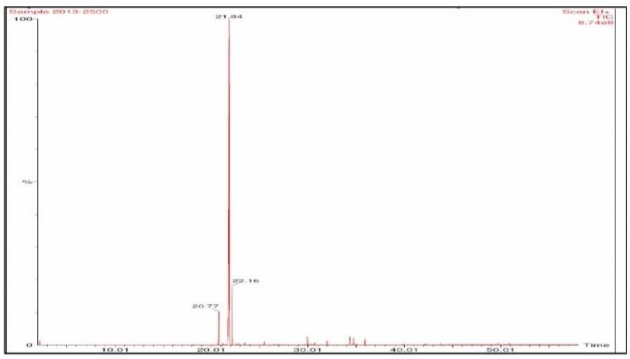

Figure 15: GC-MS analysis of Ocimum tenuiflorum

Table 4: Compounds inAzadirachta indica

Retention time	Name of the compounds	Molecular formula	Molecular weight	Activity
34.34	3,7,11,15- tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.53	Cancer-preventive Antimicrobial , Anti-inflammatory, Anti-diuretic ,Antioxidant
34.73	9,12,15- Octadecatrienoic acid	$C_{13}H_{20}O_2$	278.4296	Anti-bacterial, Anti-fungal
34.73	8,11,14-Eicosatrienoic acid	$C_{20}H_{34}O_2$	306.482	Astringent Anti-inflammatory Anticoagulant
31.99	N-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	Anti-oxidant, Nematicide, Hemolytic, Antiandrogenic
31.99	Tridecanoic acid	C12H28O2	214.344	No activity reported

Table 5: Compounds in Ocimum tenuiflorum

Retention time	Name of the compounds	Molecular formula	Molecular weight	Activity
21.84	Benzene,1,2-diemthoxy-4-(1-propenyl)	$C_{11}H_{14}O_2$	178.2	Anti bacterial, Nematicide, Insect-attractant , perfumery, Flavour
22.16	Caryophyllene	$C_{15}H_{24}$	204.3	Anti-bacterial, Anti-tumor, Analgesic, Anti-inflammatory, fungicide
20.77	Eugenol	$C_{10}H_{12}O_2$	164.2	Acaricide, Anti bacterial, Anti-inflammatory, Antioxidant, Cancer-preventive, Antispasmodic, Antiviral, Insecticide

High flavonoid concentration was found in the aqueous extract of Clinacanthus nutans leaves after GC-MS analysis (Figure 16). The chemicals were recognized by comparison to the NIST library's matching compound, which had a similarity index of at least 80%. Comparing the detected chemicals' biological potential to earlier published findings allowed for this determination. The 28 phytochemical components found in this investigation are listed, with N-(4-methoxyphenyl)-2hydroxyimino-acetamide (4.72%) having the biggest relative peak area.[48] However, as the fatty acid class made up 20.29% of the overall peak area, the detected phytochemicals primarily belonged to this group (Table 6).

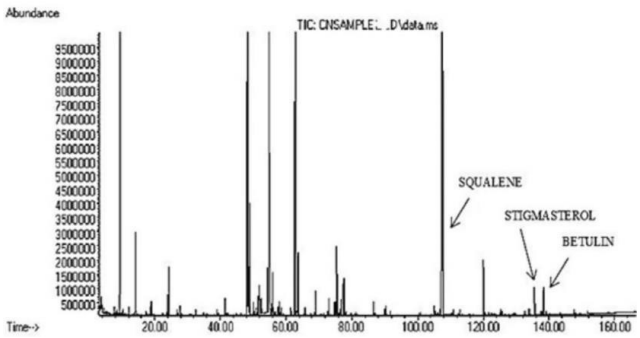

Figure 16: GC-MS analysis of Clinacanthus nutans

3.4. Formulation of herbal mouthwash

Based on the characteristic study of the plant extract, the herbal mouth wash was formulated in three distinct concentrations based on the (Table 7) (Figure 17).

3.5. Evaluation of Physical Parameters of Mouth Wash

3.5.1. PH test

The formulation's pH was discovered to be 6.6. The formulation's pH range is appropriate for oral diseases because the skin has an acidic pH of roughly 5.5.[49] Heavy metals were discovered to be absent from the formulation. The lack of microbial development after being inoculated in the agar medium proved that the formulation was devoid of microorganisms.[50]

3.5.2. Test for stability

Any pharmaceutical product's formulation and preparation are deficient without sufficient stability assessments of the manufactured product. This is carried out in order to assess the prepared product's physical and chemical stability and, consequently, its safety. Accelerated stability tests, in which the product is heated up in accordance with ICH rules, are a universal technique for forecasting the stability of any product. For a period of three months, an accelerated short-term stability assessment of the produced

Table 6: Compounds inClinacanthus nutans

Retention time	Name of the compounds	Molecular weight	Activity
4.453	Cyclohexane, isocyanato-	125.084	Anti bacterial, ,
5.078	Sulfuric acid, dimethyl ester	126.132	Anti-bacterial,
7.473	Naphthalene, 1,2-dihydro-1,1,6-trimethyl-	172.125	Acaricide, Antioxidant, ,
8.16	Cyclodecane	140.27	Nematicide,
8.479	Butylated hydroxytoluene	220.183	Anti bacterial,
9.285	2-propenoic acid, 3-(3-hydroxyphenyl)-, methyl ester	178.063	Anti-inflammatory
9.389	1-Tridecene	182.203	Anti bacterial, ,
9.84	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	180.079	Cancer-preventive
10.722	Pentadecanoic acid, 14-methyl-, methyl ester	270.256	Insecticide
10.895	n-hexadecanoic acid	256.24	Cancer-preventive
11.215	Heptadecanoic acid, methyl ester	284.272	Anti-tumor,
11.291	Triphenylmethane	244.125	Cancer-preventive
11.499	1,3-dicyclohexylurea	224.35	, Anti-inflammatory
11.569	9,12-octadecadienoic acid, methyl ester	294.256	Analgesic, Anti-inflammatory, fungicide
11.603	9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-	292.24	, Anti-inflammatory
11.652	Phytol	296.308	Antispasmodic, Antiviral,
11.694	Octadecanoic acid, methyl ester	298.287	Insecticide
11.777	7,10,13-hexadecatrienoic acid, methyl ester	264.4	Anti-tumor
11.853	Octadecanoic acid	284.484	Anti viral
12.048	1-methyl-10,18-bisnorabieta-8,11,13-triene	256.219	Cancer preventice
13.714	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	278.152	Cancer-preventive
13.915	Hexadecane	226.44	Insecticide
14.436	N-(4-methoxyphenyl)-2-hydroxyimino-acetamide	194.069	Anti bacterial
14.589	9,12-octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	354.277	Cancer preventive
14.658	Nonanoic acid, 9-(3-hexenylidenecyclopropylidene)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, (Z,Z,Z)-cont.	352.261	Analgesic, Anti-inflammatory, fungicide, Anti viral
15.248	13-Docosenamide, (Z)-	337.334	Anti-inflammatory
19.476	Vitamin E	430.381	Antispasmodic, Antiviral,
21.94	Stigmasterol	412.702	Anti inflammatory

Table 7: ormulation of herbal mouthwash

Formulations	Azadirachta indica Extract (ml)	Ocimum tenuiflorum Extract (ml)	Clinacanthus nutans Extract (ml)
A	50	25	25
B	25	50	25
C	25	25	50

Figure 17: Formulation of herbal mouthwash

formulation was conducted. The samples were stored at under the following conditions of temperature at 3-5°C, 25°C RH=60%, 40°C±2°C RH=75% (Table 8). The samples were withdrawn at regular interval monthly and the stability found to be good. The preceding study showed that formulated mouthwash exhibited better stability[51] (Figure 18).

Figure 18: Stability test of herbal mouthwash

The samples were stored at under the following conditions of 3.6 *In Vitro* Antibacterial Activity.

The anti-bacterial activity was estimated by disc diffusion method for formulations A, B, C at a concentration of 100ul. The zone of inhibition for Staphylococcus aureus were found to be 7mm, 16mm and 25mm respectively (Figure 19). The prepared formulations were contrasted against the chemical mouthwash primary ingredient Chlorohexidine and the zone of inhibition were found to be 22mm (Figure 9).The results showed that the formulated mouthwash has promising activity against the bacteria and the formulation C exhibited maximum inhibition against the oral bacteria (Figure 20).[52] However, herbal mouthwashes containing medicinal ingredients like antimicrobials may be useful for some long-term odor control.[8]

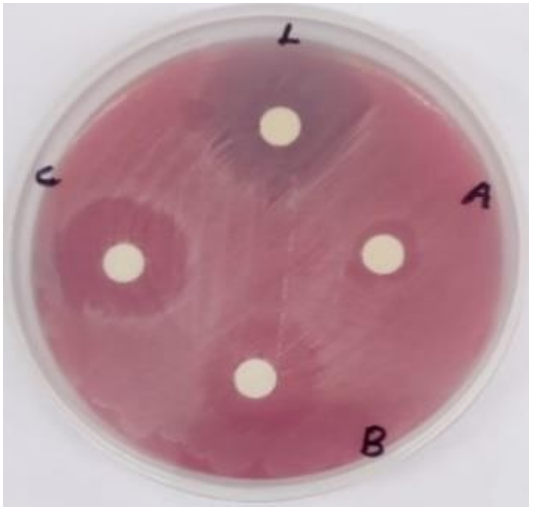

Figure 19: Anti-bacterial activity

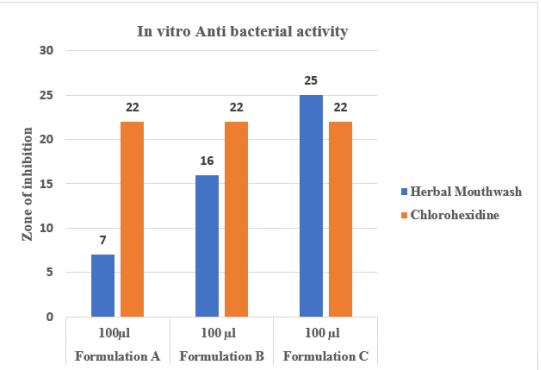

Figure 20: Anti-bacterial activity of the formulations

3.6. Toxicity Test Using Brine Shrimp:

According to the regression equation, the LC_{50} value for mortality rate in treated brime shrimp with formulations A, B, and C extracts from Azadirachta indica, Ocimum tenuilforum, and Clinacanthus nutans was determined to be (R^2= 0.5604), 16.0598 μg/ml, (R^2= 0.6366), 15.948 μg/ml and (R^2=0.7854), 16.058 μg/ml respectively. When compared to the reference potassium dichromate (LC_{50} = 16.403 μg/ml), the value was substantially higher(Fig.21,22,23).[53] The percentage of mortality value was observed to increase with an increase in concentration for all the plant extracts tested and the standard, as

Table 8: Stability studies of Herbal Mouthwash

Temperature	Evaluation Criterion	Observation (Month)				
		0	1	2	3	4
3-5°C	Visual Aspects	Dark green	Dark green	Dark green	Dark green	Dark green
	Separation of Phases	None	None	None	None	None
	Uniformity	Good	Good	Good	Good	Good
	Odour	Stable	Stable	Stable	Stable	Stable
	pH	6.5	6.5	6.6	6.6	6.6
Room Temperature (25°C RH=60%)	Visual Aspects	Dark green	Dark green	Dark green	Dark green	Dark green
	Separation of Phases	Nil	Nil	Nil	Nil	Nil
	Uniformity	Good	Good	Good	Good	Good
	Odour	Stable	Stable	Stable	Stable	Stable
	pH	6.5	6.5	6.6	6.6	6.6
40°C±2°C RH=75%	Visual Aspects	Dark Green	Dark green	Dark green	Dark green	Dark green
	Separation of Phases	Nil	Nil	Nil	Nil	Nil
	Uniformity	Good	Good	Good	Good	Good
	Odour	Olive green	Olive green	Olive green	Olive green	Olive green
	pH	6.5	6.5	6.5	6.5	6.5

Table 9: Anti-bacterial activity of the formulations

Organism	Zone of inhibition (mm)		
Staphylococcus aureus	Formulation A 100μl	Formulation B 100 μl	Formulation C 100 μl
	7	16	25
Chlorhexidine	22	22	2.2

Table 10: Toxicity test usingArtemia nauplii

S.No.	Concentration (μg/ml)	% Death of nauplii		
		A	B	C
1	10	13	15	12
2	12	15	13	10
3	14	12	11	8
4	16	10	18	20
5	18	25	22	29
6	20	30	22	32

demonstrated by Tukey's test, and there was also a significant association between the concentration of the extracts utilized and this value(Table 10).[44]

4. Conclusion

The present liquid herbal mouthwash is highly effective in aiding individuals in eliminating bad breath and other oral health concerns. Additionally, the preparation is devoid of unhealthy ingredients. The results of the physicochemical analysis indicate that the present herbal formula has an acceptable color and aroma, with a pleasant scent and superior post-effects. Furthermore, the zone of inhibition results corroborated that this herbal mouthwash is a potent plaque inhibitor. Patients preferred it for its taste, ease of use, and mouthwash duration after rinsing. It can be

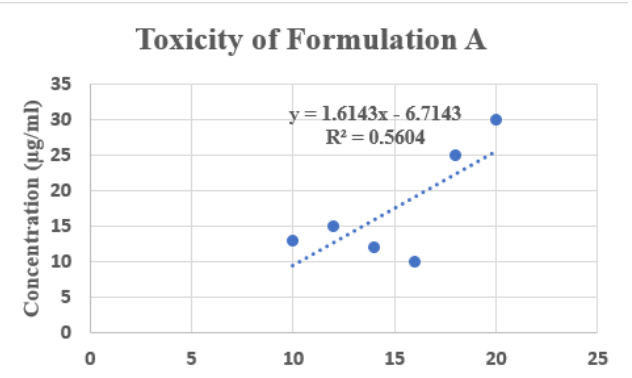

Figure 21: Toxicity of formulation A

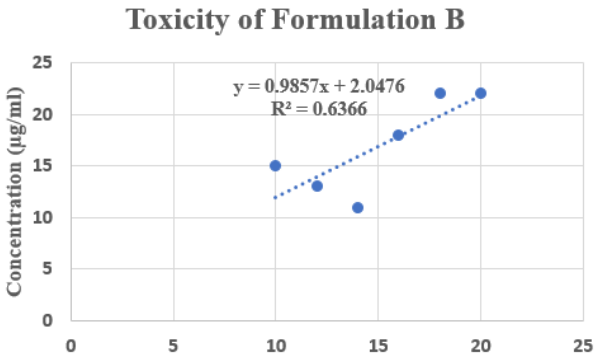

Figure 22: Toxicity of formulation B

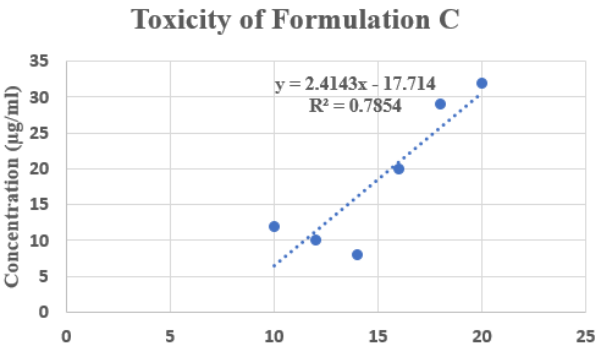

Figure 23: Toxicity of formulation C

utilized as a supplement to mechanical therapy in the treatment of plaque-induced gingivitis. The current study has a significant impact on efforts to develop an affordable and efficient herbal oral health intervention for low socio-economic populations. However, since this study was brief, more extensive studies with larger sample sizes are necessary. The natural herbs utilized in this composition have demonstrated medicinal benefits in treating oral hygiene issues and bad breath. Several studies have demonstrated the successful use of these plants throughout history. This herbal mouthwash simplifies mouth rinsing and prevents a variety of oral health problems.

5. Source of Funding

No funding has been received for the study.

6. Conflicts of Interest

The authors state that they are aware of no personal or financial conflicts that would have appeared to have an effect on the research reported in this study.

7. Data Availability

Data used in this study is available with the authors and the same will be shared on request.

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