"Comparison of Effectiveness of Moringa oleifera Leaves Extract Gel (2%) With Retino A (0.1%) Cream for Treatment of Oral Leukoplakia: Double Blinded Randomized Control Trial"

By

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Dissertation

Submitted to KLE Academy of Higher Education and Research (KAHER), Belagavi
In Partial Fulfillment of the Requirements for the Degree Of

MASTER OF DENTAL SURGERY

In

ORAL MEDICINE AND RADIOLOGY

(BRANCH - 01-)

Under the Guidance of

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2019 -2022

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DR. J.B Prasad.
Name & Signature of Biostatistician

Place: Belagavi
Date: 31/12/21

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ACKNOWLEDGEMENT

It's a really happy movement to achieve such a level when you feel overwhelmed due to the presence of that particular time, in which you are dwelling. Here is such a situation to recollect and thank all the people around me for completion of my research project.

I would like to take this opportunity to express my profound gratitude and regards towards the most fun-loving, friendly, smart, great mentor and my guide ***Dr.Shivayogi Charantimath*** *sir, the most loving, caring and head of the department* ***Dr.Vaishali Keluskar*** *ma'am, the most intelligent, smart and great mentor* ***Dr.Vasanti Jirge*** *ma'am, the respectable top gun of the department* ***Dr.Anjana Bagewadi*** *ma'am, the most fun-loving and smart* ***Dr.Arvind Shetti*** *sir, the calmest and composed* ***Dr.Zameera Naik*** *ma'am, and the most knowledgeable* ***Dr.Kajal Gokak*** *ma'am, the sweetest of all* ***Dr.Daneshwari Koshti*** *ma'am, the caring and forensic expert* ***Dr.Namrata Patil*** *ma'am for their exemplary guidance, valuable feedback and constant encouragement throughout the duration of the project. Your valuable suggestions were really helpful throughout the research work. Thank you all!!!!!!!!*

I would also like to extend my thank you to ***Dr.Sagar Mahadev Nitturkar****, M.B.B.S M.D and* ***Dr.Anuradha Patil****, professor and head of the department of biochemistry JNMC for their guidance in phytochemical analysis. I would like to thank* ***Dr.Angula Amritha*** *M.B.B.S, post graduate student, and* ***Dr.Manjula Vagarali****, professor and head from the department microbiology JNMC for guidance and help in the antimicrobial assay. I would also like to thank* ***Dr.Mahendra Kumar Chauhan*** *research associate, from KLE's Dr. Prabhakar Kore's basic science research center for his guidance in the preparation of mucoadhesive gel. Thank you,* ***Dr.Rithia C***

Uppin ***M.D.S*** ***Ph.D*****,** *KLE's Dr.Prabhakar Kore's basic science research center for guidance and assistance in MTT assay. I am thankful to* ***Dr. J. B Prasad*** *Professor, Department of epidemiology and biostatistics for helping and guiding me in statistical analysis.*

I would also like to thank ***Mr.Somaling Patil****, for helping in procuring M. oleifera leaves and* ***Mr.Manoj Allimatti*** *and* ***Mr.Vinay Mirajkar*** *and* ***Mr.Panduranga Pattar*** *for helping in drying the MO leaves.*

My thankful gratitude to my colleagues ***Dr.Lokesh Kumar S*** *and* ***Dr.Arun Panwar*** *for making the academic journey lively. I would like to thank my juniors* ***Dr.Jayraj B Malik, Dr.Anabelle Fernandes, Dr.Sridhar M*** *for their extended support of my research project, thank you for informing me about oral leukoplakia patients in OPD. Thank you,* ***Dr.Jayraj Malik****, for helping in dispensing gel in laminate tubes.*

I am highly obliged to ***Dr.(Mrs.) Alka D. Kale****,* *M.D.S, Principal, KLE VK Institute of dental sciences, Belagavi for providing necessary facilities and helping me in carrying out this project.*

Last but not least is my family. I would like to thank my Late. Mother ***Mrs. Aqeela Ansari*** *you are my inspiration and my strength, whatever I'll ever achieve in my life will be because of you. I would like to thank my father* ***Mr.Riyaz Ahmed Ansari*** *for his constant support. I would to thank my dear husband* ***Mr.Aiman Khan*** *for his unconditional support and assistance in the completion of this research project. I would to extend my thankful gratitude towards my aunt* ***Mrs.Zeenat Maqsood Ansari*** *and my dear sisters* ***Nashrah Ansari*** *and* ***Iqra Ansari****. The support of my family cannot be described in words.*

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ABBREVATATIONS

MO, M. oleifera	Moringa oleifera
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	(2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid))
VLDL	Very low-density lipoprotein
LDL	Low density lipoprotein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
TEA	Triethanolamine
DMSO	Dimethyl sulfoxide
MIC	Minimum inhibitory concentration

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INTRODUCTION

Potentially malignant disorders, commonly known as precancerous lesions of the oral mucosa, are a cluster of diseases that should be recognized at the initial stage. The most prevalent oral mucosal diseases with a high malignant transformation rate are oral leukoplakia, oral erythroplakia, and oral submucous fibrosis[1].

Leukoplakia is the terminology used for a precancerous non-scrapable white lesion and World Health Organization (WHO) describes leukoplakia as ‘‘a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”[2].

Over the past thirty years, the epidemiologic data from several countries shows that the prevalence of oral leukoplakia ranges between 1.1-11.7 %, with a mean value of 2.9 percent[3]. In Banoczy’s study, 87% of individuals with leukoplakia were having the habit of smoking tobacco, & 77% of those who developed malignancy smoked tobacco[4]. According to Gupta et al, the association of tobacco with leukoplakia ranged from 47% -73% among villagers living in India found in 10-year follow-up[5].

Tobacco is the main etiological factor for oral leukoplakia. Smoking habits in India have a varying relationship with locally established mixed tobacco habits, such as smoking, chewing, and a combination of the two (chewing betel quid and bidi smoking)[3]. Tobacco products contain many carcinogens; nitrosamines are the most carcinogenic agents. These carcinogenic agents were found to be abundant in the saliva of smokers as well as non-smokers and are linked with oral leukoplakia[6].

The conservative treatment employs local and systemic chemo-preventive agents such as beta carotene, vitamin A, systemic lycopene, and topical bleomycin (a chemotherapeutic agent) with a partial effectiveness[7]. Anti-oxidants such as Beta carotene, Retinol, Retinoids, ascorbic acid, Alpha-tocopherol, and Vitamin A are commonly used in the management of oral leukoplakia, commonly used topical preparation is Retino-A cream available commercially[8]. Among Anti-oxidants, the administration of retinoic acid and beta-carotene has some efficacy to resolve oral leukoplakia[9]. Vitamin A/tretinoin is essential for the normal pathway of epithelial cell differentiation. The use of antioxidants, such as vitamin A, reduces free radicals thus reducing oxidative stress and preventing cellular changes in oral leukoplakia[10].

Moringa oleifera is a tropical shrub native to India, which is also known as the 'drumstick tree'.[11]Moringa oleifera leaves demonstrate potent antioxidant activity[12]. A study on moringa oleifera reported that it provides 7 times more vitamin C than oranges, 10 times more vitamin A than carrots[11].

Moringa oleifera has an abundance of bio-flavonoids and which have antioxidants, anti-inflammatory, anti-cancer, and anti-diabetic properties[13]. M. oleifera is proved to have higher free radical scavenging activity evaluated by two methods DPPH and ABTS assays[14]. The extract obtained from leaves of M. oleifera proved to demonstrate anti-cancer properties causing apoptosis in breast and colorectal cell lines[15]. Moringa oleifera leaf extract is a potential oral anticancer drug contender, which induces apoptosis in cell lines of human hepatocellular carcinoma[16].

Thus, this study intended to evaluate the efficacy of Moringa oleifera mucoadhesive gel (2%) in the treatment of oral leukoplakia.

AIM AND OBJECTIVES

Aim:

The study aims to evaluate and compare the efficacy of Moringa oleifera leaves extract gel (2%) & Retino A cream (0.1%) in the reduction of the size of the lesion in oral leukoplakia.

Objectives:

1. To assess the efficacy of Moringa oleifera mucoadhesive gel (2%) in reducing the size of lesion among oral leukoplakia patients.
2. To assess the efficacy of Retino-A cream (0.1%) in reducing the size of the lesion among oral leukoplakia patients.
3. To compare the efficacy of Moringa oleifera mucoadhesive gel (2%) and Retino-A cream (0.1%) in reducing the size of the lesion among oral leukoplakia patients.

REVIEW OF LITERATURE

Leukoplakia is derived from the Greek words Leucos, which means "white," and plakia, which means "plate." Schwimmer created the name leukoplakia in 1877, and it was first used in 1978"[17].

DEFINITIONS:

WHO (2005): "A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer "[18].

Etiological factors:

Leukoplakia has a multifactorial etiology but the most important factors are tobacco and alcohol, separately and synergistically[7].

Tobacco:

Various forms of tobacco i.e., chewing, smoking, smelling cause adverse effects on the oral cavity and result in life-threatening diseases such as oral malignancies and leukoplakia. Individuals who smoke are more likely to suffer from oral leukoplakia than non-smokers[19].

Tobacco usage in various forms is common in India, and approximately between 47 percent to 73 percent of the population engaged in the habit, which is rather high when compared to the United States, where approximately 36 percent of the population engages in the habit.[20].

Smokeless tobacco contains a combination of 4,000 chemical carcinogens, including 30 or more that are linked to cancer. These chemicals consist of heavy metals such as cadmium, lead, and nickel; as well as arsenic, a chemical used in

pesticides; formaldehyde, which is used in preserving fluid; and N-Nitrosonornicotine (NNN), among others[21].

Irritation and the heat from the combustion of tobacco in smoking form cause reaction with the oral tissues which results in leukoplakia[22].

Chronic irritation:

Carious teeth having sharp cusps, sharp edges of the prosthesis, lingual bars, and other dental appliances; habitual cheek and lip biting and hot and spicy foods are etiological factors in the cases of oral leukoplakia[22].

Alcohol:

Alcohol has a synergistic effect with tobacco in the etiology of oral leukoplakia. There is an increased risk of oral leukoplakia among drinkers as it causes the dehydrating effect on the oral mucosa. Among all alcoholic beverages, spirit drinking has been linked to an elevated risk of leukoplakia[23].

Nutritional deficiencies:

Vitamin A, vitamin B complex, zinc deficiencies have been linked to the development of oral leukoplakia. Studies have suggested that the patients who developed oral leukoplakia have low blood levels of these vitamins & trace elements[24].

Age:

Oral leukoplakia usually occurs in the 5th decade of life. The mean age of occurrence is 56.93 years [25].

Gender predilection:

PC Gupta et al reported that oral leukoplakia is more prevalent in males as compared to females[26].

Classification of leukoplakia:]

1. Depending upon clinical appearance[7]

a) Homogenous leukoplakia

b) Non-homogenous leukoplakia –

i. Speckled

ii. Nodular

iii. Verrucous

2. Depending upon the etiology[27],[28]

a) Tobacco-associated leukoplakia

b) Candidal leukoplakia (chronic hyperplastic candidiasis)

c) Hairy leukoplakia

d) Syphilitic leukoplakia

e) Sanguinaria-associated leukoplakia

3. According to Banoczy J et al (1982)[29]

a) **Leukoplakia Simplex -** A slightly elevated white homogeneous keratinized lesion.

b) **Leukoplakia verrucosa** - A wrinkled white verrucous lesion.

c) **Leukoplakia erosiva** - Erythematous, erosive, and fissured white lesion.

4. According to Axell et al (1984)[30]

a) **Idiopathic** – unclear etiology

b) **Tobacco-related leukoplakia -** This type of leukoplakia is caused by tobacco.

5. Clinical appearance -International symposium held in Sweden (1994)[29]

a) **Homogenous** – Primarily white, with a cracked/wrinkled/smooth surface but a uniform texture throughout.

b) **Non-homogenous Leukoplakia** –

i. **Erythroleukoplakias** – This is a red and white lesion.

ii. **Nodular** – This is a red and white lesion. Slightly raised, rounded, red, and/or white excrescences.

iii. **Exophytic** – a growth that is irregular, blunt, or sharp.

MORINGA OLEIFERA

Introduction

Moringa oleifera, a member of the Moringaceae family, is often known as the "drumstick tree" or "horseradish tree." Moringaceae is found in India, Afghanistan, Bangladesh, and Pakistan's sub-Himalayan highlands. Moringa oleifera is a miraculous tree that is commonly planted in India. Moringa's seeds, leaves, and pods are packed with nutrients due to the presence of different phytochemicals. Moringa is thought to have seven times the vitamin C of oranges, fifteen times the potassium of bananas, ten times the vitamin A of carrots, seventeen times the calcium of milk, nine times the protein of yogurt, and twenty-five times the iron of spinach[31].

Moringa oleifera is known for its hypolipidemic, antioxidant, anti-inflammatory, immunomodulatory, anti-diabetic, hepatoprotective, anti-hypertensive, and anti-cancer properties[31]. Phytochemical analysis of different parts of Moringa oleifera has shown that the leaves contain a higher amount of nutrients, antioxidants and minerals, and bioflavonoids than other parts of the tree[32]. Leaves of MO are rich in essential amino acids such as cysteine, methionine, tryptophan, and lysine[33].

Chemical composition[34]

Carbohydrates	9.1 g
Dietary fiber	2.1 g
Fat	1.7 g
Protein	8.1g

Table-1: Nutritional value per 100 g (3.5 oz) of Moringa oleifera leaves extract [34].

Vitamin A equiv.	80 μg
Thiamine (B1)	0.103 mg
Riboflavin (B2)	0.112 mg
Niacin (B3)	1.5 mg
Pantothenic acid (B5)	0.48 mg
Vitamin B6	0.129 mg
Folate (B9)	41 μg
Vitamin C	8.6mg

Table-2: Vitamin's content of Moringa oleifera L. all vitamins in 10%[34].

Calcium	99.1 mg
Iron	1.3 mg
Magnesium	35.1 mg
Manganese	0.119 mg
Phosphorus	70.8 mg
Potassium	471 mg
Sodium	70 mg
Zinc	0.85 mg

Table-3: Mineral's content of Moringa oleifera L. trace metals all in 10% [34].

Threonine	36.77
Valine	22.1
Methionine	2.13
Leucine	20.50
Isoleucine	31.8
Phenylalanine	36.8
Histidine	30.88
Lysine	27.67
Arginine	21.45

Table-4: Essential amino acids content of Moringa oleifera L. (ug/ml) [34]

Properties of Moringa oleifera leave extract:

1) Antioxidant property

Moringa oleifera is rich in anti-cancer agents and bioflavonoids. It prevents the damaging impacts of free radicals in the human body. The ethanolic concentrate of leaves showed the best scavenging activity to DPPH radical. Free radical scavenging activity with IC50 value of 49.30μg/mL in DPPH assay and 11.73μg/mL in ABTS assay[35]. **Quercetin-**3-O-d-glucoside is detected in dried Moringa oleifera leaves with quantities of 100 mg/100 g. Quercetin is a powerful antioxidant with a wide range of medicinal applications. It has hypolipidemic, hypotensive, and anti-diabetic actions in obese Zucker rats with metabolic syndrome.[31]

The ethanolic concentrate of leaves showed the best scavenging activity to DPPH radical. The antioxidant properties of the M. oleifera plant provide evidence to use as many parts of this plant to be employed in the human diet as natural antioxidants[36]. In-vitro antioxidant activity of Moringa oleifera ranges from 22.36% to 89.35%. Moringa oleifera leaves exhibit potent scavenging activity for superoxide anion radicals. Moringa oleifera leaves extract showed protective activity against DNA damage in an in-vitro study on Male Sprague–Dawley rats[37].

2) Anti-cancer property

Moringa oleifera has a potent cancer activity. Each part of the tree bears anti-cancer property including barks, roots, seeds, and leaves however leaves have been studied extensively for exhibiting anti-cancer properties[38]. Moringa leaf extracts on the human B-lymphocyte plasmacytoma cell line showed anti-cancer activity, can inhibit the propagation of Multiple myeloma[39]. Another study tested the anti-cancer

activity of Moringa leaf on cervical cancer cell lines showed anti-cancer properties in higher concentrations[40].

3) Anti-diabetic property

Moringa oleifera has a potent anti-diabetic property. The antioxidants present in the leaf extract of *M. oleifera* enhance the antioxidant defense mechanism at the cellular level thus protecting the pancreas against reactive oxygen species damage and having an antihyperglycemic effect[41].

4) Anti-hypertensive property

There are several studies on *M.* oleifera regarding the anti-hypertensive property. Thiocarbamate glycosides were formerly isolated from leaves of *MO* and identified as anti- hypertensive agents. Mice treated with crude methanol and ethyl acetate extracts of M. oleifera showed a substantial reduction in systolic blood pressure in hypertension-induced mice, according to an in vivo investigation.[42].

5) Hypolipidemic property

Hypolipidemic property Moringa oleifera is supposed to lower the serum cholesterol, triacylglyceride, VLDL, LDL, and atherogenic index. Moringa oleifera was found to increase the excretion of fecal cholesterol in male albino Wister rats[43]. An in-vivo study on high-fat diet-induced obese rats demonstrated that Moringa oleifera causes a reduction in total cholesterol, triglycerides, and LDL level along with a significant reduction in body weight[44].

6) Anti-inflammatory property

Several studies suggest that Moringa oleifera leaf is a potent anti-inflammatory agent, reduces signs and symptoms of inflammation. The MO leaves are a potent natural source of antioxidants and anti-inflammatory agents, and capable of developing into the health-enhancing dietary supplement[45]. Amit et al. (2017) conducted in-vivo research on Albino Wister rats with edema and granuloma who were given an aqueous extract of Moringa oleifera and found that the signs and symptoms of inflammation were significantly reduced.[46].

7) Hepatoprotective property

Moringa oleifera leaf extract has been found to have hepatoprotective qualities in numerous in vitro and in vivo experiments. An in-vivo study on Carbon tetrachloride-induced Hepato-Toxicity in albino rats reported M. oleifera has a hepatoprotective effect; it is due to the presence of Quercetin and kaempferol[47]. In another study *M. oleifera* was tested in paracetamol-induced hepatoxicity in rats, *M. oleifera* was equally effective as a standard hepato-protective agent i.e., silymarin[48].

METHODOLOGY

SOURCE OF DATA:

This in-vivo study will be conducted in:

1. Patient reporting to the Department of Oral Medicine and Radiology, KLE VK Institute of dental sciences, KLE Academy of Higher Education & Research Belagavi.

STUDY DESIGN:

It is a double-blind randomized control trial.

STUDY PERIOD: Two years from December 2019 to December 2021

METHOD OF DATA COLLECTION:

Clinically diagnosed cases of Oral Leukoplakia were included in this study. The sample size is 72. Using a stratified randomized sample procedure, 72 individuals with oral leukoplakia were evenly divided into case and control groups. Using a chit method, 36 patients with lesion sizes ranging from 2 to 4 cm and 36 patients with lesion sizes ranging from 4.1 to 6 cm were randomly assigned to the case and control groups. So, case and control both groups had 36 patients with an equal number of size range lesion.

1. **Case group** (Moringa oleifera leaves extract group): Subjects were treated with Moringa oleifera leaves extract mucoadhesive gel.
2. **Control group** (Retino-A 0.1% cream): Subjects were treated with Retino-A 0.1% cream.

SELECTION CRITERIA:

INCLUSION CRITERIA:

- Homogenous leukoplakia of size range 2-6 cm in greatest dimension.
- Patients above 18 years.
- Patients who are willing to participate in this study.

EXCLUSION CRITERIA:

- Non-homogenous leukoplakia.
- Leukoplakia with a maximum dimension of more than 6 cm.
- Leukoplakia involving the tongue and floor of the oral cavity.
- Patients who are being treated for any other potentially malignant diseases except oral leukoplakia.
- Pregnant and lactating females.

MATERIALS:

- Informed consent
- Case history Proforma
- Patient information sheet

ARMAMENTARIUM:

- Dental Vernier caliper
- Mouth mirror
- Probe
- Tweezer
- Kidney tray

- Cheek retractor
- Patient apron
- Gloves
- Mouth mask
- Moringa oleifera leaves mucoadhesive gel (2%)
- Retino-A (0.1%) cream

CLINICAL TRIAL REGISTRATION:

This RCT is registered in Clinical trial registry India (CTRI) with registration no: CTRI/2020/10/028529.

METHODOLOGY

Clinically diagnosed cases of Oral Leukoplakia were included in this study. The sample size is 72. Seventy-two patients were equally divided into case and control groups using a stratified randomized sampling method. 36 patients with lesion sizes ranging from 2- 4 cm and 36 patients with lesion sizes ranging from 4.1 – 6 cm were equally distributed in the case and control group using the chit system. So, case and control both groups had 36 patients with an equal number of size range lesions. The intervention was dispensed in sterile laminate tubes (Figure-1).

The participants in the case and control group were advised topical application of the intervention and Retino-A (Topical vitamin-A/tretenoin) respectively thrice-daily using a sterile cotton bud.

The participants were advised not to eat or drink anything for at least half an hour after the application of topical preparations.

CLINICAL ESTIMATION OF LESION:

In the present study, the response of the treatment was assessed by quantitative unidimensional measurement using dental vernier caliper and measurement of the longest dimension of the lesion was done pre and post-treatment in both case and control groups.

This study was double a blind randomized controlled trial the interventions were dispensed by 1st observer and the size of the lesion was measured by 2nd observer who was unaware of the interventions prescribed to the patients.

The measurement was done at baseline and after 1, 2, 3 months of the therapy. The pre and post-treatment measurements were compared and statistical analysis was done for the final assessment.

PREPARATION OF THE MUCOADHESIVE GEL

Leaves of M. oleifera were procured from Kakati, Belagavi (altitude-752 meters above sea level, humidity-13%, temperature- 34^{o}C) (Figure-2). Leaves were washed with distilled water and shade dried for 4-5 days (Figure-3). Dried leaves were coarsely powdered in the grinder. Extraction of leaves was done by maceration procedure using a hydroalcoholic method using 70:30 ethanol: distilled water (Figure-4). According to an in-vitro investigation, extracting dried leaves with 70% ethanol produced an extract with the highest production of flavonoids and total phenolic components[49], thus this method was used for extraction. Leaves were kept in a flask dipped in 70% aqueous ethanolic solution for 72 hours in a shaker at 75 rpm & room temperature and extracted repeatedly (Figure 5).

Whatman's filter paper no.42 was used to filter the solution (Figure-6). The obtained filtrate was transferred to a crucible and dried over a water bath at 40^{o}C (Figure-7). The obtained Dry content obtained was weighed to the weighing machine. The extract obtained was stored at 2-8^{o}C temperature. (Figure-8).

100 grams of leaves

↓

Washed & shade dried for 4-5 days

↓

Grounded into coarse powder

↓

Extraction using hydroalcoholic method (70:30 ethanol: water)

↓

Filtration using Whatman's filter paper no.42

↓

Drying in evaporator at 40 ^{o}C

↓

% Yield obtained

↓

Stored at 2-8 ^{o}C

DETERMINATION OF % YIELD:

Blank weight of tube=3.67 g

Sample with extract =14.97 g

Yield= 11.3 g

$$\% \text{ yield} = \frac{\text{X grams of extract}}{\text{100 grams of plant}} \times 100$$

$$= \frac{11.3}{100} \times 100$$

$$= 11.3\%$$

PHYTOCHEMICAL TESTS[50,51] :

The phytochemical tests were carried out in the department of Biochemistry J.N.M.C Belagavi.

Table 5: **Phytochemical tests of the M. oleifera extract:**

Phytochemical	Test	Procedure and Observations	Results
1) Alkaloids	1) Mayer's test (Figure-9)	2-3 ml filtrate + few drops of Mayer's reagent- Appearance of cream-colored precipitate	**+ve**
2) Phenolic compounds	1) H2SO4 test	Extract + Conc.H2SO4- Formation of orange color	**+ve**
3) Detection of flavonoids	1) Aqueous sodium hydroxide test (Figure-10)	Extract + aqueous NaOH – Yellow orange color	**+ve**
	2) H2SO4 test (Figure-11)	Extract + conc.H2SO4- Formation of orange color	**+ve**
4) Saponins	1) Foam test (Figure-12)	Extract + water + Sodium bicarbonate vigorously shaken – Honeycomb like froth	**+ve**
5) Test for sugars	1) Benedict's test (Figure-13)	Extract + Benedict's solution - heated in boiling water bath- Formation of red precipitate	**+ve**
6) Terpenoids	(Figure-14)	5 ml of extract + 2 ml of chloroform + slow addition of H2SO4 -formation of reddish-brown color layer at the junction.	**+ve**
7) Steroids	1) Salwoski test	Extract + chloroform + few drops of conc.H2SO4- Appearance of red color chloroform layer	**-ve**

8) Tannins	1) Maxson + Rooney Method (Figure-15)	1 ml of extract + 2ml of FeCl- dark green color	**+ve**
9) Anthocyanosides		1ml of extract + 5 ml of dilute HCL-Pale pink color	**-ve**
10) Cyanogenic glucoside	1) Sodium picrate test	0.5g extract + 10 ml of sterile water+ sodium picrate-heat- appearance of brick color	**-ve**
11) Cardiac glucoside	1) Killer-kiliani Test (Figure-16)	0.5 g of extract + 2 ml acetic anhydrate + H2SO4- reddish brown layer-turns bluish green	**+ve**
	2) Baljet test (Figure-17)	Extract dip into sodium picrate solution- appearance of yellow orange color	**+ve**
12) Protein	1) Ninhydrin Solution test (Figure-18)	3ml of extract + 3 drops of 5% Ninhydrin solution heated in boiling water bath for 10 mins- appearance of purple or bluish color	**+ve**
	2) Biuret test (Figure-19)	extract + 3ml of NaOH + few drops of 1% CuSo4-appearance of violet or pink color	**+ve**
13) Coumarin glycoside	1)Ferric chloride test (Figure-20)	Extract + few drops of FeCl3 solution appearance of dark green color- turned to yellow after some time on addition of conc HNO3	**+ve**

+ve- Present, -ve- Absent

Table 6 :SUMMARY OF QUALITATIVE ANALYSIS OF M.OLEIFERA

Alkaloids	**+ve**
Phenols	**+ve**
Flavonoids	**+ve**
Saponins	**+ve**
Sugars	**+ve**
Terpenoids	**+ve**
Steroids	**-ve**
Tannins	**+ve**
Proteins	**+ve**
Cardiac glucoside	**+ve**
Coumarin glucoside	**+ve**

+ve- Present, -ve- Absent

Test for flavonoids: Flavonoids were extracted from about 10 grams of M. oleifera leaves using 100 ml of 80 percent aqueous methanol at room temperature. Whatman filter paper No.42 was used to filter the entire solution. The filtrate was then transferred to a crucible and evaporated until dry over a water bath before being measured with a weighing machine. Flavonoids in the amount of 4.2 grams were obtained[52].

Table 7 :Composition of Moringa oleifera mucoadhesive gel (2%) [53]

Ingredients	For 100 ml of gel
1) Carbopol 940	1300mg
2) Distilled water	100
3) M. oleifera leaves extract	2000mg
4) TEA(Triethanolamine)	qs
5) Methyl paraben	18 mg
6) Ethyl paraben	2mg

The mucoadhesive gel was prepared in laminar air flow under sterile conditions(Figure-21). The laminar airflow was UV irradiated for half an hour before the preparation of gel. Carbopol 940 was used as a gelling agent.2% of gel was prepared by adding 2gm of extract / 100 ml of distilled water.TEA was used to enhance the gelling property. Methyl and ethyl paraben were used as apreservatives.The obtained gel (Figure-22) was stored in a sterile condition below 8^{o} C of temperature. The composition of the gel is given above in table-3.

TESTS FOR PHYSICAL PROPERTIES OF GEL[53]:

1) pH Determination in gel formulation

The pH was measured with a pH meter, which was standardized with buffer solutions at pH 7 and 4 before each use. One gram of gel formulation was precisely measured and dispersed in ten milliliters of distilled water. The electrode was put into the solution 10 minutes before the readings were taken to ensure that the readings were taken at room temperature. Each formulation's pH was measured three times, and average values were calculated. pH measurements were taken 48 hours, 1 week, 2 weeks, 1 month, 3 months, and 6 months following preparation to see if there was any pH fluctuation over time. After 48 hours, the pH was 6.7 + 0.3 (Mean + SD).

Centrifugal test

The gel was relocated into a tube and placed in the centrifuge machine 48 hours later to investigate the stability of the gel preparation to withstand the centrifugal force. The gel was centrifuged at 2000 rpm for 60 minutes and the stability of the preparation was evaluated at the time intervals of 5, 15, 30, and 60 minutes. After this test, the gel was stable.

Thermal test

Three gel samples were placed at 4°C, 25°C, and 45°C 48 hours after preparation to examine the stability of the gel formulation in varied weather conditions. Gel compositions were tested for 24 hours, one week, 2 weeks, 4 weeks, 12 weeks, and 24 weeks.

Freezing and thawing test

To test the formulation's stability in extremely cold temperatures, a 10 grams gel was placed at –8℃ for 48 hours after preparation, followed by 48 hours at 25℃ for six time periods. After that, the gel compositions' stability was assessed.

Cooling and heating test

48 hours after preparation, 10 gel was placed at 45℃ for 48 hours and then 48 hours at 4℃ for six time periods to evaluate the stability of the gel formulation against extreme temperature changes. The gel formulation's stability was next assessed.

Determination of viscosity

The prepared mucoadhesive gel's viscosity was determined using a viscometer at 100 rpm and spindle number 7 at 25°C. Before the measurements, the gel sample was allowed to settle for 30 minutes at room temperature. On the formulation, the test was repeated three times. The gel's viscosity was 5210 + 50 (Mean + SD).

ANTIMICROBIAL ASSAY[54]**:**

The agar-well diffusion method was used to test the gel's antibacterial capabilities. Using a sterile inoculating loop, old broth cultures of the test organisms were streaked over a sterile Cation activated Mueller Hinton agar in Petri dishes for 24 hours. To form wells on the agar plate, a sterile cork borer with a diameter of 6 mm was employed. 0.5 ml of varying concentrations of gel were poured into the perforations. Each well was given different codes to reflect a different concentration.

Minimum inhibition concentration(MIC) determination:

The minimal inhibitory concentration must be determined (MIC) following the antimicrobial test, the minimum inhibitory concentration against bacteria was established. The microbial isolates were cultivated on Meuller Hinton agar, which was done by well diffusion method. After streaking the bacterial strain to be tested, a sterile cork borer with a 6 mm diameter was used to produce wells on the agar plate. These plates were left at room temperature for an hour before being incubated at 37° C. After 24 hours, the results were interpreted.

Table 8: Antibacterial effect of Moringa oleifera gel (2%) on Staphylococcus aureus (Figure-23)

Moringa oleifera gel	**0.1g/ml**	**0.2g/ml**	**0.4g/ml**	**0.6g/ml**
Zone of inhibition(mm)	8.0	10.0	12.0	14.0

Table 9: Antibacterial effect of Moringa oleifera gel (2%) on Psuedomonas (Figure-24)

Moringa oleifera gel	**0.1g/ml**	**0.2g/ml**	**0.4g/ml**	**0.6g/ml**
Zone of inhibition(mm)	9.0	4.0	3.0	3.0

Table 10 : Antibacterial effect of Moringa oleifera gel (2%) on Candida species(Figure-25)

Moringa oleifera gel	**0.1g/ml**	**0.2g/ml**	**0.4g/ml**	**0.6g/ml**
Zone of inhibition(mm)	12.0	8.0	7.0	5.0

Cell viability assay of M.oleifera gel(2%)[55,56] **:**

For evaluating cell viability of MO mucoadhesive gel MTT assay was employed. For this assay mouse fibroblast cell line(L929) was employed. The reaction of live cells' mitochondrial dehydrogenase enzymes with the tetrazolium rings of a soluble Dimethyl sulfoxide (DMSO) are used to dissolve crystals. The color of the solution represents the quantity of surviving cells, which is proportional to the amount of formazan. A multi-well scanning spectrophotometer is used to measure the color.4 µl of cell suspension was seeded in a microplate and incubated for 24 hours. Later M. oleifera gel (10 µl) was added and DMSO 10% was used as a negative control and ELISA microplate was incubated for 48 hours. A spectrophotometer was used to check the vitality of the cells 48 hours later. Each well was filled with MTT solution and incubated for 3 hours to measure cell survival. Then, after gently replacing 150 µl of MTT medium with DMSO and pipetting to remove formazan crystals that had formed, the absorbance at 540 nm was measured using an ELISA plate reader (Figure 26). The procedure was repeated 3 times. The results of the MTT assay are given below. (Table-7)

Table 11 : Result of MTT assay

Concentration	Optical Density	Mean	% Cell viability
	0.455		
	0.339		
Negative control	0.31	0.368	100
	0.5		
	0.331		
M. oleifera gel (2%)	0.421	0.377	102.4457

PHOTOGRAPHY

Figure 1: Sterile laminate tubes for gel

Figure 2: Fresh M. oleifera leaves procured.

Figure 3: M. oleifera leaves kept for drying for 4-5 days

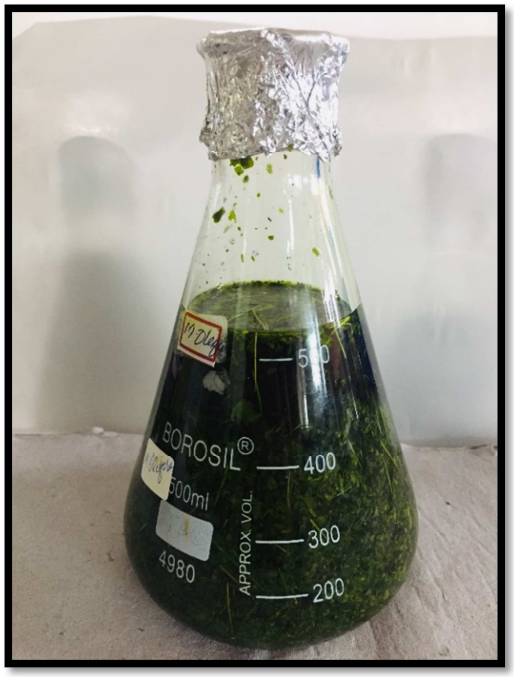


Figure 4: Leaves kept for extraction in 70% ethanol

Figure 5: Dried leaves placed with 70% ethanol in shaker for 72 hours at 75 rpm

Figure 6: Filtration of extract using Whatman's filter paper 42

Figure 7: Extract kept over water bath for evaporation

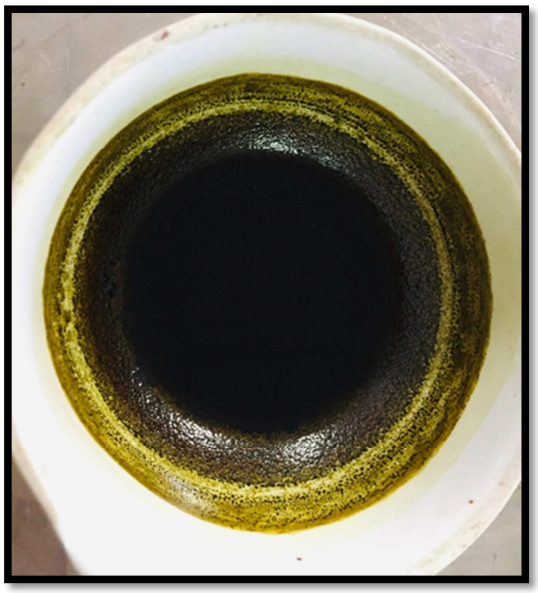


Figure 8: M. oleifera extract obtained.

Figure 9: Mayer's test – Cream colored precipitate seen +ve for alkaloids

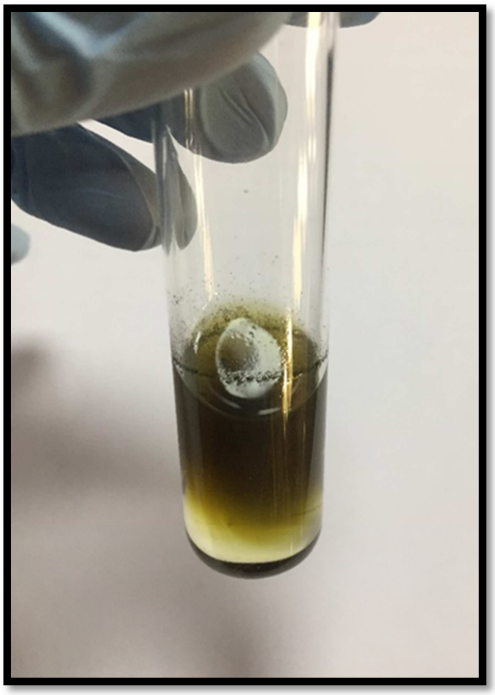


Figure 10: Aqueous sodium hydroxide Test- Yellow orange colored precipitate at bottom +ve for flavonoids

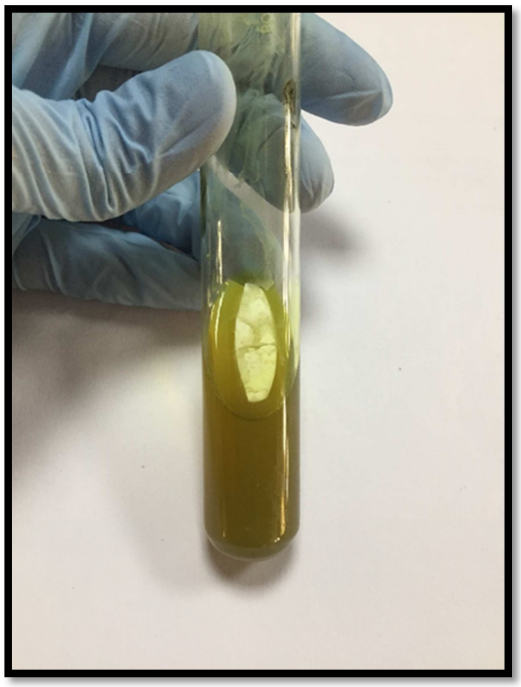


Figure 11: H2SO4 Test- Orange color formation +ve for flavonoids

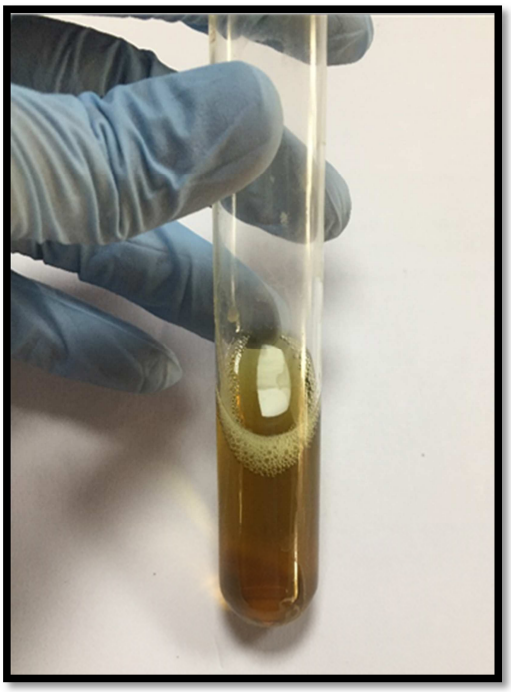


Figure 12: Foam Test-Frothing seen +ve for saponins

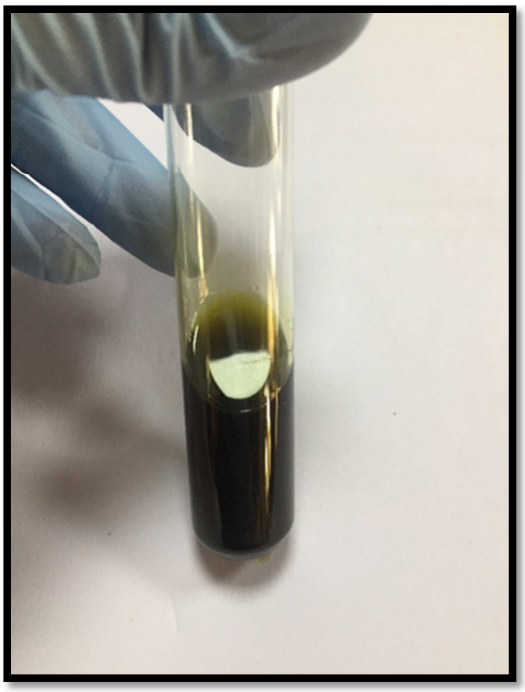


Figure 13: Benedict's test- red precipitate at bottom +ve for sugars

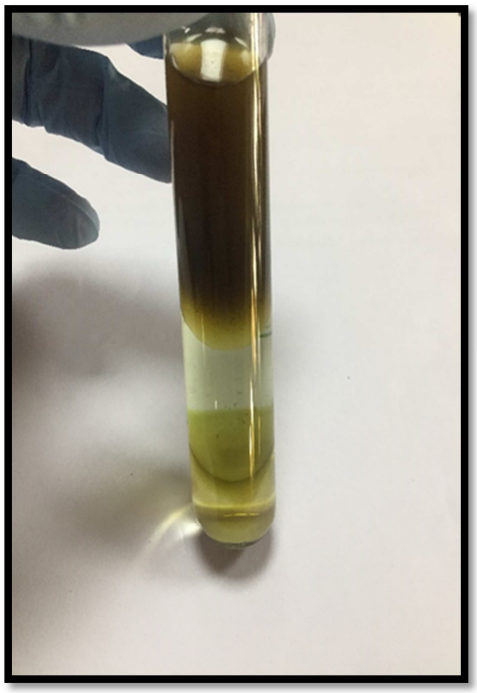


Figure 14: Formation of reddish-brown ring at the junction +ve for terpenoids

Figure 15: Maxson & Rooney test- Dark green color +ve for tannins

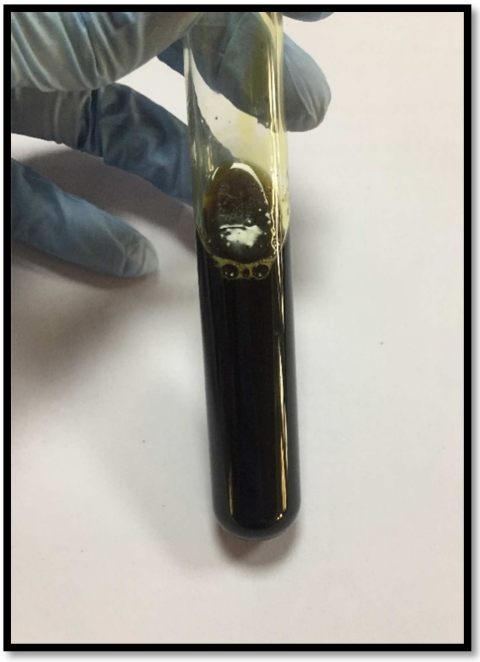


Figure 16: Killer kiliani test- Bluish green color +ve for cardiac glucoside

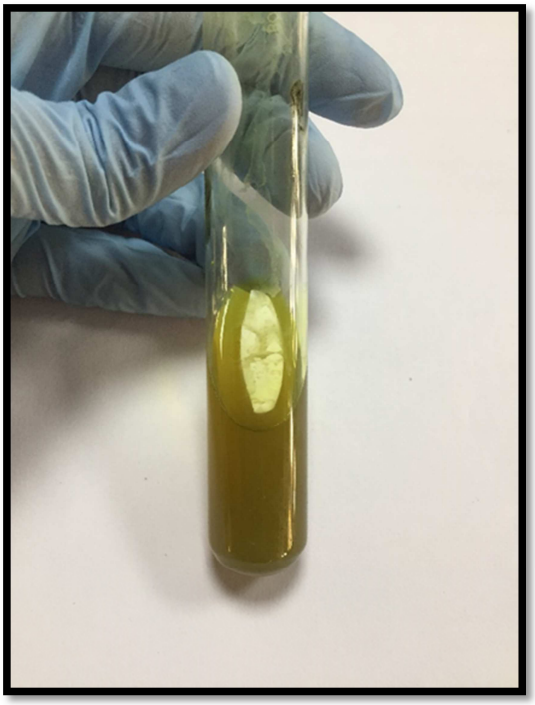


Figure 17: Baljet test- Appearance of yellow orange color +ve for Cardiac glucoside

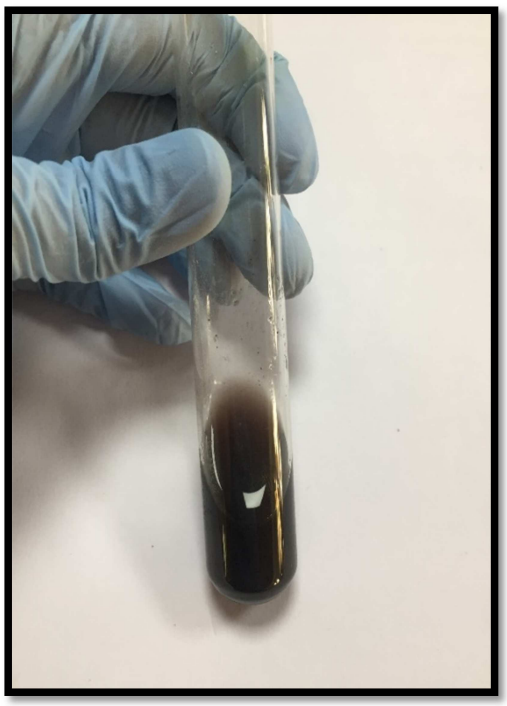


Figure 18: Ninhydrin solution test- Appearance of purple color +ve for protein

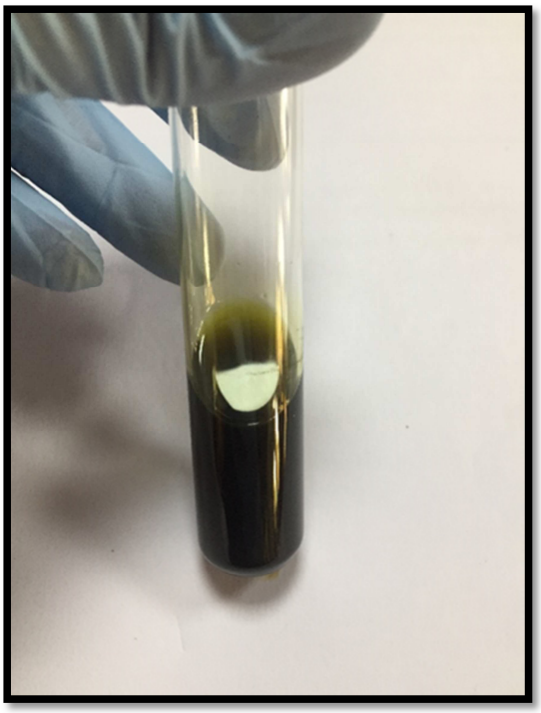


Figure 19: Biuret test- Appearance of violet color +ve for proteins

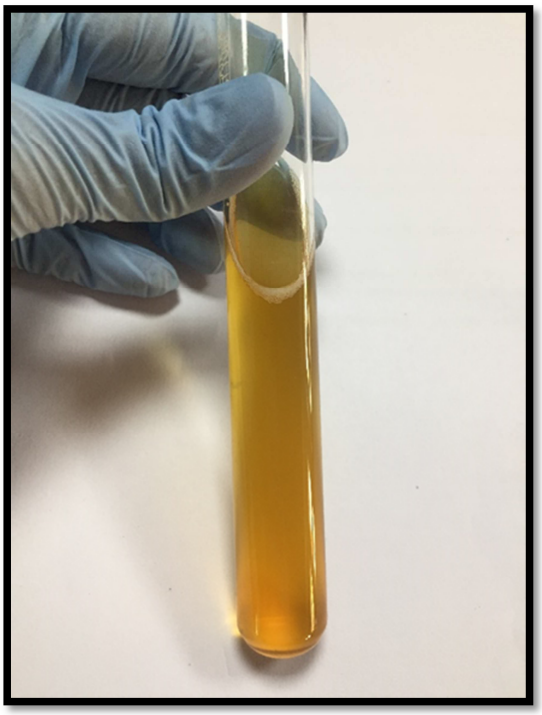


Figure 20: Ferric chloride test- Appearance of yellow color +ve for Coumarin glycoside

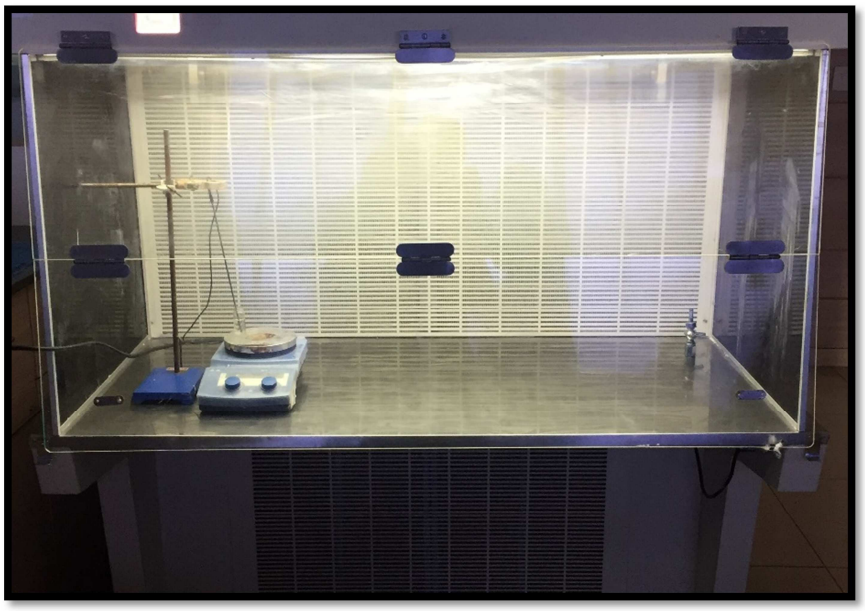


Figure 21: Laminar airflow used for preparation of gel

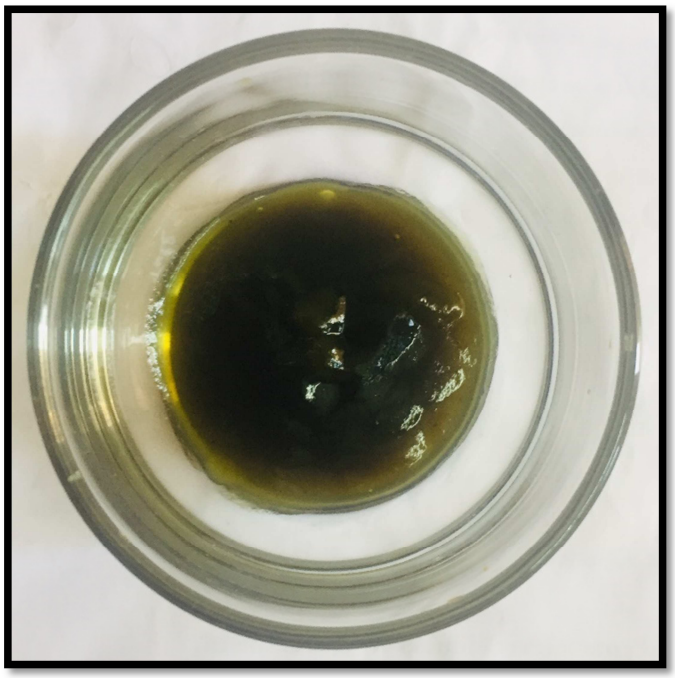


Figure 22: M. oleifera mucoadhesive gel (2%)

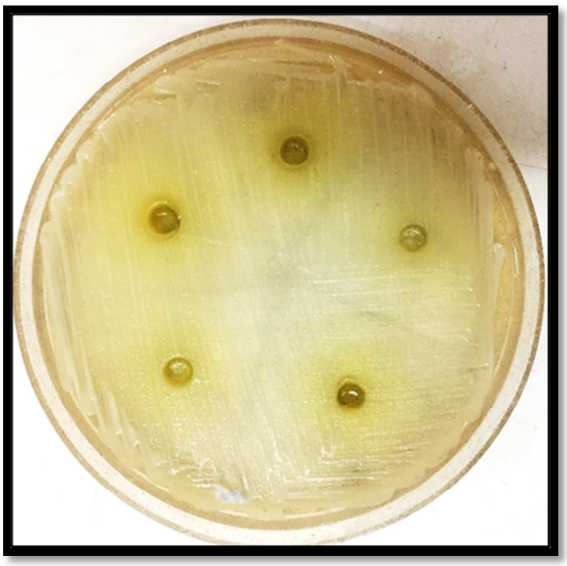


Figure 23: Minimum inhibitory concentration against Staphylococcus aureus

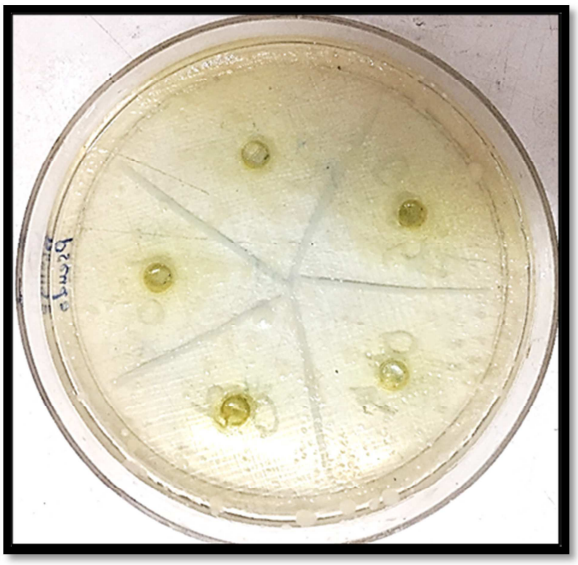


Figure 24: Minimum inhibitory concentration against Pseudomonas

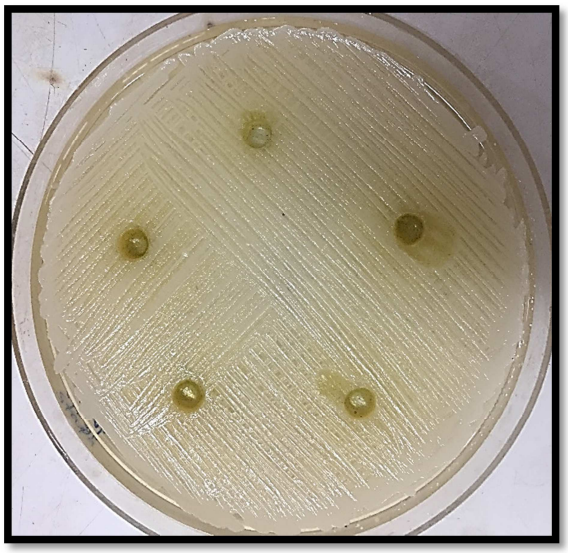


Figure 25: Minimum inhibitory concentration of M. Oleifera against Candida species

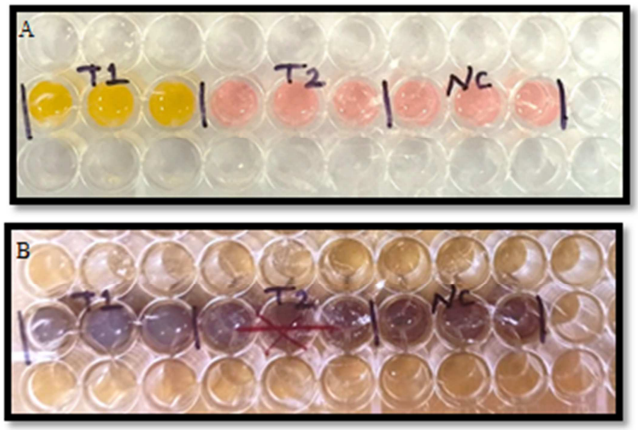


Figure 26: MTT Assay A- At baseline B- After 48 hours

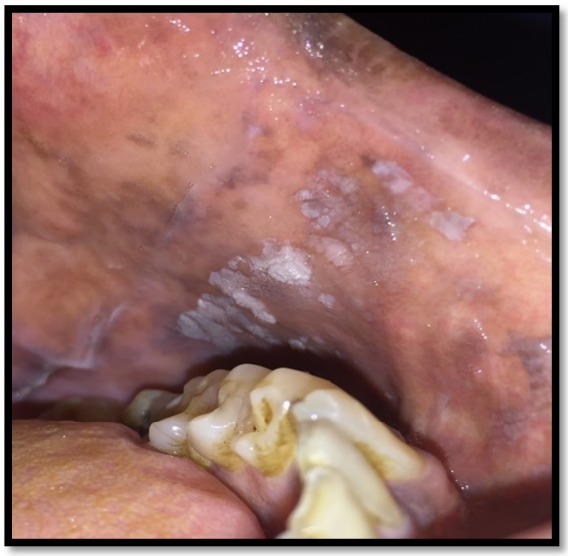


Figure 27: Clinical presentation at baseline in M. oleifera gel (2%) group

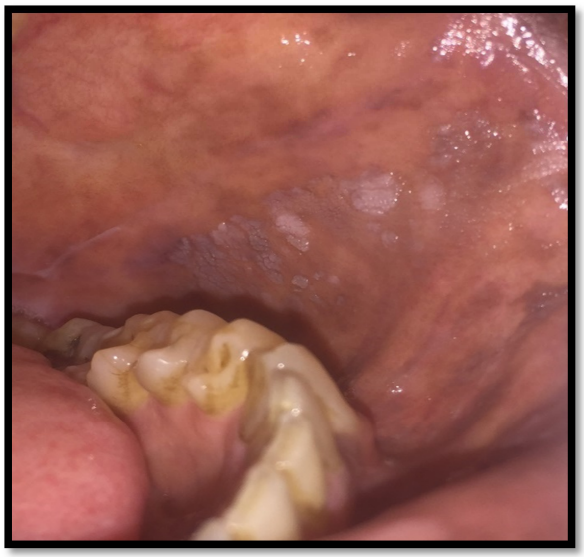


Figure 28: Clinical presentation post-treatment in M. oleifera gel (2%) group

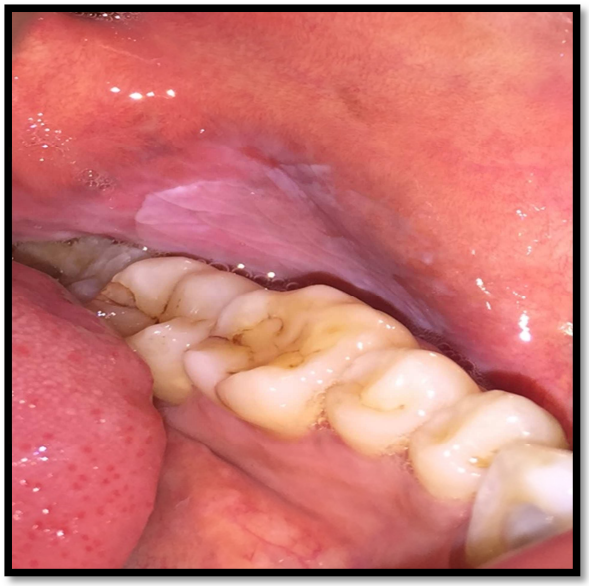


Figure 29: Clinical presentation at baseline in Retino-A cream (0.1%) group

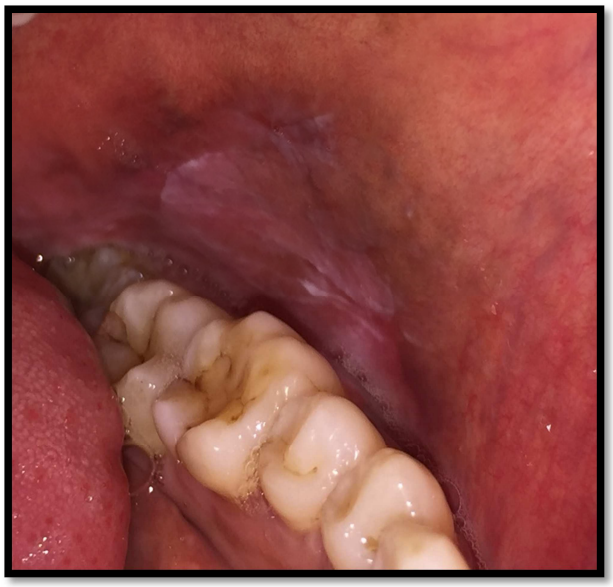


Figure 30: Clinical presentation post-treatment in Retino-A cream (0.1%) group

RESULTS AND OBSERVATIONS

In this study, a total of 72 participants were enrolled.36 in the control group (Retino-A)and 36 in the case group (M. oleifera) respectively. Lost of follow-up of 3 patients from the M.oleifera group and 4 patients from the Retino-A group after 1st visit occured.

Results were based on the inter-group comparison of the change in size in the M.oliefera group and Retino-A group (Pre and post-treatment) and intragroup comparison at baseline and post-therapy.

Statistical analysis was done using SPSS version 26 software. Unpaired t-test was used to compare the statistical difference between data distribution of case and control group at baseline.The resolution of the lesion in the case and control groups was compared using a paired t-test at baseline and post-therapy. The intergroup analysis of Retino-A (0.1%) and M.oleifera gel (2%) post-treatment was done using an unpaired t-test for post-treatment values and a separate unpaired t-test was done using baseline (B) values and post-treatment (PT) values difference(B - PT) to determine which group caused the most reduction in the size of the lesion.

Table 12: **Distribution of subjects according to gender.**

Gender	M. oleifera (2%) group		Retino-A (0.1%) group	
	No.of cases	Percentage (%)	No.of controls	Percentage (%)
Male	25	75.75	23	71.87
Female	8	24.24	9	28.12
Total	33	100	32	100

This study had 25 males and 8 females in the M.oleifera group and 23 males and 9 females in the Retino-A group.

Table 13 : **Distribution of subjects according to age.**

Groups		Frequency	Percentage (%)
M. oliefera (25) Mucoadhesive gel Group	25-34 years	4	12.12
	35-44 years	6	18.18
	45-54 years	10	30.30
	>55 years	13	39.39
	Total	**33**	**100**
Retino-A (0.1%) cream	25-34 years	5	15.62
	35-44 years	6	18.75
	45-54 years	4	12.5
	>55 years	17	53.12
	Total	**32**	**100**

The maximum number of subjects in the M.oleifera group was above 55 years (39.39%), followed by 45-54 years (30.30%), and least in the younger age group of 25-34 years (12.12%). The Retino-A group had similar distribution i.e; maximum subjects in the age group above 55 years (53.12%), followed by 35-44 years age group (18.75), and least in 45-54 years age group (12.5%).

Table 14: Mean (± standard deviation) at baselines of Retino-A and M. oleifera group using unpaired t-test

Group	No. of participants	Mean(mm)	Std. Deviation
M. oleifera Group	33	36.818	14.629
Retino-A Group	32	35.531	15.574

Unpaired t-test was employed to compare the baselines of both the groups, there was no statistical difference found in the size of the lesion between both the groups at baseline and there was normal distribution of data among both the groups.

Table 15:Mean (± Standard deviation) at baseline and post-therapy using paired t-test in case and control group

Variables	Case group		Control group	
	M. oleifera (2%) group		Retino-A (0.1%) group	
	N=33		N=32	
	Baseline	Post therapy	Baseline	Post therapy
Mean (mm)	36.818	8.424	35.531	17.843
Std. deviation	14.629	5.612	15.574	8.621

Paired t-test was used for intra-group analysis to compare the baseline and post-therapy regression in the size of the lesion in each group.

In the Moringa oleifera group, the mean value and standard deviation at baseline were (36.818± 14.629), and post treatment was (8.424 ± 5.612), the result was statistically significant at p-value ($p<0.001$).

In the Retino-A group, the mean value and standard deviation at baseline were (35.531±15.574) and the post-treatment was (17.843 ± 8.621), the result was statistically significant at p-value ($p<0.001$).

Table 16: Mean (± Standard deviation) of post-therapy using unpaired t-test among case and control group

Groups	No.of participants	Mean (mm)	Std. Deviation
M. oleifera group	33	8.424	5.612
Retino-group	32	17.843	8.621

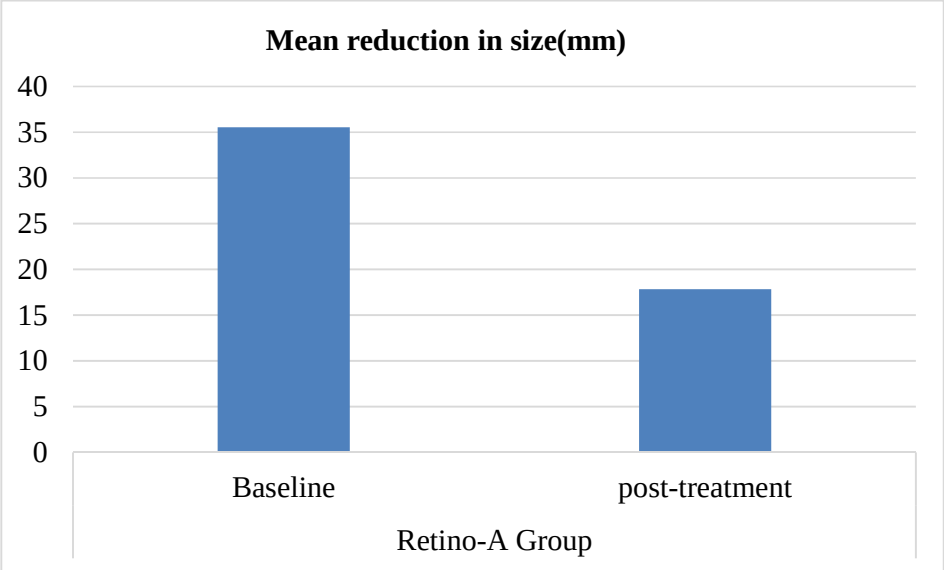
To compare the efficacy of the M.oleifera group and Retino-A group post-treatment unpaired t-test was used. The mean and standard deviation of the M.oleifera and Retino-A group were (8.424 ± 5.612) and (17.843 ±8.621) respectively, M. oleifera was found more effective in reduction in the size of the lesion.

Table 17 :Mean (± Standard deviation) of reduction difference of size ((B-PT) at Baseline(B) and post-therapy(PT) in M. oleifera(2%) and Retino-A (0.1%) groups using unpaired t-test

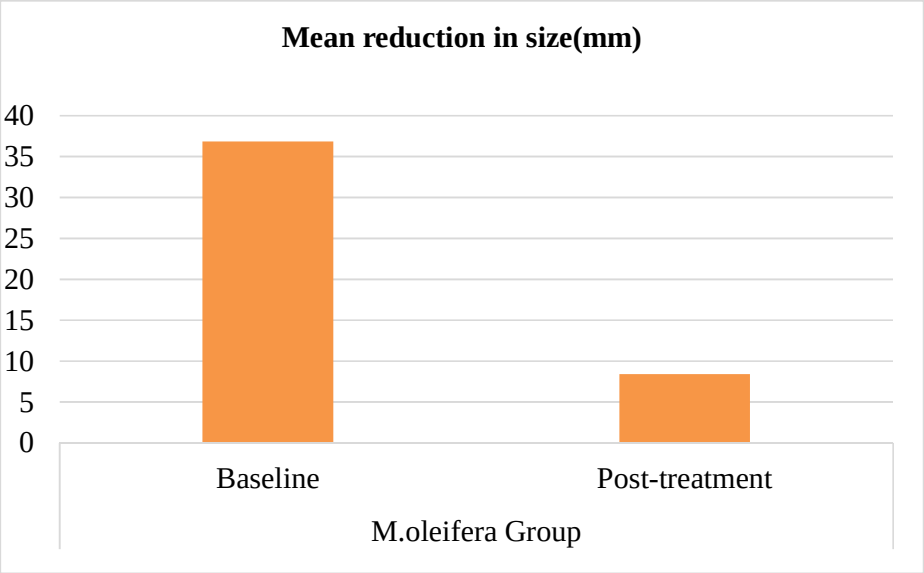
	Group	N	Mean (mm)	Std.Deviation	t-value	p-value
Reduction difference at baseline and post-therapy	**M. oleifera group**	33	28.393	9.930	4.765	0.000012
	Retino-A group	32	17.687	8.054		

To compare the effectiveness of both the group difference in the size of the lesion at baseline and post-treatment were taken from each group and analyzed using an unpaired t-test. In the M. oleifera group mean and standard deviation values were (28.393 +9.930) respectively, whereas in the Retino-A group the values were (17.687+8.054). There was a statistical difference between both the group and the M. oleifera group was found to be more effective in reduction of the size of oral leukoplakia at the mean difference of (10.71+1.876) at p-value ($p<0.001$).

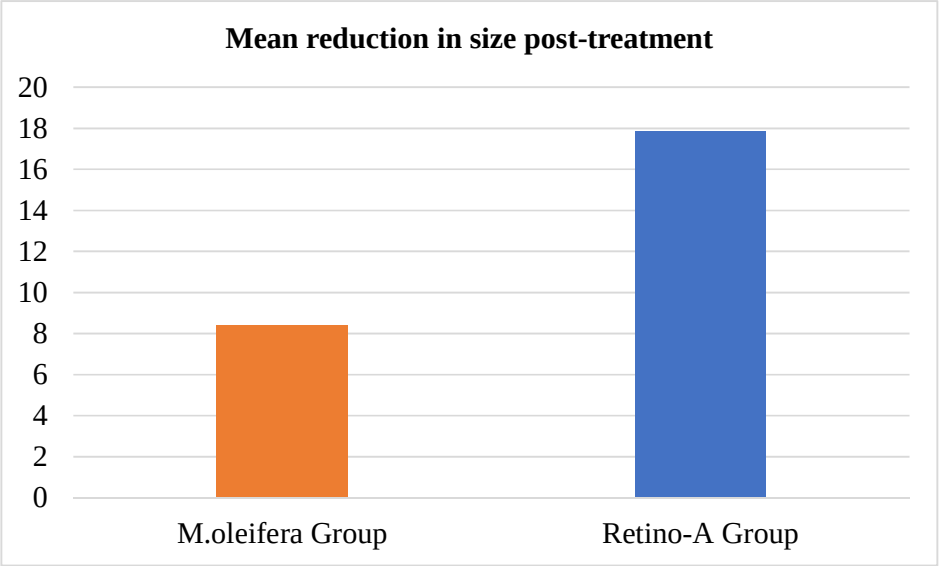
GRAPHS



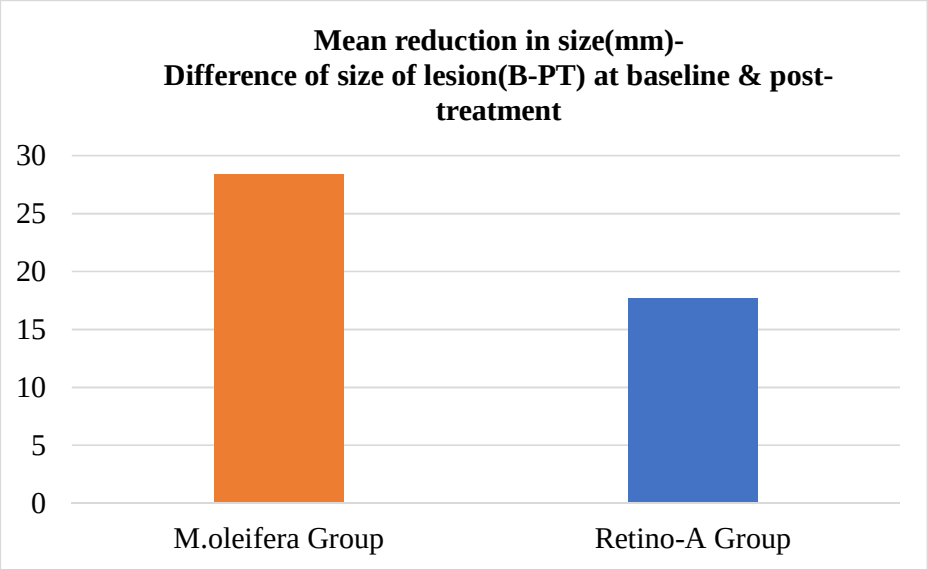
Graph 1: Mean reduction in size (mm) in Retino-A group at baseline and post-treatment



Graph 2: Mean reduction in size (mm) in M. oleifera group at baseline and post-treatment



Graph 3: Mean reduction in size (mm) post-treatment in M. oleifera and Retino-A group



Graph 4: Mean reduction in size (mm) -Difference of size of lesion at baseline & post-treatment among M. oleifera and Retino-A group

DISCUSSION

''Oral leukoplakia is a whitish non-scrapable patch, that cannot be diagnosed clinically or pathologically as any other disease and is not caused by physical or chemical causes, with the exception of tobacco habits''. The malignant potential of leukoplakia was first suggested by Sugar and Benoczy in 1957[4''].

Recent literature has shown the use of various treatment modalities such as topical and systemic antioxidants such as lycopene, tretinoin etc[8], M. oleifera mucoadhesive gel is nevertheless an ideal topical agent as a chemopreventive agent in management because it has higher efficacy than tretinoin, potent anti-oxidant activity, low cell toxicity, well-tolerated, cost-effective and readily available in India. M. oleifera gel can be used in patients with systemic disease such as diabetes, hypertension, and patients with liver disorders without any adverse effects as it has anti-hyperglycemic,anti-hypertensive and hepatoprotective properties[31].

The antioxidants present such as bioflavonoids i.e quercetin, Kaempherol reduces oxidative stress by scavenging reactive oxygen species in the cell[12]. It also has anti-cancer properties which provide added therapeutic effects in premalignant conditions such as leukoplakia[14].

In the present randomized controlled trial, we have evaluated the efficacy of M. oleifera gel (2%) for topical application among patients with oral leukoplakia and compared it with Retino-A cream (0.1%).Seventy-two patients with clinically diagnosed cases of oral leukoplakia were included in the study. Thirty-six patients were enrolled in each group. The test group received M. oleifera gel (2%) and the control group received Retino-A cream(0.1%). Although there was a loss of follow-up

of 3 patients in the test group and 4 patients in the control group, so a total of 33 patients received M. oleifera gel (2%) treatment and 32 patients received Retino-A cream (0.1%) treatment.

In this RCT around 73.84% of patients were males and 26.15% were females. According to some studies oral leukoplakia is predominant in men[57], although other studies found no gender predilection [58,59]. Around 46.15% of the patients were above 55 years, these findings were consistent with the findings of Amagsa et al[60].Retino-A cream (0.1%) i.e tretinoin is an anti-oxidant most commonly used in chemopreventive agents, it is most commonly employed for the management of oral leukoplakia.

The choice of M. oleifera as a chemopreventive agent was based on the preclinical in-vitro studies demonstrating its potent antioxidant activity and anti-cancer properties.M. oleifera was found to be effective against and caused apoptosis of colorectal cell lines, hepatocellular carcinoma, and breast cancer cell lines[38,39,40].

In the previous studies, many herbal agents as a topical preparation have been employed in the management of oral leukoplakia, such as curcumin, Calendula Officinalis, green tea extract, and lycopene [61].This is the first study that demonstrates the therapeutic efficacy of M. oleifera in the form of a mucoadhesive gel in vivo.Although tretinoin is most commonly used in the management of oral leukoplakia, according to literature M. oleifera is 10 times more potent than tretinoin as an anti-oxidant with no cellular toxicity[11]. Retino-A cream is commonly used for the management of acne vulgaris[61] as there is no topical chemotherapeutic agent available for the treatment of oral leukoplakia oral physicians prescribe topical tretinoin cream as an alternative, M. oleifera gel is a 1st mucoadhesive gel which is

made for the management of oral leukoplakia, thus it has better tolerability, better therapeutic efficacy and it is more palatable as compared to Retino-A cream.

The scope for further research is wide open to evaluating the effect of M. oleifera in non-homogenous leukoplakias and other pre-malignant diseases such as lichen planus etc as M. oleifera is a potential candidate as a chemopreventive agent.Also, long-term follow-up of these patients is required to evaluate the long-term therapeutic effects on oral leukoplakia and to evaluate the recurrence of this is pre-malignant lesion.

SUMMARY & CONCLUSION

Potentially malignant disorders like oral leukoplakia are one of the most commonly encountered in the oral cavity. Previous epidemiological have proven the use of antioxidants as a potent treatment modality as a topical form. One such herbal therapeutic agent is M. oleifera, which is most commonly found in India and known for its potent anti-oxidant property reported in various in-vitro studies. Also, it has anti-cancer properties it can be used as a chemotherapeutic agent in the future. It is proven to be effective in in-vitro studies against hepatocellular carcinoma, colorectal carcinoma, and breast cancer. As the commercial form of M. oleifera in topical form is not available it was formulated in this RCT.

The present RCT assessed the effectiveness of M. oleifera gel in oral leukoplakia patients. A total of 72 patients were randomly selected which were randomized into two groups. The M. oleifera group (n=36) and Retino-A group (n=36), although we lost follow-up of 3 patients in the M.o leifera group and 4 patients in the Retino-A group. So the total number of individuals in the M. oleifera group was 33 and in the Retino-A group were 32 individuals. The therapy was instituted for 3 months. The longest dimension of the lesion was measured with vernier caliper at baseline and after 3 months and was statistically compared at the end of the therapy using SPSS version 26.

The present clinical trial concluded that patients who received M. oleifera mucoadhesive gel (2%) presented with a significant reduction in the size of the lesion as compared to Retino-A cream (0.1%). This could be attributed to the potent anti-oxidant property of M. oleifera which reversed the hyperkeratosis, the earliest change in oral leukoplakia. All patients responded to M. oleifera gel. None of the patients in this study reported local irritation or adverse effect with the use of M. oleifera gel.

LIMITATIONS OF THE STUDY:

Long term follow-up of oral leukoplakia patients are required to evaluate long term effectiveness of M. oleifera mucoadhesive gel in oral leukoplakia patients.

FUTURE IMPLICATIONS:

M. oleifera appears to be a safe and promising antioxidant as a topical treatment for oral leukoplakia. Further studies should be conducted with concentration variations and a longer follow-up period. Even an initiative has to be taken to introduce the herbal gel at the commercial level. It can be employed in studies with other potentially malignant oral disorders such as lichen planus and OSMF. The development of chemotherapeutic drugs for the treatment of cancer from MO should be taken into consideration.

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ANNEXURE - I - ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
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FAX: 0831-2470640
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E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1331

CERTIFICATE

This is to Certify that the synopsis titled

COMPARISON OF EFFECTIVENESS OF MORINGA OLEIFERA LEAVES EXTRACT GEL [2%] WITH RETINO-A [0.1%] CREAM FOR TREATMENT OF ORAL LEUKOPLAKIA - DOUBLE BLINDED RANDOMIZED CONTROL TRIAL

Submitted by Dr. ANSARI SULEM RIYAZ AHMED *P. G. Student / Staff, Guided by* DR. SHIVAYOGI CHARANTIMATH *from Department of* ORAL MEDICINE & RADIOLOGY *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 04/07/2020

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

ANNEXURE - II - CASE HISTORY PROFORMA

KLE V.K. INSTITUTE OF DENTAL SCIENCES, BELGAVI.

DEPARTMENT OF ORAL MEDICINE AND RADIOLOGY

"Comparison of Effectiveness of Moringa oleifera Leaves Extract Gel (2%) With Retino- A (0.1%) Cream for Treatment of Oral Leukoplakia: Double Blinded Randomized Control Trial"

CASE HISTORY PROFORMA

Name of the patient: **Age/ Sex:**

Address: **Occupation:**

Chief complaint:

History of present illness:

Past medical and dental history:

Personal history:

Tobacco habits: Yes/ No

Type: Smoking Smokeless tobacco Spit/ Swallow

Frequency: **Duration:**

General physical examination:

Extraoral examination:

Intraoral examination:

Hard tissue examination:

Soft tissue examination:

Examination of specific lesion: (Oral leukoplakia)

Inspection of Oral mucosa:

White patch: Yes/ No **Side:** Right side/ Left side/ Both

Site: Buccal mucosa/ Labial mucosa/ Floor of mouth/

Upper buccal sulcus/ Lower buccal sulcus/ tongue

Extension:

Longest dimension of lesion: **Corrugated appearance:** Yes/No

Scrapable: Yes/No

Provisional diagnosis:

ANEXURE – III - CONSENT FORM

KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES,

BELAGAVI

DEPARTMENT OF ORAL MEDICINE AND RADIOLOGY

I, _____ aged_____ years old have been informed about my involvement in the study.

1) I agree to give my personal details like name, age, sex, address, previous dental history, medical history and the details required for the study to the best of my knowledge
2) I will cooperate with the dentist for my intraoral and/or extra oral examinations.
3) I permit the dentist to utilize the information given by me and results obtained from this study for presentation and publication purpose.
4) I permit the dentist to do blood investigations and/or biopsy and utilize the same for the study purpose.
5) I will not claim any returns for my cooperation in the study, even if it is being sponsored by any agency. I am participating with my own will and wish.

In my full consciousness and presence of mind, after understanding all the procedure in my vernacular language, I am willing and giving my consent to participate in this study.

Signature of the patient: …… Signature of the dentist: …….

Date: …… Name of the witness: …….....

Place: …… Signature of the witness: ……...

ANEXURE – IV - MASTER CHART-I

M. oleifera group

SI. No.	Age/Sex	Longest dimension of lesion at baseline (B) (mm)	Longest dimension of lesion at the post therapy (PT) (mm)	Difference in the size of the lesion (B-PT) (mm)
1.	54/M	25	0	25
2.	35/M	15	5	10
3.	45/M	40	15	25
4.	28/M	32	8	24
5.	26/M	10	0	10
6.	70/M	28	5	23
7.	58/M	38	8	30
8.	58/M	20	2	18
9.	36/M	28	3	25
10.	54/M	18	1	17
11.	65/M	35	8	27
12.	62/M	12	0	12
13.	34/M	25	2	23
14.	60/F	39	11	28
15.	40/M	40	10	30
16.	52/M	18	1	17
17.	45/F	39	7	32
18.	68/F	28	5	23

19.	41/F	18	2	16
20.	32/M	42	12	30
21.	65/M	55	10	45
22.	35/M	60	20	40
23.	60/M	45	18	27
24.	49/F	53	15	38
25.	70/M	46	12	34
26.	50/F	47	10	37
27.	65/M	55	15	40
28.	45/M	43	12	31
29.	60/M	58	13	45
30.	32/F	47	10	37
31.	54/M	45	12	33
32.	62/M	59	15	44
33.	50/F	52	11	41

ANEXURE – V - MASTER CHART- II

Retino-A group

SI. No.	Age/Sex	Longest dimension of lesion at baseline (B) (mm)	Longest dimension of lesion at the post therapy (PT) (mm)	Difference in the size of the lesion (B-PT) (mm)
1.	68/M	40	25	15
2.	27/M	39	20	19
3.	67/M	12	7	5
4.	58/M	17	10	7
5.	34/M	28	15	13
6.	65/M	35	21	14
7.	68/M	18	9	9
8.	63/M	14	5	9
9.	55/F	27	15	12
10.	55/M	20	8	12
11.	55/M	38	15	23
12.	60/M	19	9	10
13.	62/M	24	14	10
14.	45/F	39	4	9
15.	58/M	32	14	18
16.	42/M	10	3	7
17.	39/F	40	17	23
18.	60/F	33	17	16
19.	34/F	19	8	11

20.	52/M	60	32	28
21.	67/M	53	20	33
22.	29/M	42	22	20
23.	52/M	57	25	32
24.	44/F	45	20	25
25.	38/F	58	30	28
26.	40/F	55	25	30
27.	25/M	59	30	29
28.	45/M	46	21	25
29.	63/M	49	32	17
30.	65/F	41	25	16
31.	44/M	52	31	21
32.	62/M	42	22	20