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RESEARCH

Assessment of antioxidant efficacy of saffron-withania somnifera herbal gel against oral ulcers: an invitro study

Safran- withania somnifera bitkisel jelinin ağız ülserlerine karşı antioksidan etkinliğinin değerlendirilmesi: bir invitro çalışma

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Abstract

Purpose: Recurrent aphthous stomatitis (RAS) is the most commonly seen ulcerative lesion of the oral mucosa. The lesions are generally recurrent, painful and can affect the quality of life. The aim of this study was to evaluate the efficacy of herbal extracts of saffron, withania somnifera, Dry ginger, Tulsi as an antioxidant agent in RAS.

Materials and Methods: 1g powder of tulsi, withania somnifera, dry ginger was mixed with 100ml of distilled water separately and boiled in a heating mantle at 50 to 60 degree for 15 minutes. Anti-inflammatory, antimicrobial, antioxidant,cytotoxic activities were tested by adding 5 ml extract to empty gel containing a combination of carboxylate methyl cellulose 5g, carbapel 5g. Antioxidant property of withania somnifera, saffron, dry ginger and tulsi were evaluated using DPPH assay, H2O2 assay and FRAP assay.

Results: Our study revealed highest zone of inhibition of 90.22, 84.3, 88.93% at a concentration of 50 microgram per milliliter which is near to that of standard value

Conclusion: This study gives an insight that the naturally occurring constituents of medicinal herbs will be able to resolve oral ulcers thereby improving the quality of life of the patients. Hence, herbal medicines can be used as an adjunct to synthetic drugs available commercially.

Keywords:. Withania somnifera, tulsi, dry ginger, saffron, ulcer, medicine, quality of life

Öz

Amaç: Tekrarlayan aftöz stomatit (RAS), oral mukozanın en sık görülen ülseratif lezyonudur. Lezyonlar genellikle tekrarlayan, ağrılı olup yaşam kalitesini etkileyebilir. RAS'ta antioksidan ajan olarak safran, withania somnifera, kuru zencefil, tulsi bitkisel ekstraktlarının etkinliğini değerlendirmek.

Gereç ve Yöntem: 1 g tulsi, withania somnifera, kuru zencefil tozu, 100 ml damıtılmış su ile ayrı ayrı karıştırıldı ve 50 ila 60 derecede bir ısıtma mantosunda 15 dakika kaynatıldı. Karboksilat metil selüloz 5 g, karbapel 5 g kombinasyonunu içeren boş jele 5 ml ekstrakt eklenerek antiinflamatuar, antimikrobiyal, antioksidan, sitotoksik aktiviteler test edildi. Withania somnifera, safran, kuru zencefil ve fesleğenin antioksidan özelliği DPPH testi, H2O2 testi ve FRAP testi kullanılarak değerlendirildi.

Bulgular: Çalışmamız, mililitre başına 50 mikrogram konsantrasyonda %90.22, 84.3, 88.93'lük en yüksek inhibisyon bölgesini ortaya koydu; bu, standart değere yakın bir değerdir.

Sonuç: Bu çalışma, tıbbi bitkilerin doğal olarak oluşan bileşenlerinin ağız ülserlerini çözebileceği ve böylece hastaların yaşam kalitesini artırabileceği konusunda bir fikir vermektedir. Dolayısıyla, bitkisel ilaçlar ticari olarak temin edilebilen sentetik ilaçlara ek olarak kullanılabilir.

Anahtar kelimeler: Withania somnifera, tulsi, kuru zencefil, safran, ülser, ilaç, yaşam kalitesi

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INTRODUCTION

The most frequent ulcerative illness of the oral mucosa, recurrent aphthous stomatitis (RAS), manifests as painful, circular, shallow lesions with a well circumscribed erythematous rim and a yellowish-gray pseudomembranous center. Before an ulcer develops, RAS is characterized by a prodromal phase with burning sensation that lasts for 24 to 48 hours[1]. Herbal remedies are a common form of alternative therapy that have been tested in the management of multiple oral lesions, including RAS and many different herbal concoctions are in use[2]. The pharmacological benefits of roots of Withania somnifera, commonly known as Ashwagandha, has effects like anti-aging, cardioprotection, and antioxidant. The presence of withanolides such withaferin A and withanolide A is thought to be responsible for these[3]. The medicinal properties of Ashwagandha include anticancer, antioxidant, anti-inflammatory, and immunomodulatory. Sitoindosides and acyl steryl glucosides in Ashwagandha are anti-stress agents[4].

The Iridaceae family includes the perennial herb saffron (Crocus Sativus Linnaeus). Significant hydrogen peroxide scavenging activity and an inhibitory effect on lipid peroxidation are both seen in saffron extract[5]. Crocin, a water-soluble carotenoid with the chemical components crocetin and gentiobiose, is one of the extracts of saffron. It appears to have potent free-radical scavenging activity as well as a variety of pharmacological activity, including anti- inflammatory, antioxidant, anticancer, hypolipidemic, cytotoxic and antiatherosclerotic actions[6]. Because of its spiritual holiness, holy basil/ tulsi (Ocimum sanctum L) is frequently produced in India. It offers a variety of therapeutic benefits and is also regarded as an adaptogen because it aids in stress adaptation[7]. Tulsi has a diverse range of medicinal properties and have been studied in hundreds of scientific studies including *in vitro*, animal and human experiments. These studies reveal that tulsi has a unique combination of actions including anti-oxidant, antimicrobial, anti-inflammatory and chemopreventive effects[8].

Because of its beneficial qualities as a spice or herb and its usage in traditional medicine, ginger is widely farmed in Asia, Europe, and the Middle East[9]. Powerful antioxidants called polyphenols are abundant in the plant. Significant oxidation-related disorders are prevented by these substances, which function as an active phytochemical[10]. The aim of this study was to evaluate the antioxidant property in a combination herbal gel containing saffron, withania somnifera, tulsi and dry ginger and its efficacy against recurrent aphthous stomatitis.

MATERIALS AND METHODS

The study was approved by the Scientific Review Board (Ref No. SRB/SDC/OMED-2204/23/136). Checklist for Reporting *in-vitro* Studies (CRIS) guidelines was followed for conducting this study. No subjects or participants were included in the study.

Figure 1 shows the items in CRIS guidelines.

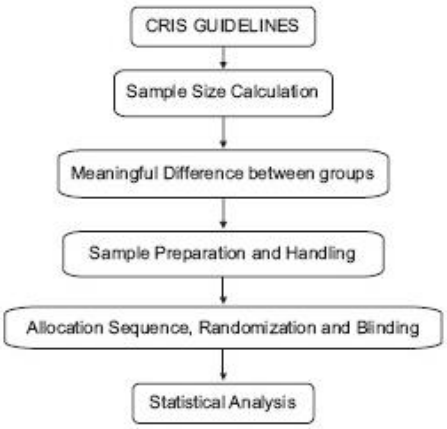

Figure 1. Items in CRIS (checklist for reportini *in vitro* studies*)* guideline.

The extract is prepared from Crocus sativus, Withania somnifera, Ocimum sanctum, Gingiber officinale roscole which have therapeutic properties and potential benefits to health.

Sample preparation and handling

This herbal formulation consists of 4 components - Saffron, Withania somnifera, Dry ginger, and tulsi. One gram powder of tulsi, withania somnifera, dry ginger were mixed with 100ml of distilled water separately and boiled in a heating mantle at 50 to 60 degree for 15 minutes then filtered and 90ml of extract was obtained. On the other hand, 0.5 g of saffron was added to 200ml of distilled water, kept in an orbital shaker for 24hrs and then boiled at 50 to

60 degree for 15 min and then filtered, extract was obtained. 50 ml of all four components are combined and concentrated for 20ml. Finally anti inflammatory, antimicrobial, antioxidant, cytotoxic activities were tested by adding 5 ml extract to empty gel containing a combination of carboxylate methyl cellulose 5g and carbapel 5g.

Figure 2. Extract preparation of Withania somnifera, dry ginger, tulsi, saffron.

Figure 3. Extract added to carboxylate methyl cellulose and carbapel gel.

Antioxidant property: The antioxidant property of herbal gel was evaluated using DPPH assay, H2O2 assay, FRAP assay.

DPPH assay

To serve as the stock solution, a 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was first produced in methanol. Before starting any test, the stock solution was carefully diluted to a final concentration of 20 μM in methanol, and a new working solution was made. Saffron, Withania somnifera, tulsi, and dried ginger extracts in varying quantities (10, 20, 30, 40, and 50 μg/mL) were added to 200μL of the DPPH working solution that had been carefully placed in a 96-well plate. After that, the plate was allowed to sit at room temperature for thirty minutes while it was incubated in the dark. The samples' absorbance was then measured at 517 nm using a microplate reader, and methanol was used as a blank. The proportion of DPPH scavenging activity was computed as follows:

(A_control – A_sample) / A_control] × 100 = % DPPH Scavenging Activity

where A_sample denotes the absorbance of the sample (DPPH solution containing the green produced silver nanoparticles) and A_control denotes the absorbance of the control (DPPH solution without the sample). Ascorbic acid (1 mg/mL) was administered to the positive control group.

H2O2 assay

The assay used in this study for hydroxyl radical scavenging adhered to the guidelines provided by Halliwell et al. A reaction mixture was made with a volume of 1 mL and 100 μL of 28 mM 2-deoxy-2-ribose. Next, different amounts of saffron, withania somnifera, tulsi, and dry ginger (10–50 μg/mL) were added to the blend. 100 μL of ascorbic acid, 200 μL of EDTA, and 200 μL of 200 μm ferric chloride were also added. At 532 nm, optical density was evaluated in relation to the blank solution after an hour of incubation at 37 °C. An example of a positive control was vitamin E. The formula [(Ablank − Asample)/Ablank] × 100 was used to compute the hydroxyl radical scavenging activity (%), where Ablank is the absorbance of the control reaction (without sample) and Asample

FRAP assay

Acetate buffer (300 mM, pH 3.6), which is made by dissolving 3.1 g of sodium acetate trihydrate and adding 16 ml of glacial acetic acid, along with distilled water to form a volume of 1 L, is one of the reagents used for the FRAP experiment. FeCl3.6H2O at a concentration of 20 mM and TPTZ (2, 4, 6-tripyridyl-s-triazine) at a concentration of 10 mM in 40 mM HCl are also required. Just before usage, combine all three ingredients in a 10:1:1 ratio to create the functioning FRAP reagent. For calibration, a FeSO4.7H2O standard solution with concentrations ranging from 0.1 to 1.5 mM in methanol is used.

3.6 mL of the FRAP solution and 0.4 mL of distilled water are combined, and the mixture is then incubated for 5 minutes at 37°C. This mixture is then mixed with 80 milliliters of a particular plant extract concentration and incubated for an additional 10 minutes at 37°C. Next, the reaction mixture's absorbance is measured at 593 nm. Five concentrations of FeSO4.7H2O (0.1, 0.4, 0.8, 1, 1.12, and 1.5 mM) are used to create calibration curves by measuring absorbance values in a way that is comparable to that of the sample solutions.

Statistical analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). The percentage of antioxidant activity (% scavenging) for DPPH, H_2O_2, and FRAP assays was calculated for each concentration (10, 20, 30, 40, and 50 μg/mL). Statistical significance between different concentrations was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS Version 23.

RESULTS

Antioxidant activity of ingredients like saffron, tulsi, withania somnifera and dry ginger has been tested after preparing a gel and testing it against pathogens like S. aureus, E. coli, E. Faecalis, P. Acidophilus against a standard antibiotic. The results of a DPPH assay, shows the percentage of inhibition (Y-axis) at various concentrations (X-axis) ranging from 10 to 50 μg/mL. The chart compares the antioxidant activity of a standard, Vitamin C (blue bars) versus a gel formulation (orange bars) containing natural ingredients. As the concentration increases, both the standard and the gel demonstrate higher percentages of inhibition, with the values for both remaining relatively close across all concentrations. This indicates similar antioxidant efficacy between the gel and the standard, especially at higher concentrations (Figure 4, Table 1). At 10 μg/mL, statistical analysis by one-way ANOVA revealed an F-value of 0.224 and a p-value of 0.649, indicating no statistically significant difference between the herbal gel and the standard antioxidant ($p > 0.05$). Although a slight decrease in scavenging activity was observed for the herbal formulation compared to the standard at all concentrations, the herbal gel demonstrated comparable antioxidant efficacy in a dose-dependent manner.

The results of an H2O2 assay (Figure5, Table 2), shows the percentage of inhibition at different concentrations (10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, and 50 μg/mL) for both a standard and a gel formulation. As the concentration increases from 10 μg/mL to 50 μg/mL, the percentage of inhibition also increases for both the standard and gel, indicating a dose-dependent response. At lower concentrations (10 μg/mL and 20 μg/mL), both the standard and gel show similar inhibition percentages, with only slight variation. However, at higher concentrations (30 μg/mL to 50 μg/mL), both formulations exhibit near-identical inhibition percentages, with the highest inhibition (~80-90%) observed at 50 μg/mL. Overall, the data suggest that both the standard and gel are effective at inhibiting H2O2, and their effectiveness improves with higher concentrations, showing comparable performance (Figure 5, Table 2). At 10 μg/mL, one-way ANOVA analysis revealed an F-value of 0.178 and a p-value of 0.685, indicating no statistically significant difference between the herbal gel and the standard ($p > 0.05$). Although the herbal gel consistently showed slightly lower scavenging activity compared to the standard across all concentrations, the differences were minimal, especially at higher concentrations, suggesting that the herbal gel possesses effective hydrogen peroxide scavenging potential comparable to that of the standard.

The results of a Ferric Reducing Antioxidant Power (FRAP) assay (Figure 6, Table 3), comparing the percentage of inhibition at various concentrations (10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, and 50 μg/mL) for both a standard and a gel formulation. As the concentration increases from 10 μg/mL to 50

µg/mL, both the standard and gel exhibit an increasing percentage of inhibition, demonstrating a dose-dependent response. At all concentrations, the gel shows comparable inhibition to the standard, with both formulations reaching around 80-90% inhibition at the highest concentration (50 µg/mL). The two formulations have nearly identical values at each concentration, indicating similar antioxidant activity. In summary, the graph suggests that both the standard and gel possess strong and comparable antioxidant properties, which improve with higher concentrations (graph 3, table 3). In the FRAP assay, the antioxidant reducing power of the herbal gel (Saffron + Dry ginger + Withania somnifera + Tulsi) increased progressively with concentration, closely paralleling the standard. At 10 µg/mL, one-way ANOVA showed an F-value of 0.643 and a p-value of 0.446, indicating no statistically significant difference between the herbal gel and the standard ($p > 0.05$). Across all concentrations (10–50 µg/mL), although the herbal gel consistently demonstrated slightly lower FRAP values compared to the standard, the differences were minimal.

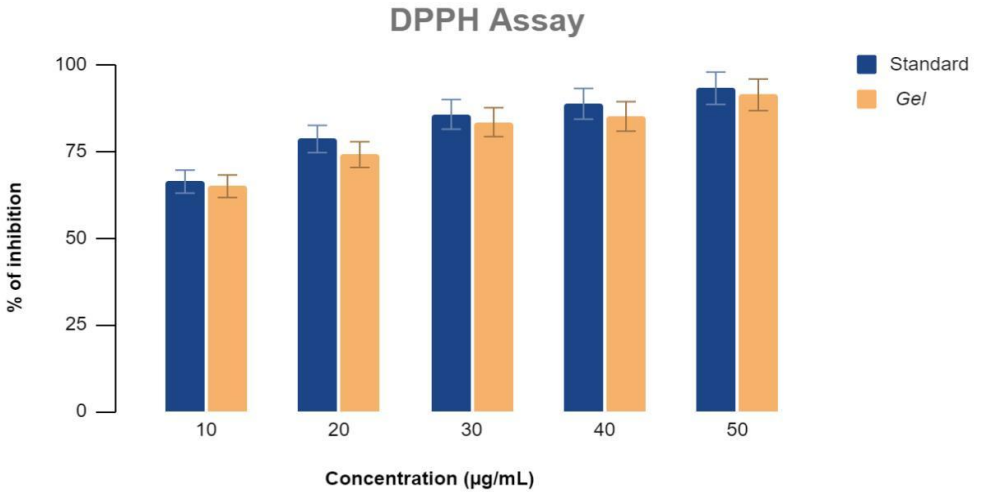

Figure 4. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using DPPH assay.

Table 1. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using DPPH assay.

DPPH	Standard	Saffron + ginger+ W.somnifera + tulsi (gel)	F value	p-value
10	66.25	62.73	0.224	0.649
20	78.52	74.38		
30	85.63	83.04		
40	88.68	85.84		
50	93.15	90.22		

NS -Not statistically significant at $p>0.05$, One-Way ANOVA

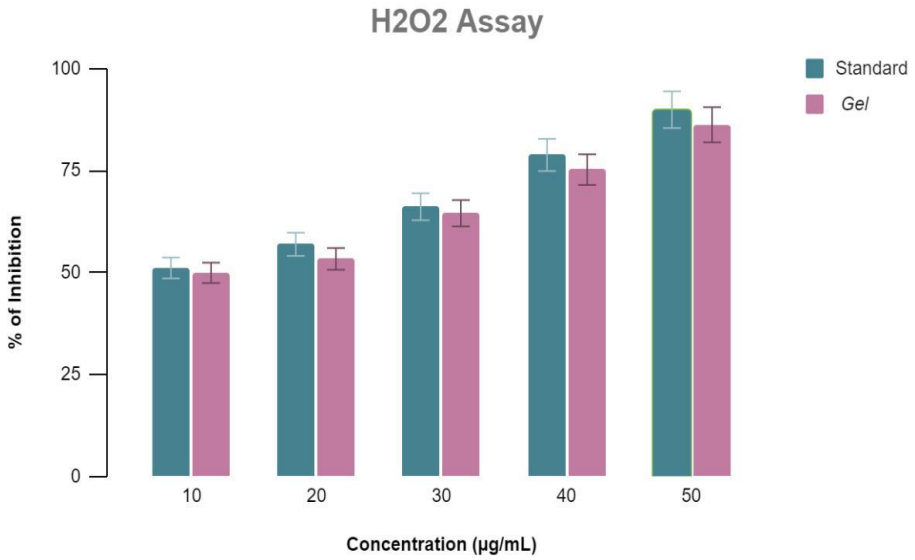

Figure 5. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using H2O2 assay.

Table 2. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using H2O2 assay

H2O2	Standard	Saffron + ginger+ W. somnifera + tulsi (gel)	F value	p-value
10	51.1	49.9	0.178	0.685
20	56.9	52.2		
30	66.1	61.4		
40	78.8	74.6		
50	89.9	84.3		

NS -Not statistically significant at p>0.05, One-Way ANOVA

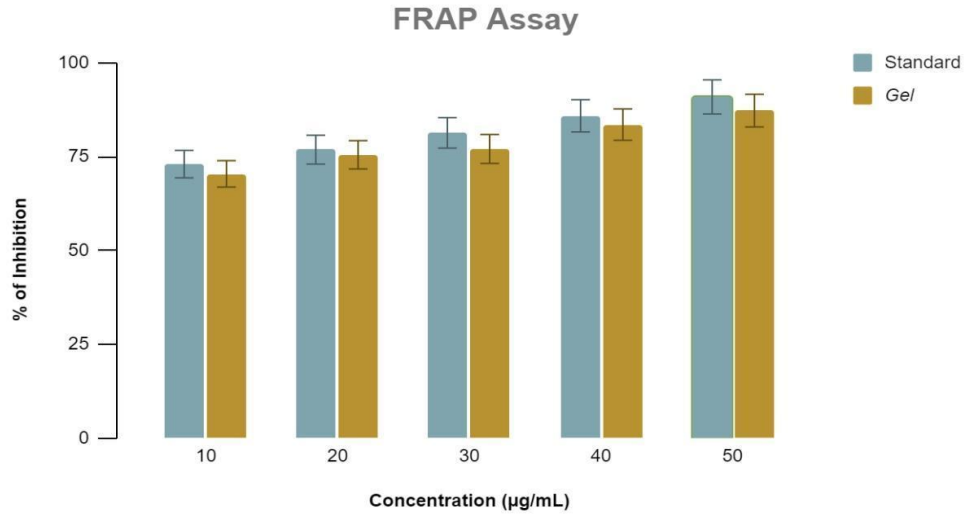

Figure 6. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using FRAP assay.

Table 3. The antioxidant activity of withania somnifera, saffron, dry ginger and tulsi using FRAP assay

FRAP	Standard	Saffron + ginger+ W. somnifera + tulsi (gel)	F value	p-value
10	72.98	69.38	0.643	0.446
20	76.84	72.54		
30	81.31	78.334		
40	85.84	80.09		
50	90.89	88.93		

NS -Not statistically significant at p>0.05, One-Way ANOVA

The results indicate that in DPPH assay at concentration of 10,20,30,40,50 micrograms/ml percentage of inhibition are 62.73, 74.38, 83.04, 85.84, and 90.22 respectively. In H2O2 assay percentage of zone of inhibition at the same concentrations mentioned above are 49.9, 52.2, 61.4, 74.6, 84.3 and in FRAP assay it is 69.38, 72.54, 78.34, 80.09, and 88.93respectively.

P value of DPPH assay is 0.649, H2O2 assay is 0.685, and for FRAP assay it is 0.446 which indicates that the test does not show a statistically significant difference but the values of the ingredients used were closer with that of the standard.

DISCUSSION

The purpose of this study is to determine whether using various traditional herbal components can help in treating recurrent aphthous stomatitis.

Despite thorough examination, the origin of the frequent oral mucosal condition, recurrent aphthous stomatitis still remains unknown, and treatment options are limited[11].Finding a suitable formulation is challenging becauseRAS is a recurrent illness with a complex etiology. Traditional medicine has been using naturally available constituents for a number of years. Aphthae is now being treated with a few natural therapies that are being investigated. Herbal therapy can treat aphthous ulcers by reducing pain, inflammation, and infection[12]. There are several benefits to traditional medicine, including lower side effects, elimination of resistance and cost-effectiveness.

Alterations in antioxidant enzyme activities, catecholamine content, dopaminergic D2 receptor binding, and tyrosine hydroxylase expression were shown to be prevented by pretreatment with ashwaganda extract[13]. Comparing the traditional formulation of ashwagandharishtha and woodfordia fruticosa flowers (ASG-WFS) to other formulations and standard ascorbic acid, it demonstrated the highest activity ($p < 0.001$). Significant DPPH hydrogen peroxide scavenging (69.62%) and free radical scavenging (78.75%) were demonstrated by ASG-WFS at concentrations of 1000 g/mL and 100 g/mL, respectively[14].

The scavenging rate of the DPPH radical was almost 100% when the concentration of ethanol extracts reached 5 mg/mL. These results are especially noteworthy because saffron has several times stronger antioxidant activity than berry extracts and red wines[15]. Trolox equivalent antioxidant capacity (TEAC) assay values demonstrated the high antioxidant activity of whole flowers of crocus sativus, all of their isolated sections, including floral bio-residues. At 6 minutes, the TEAC values of stigmas, stamens, floral bio-residues, and tepals were greater than those of styles and complete flowers[16]. According to Yang Chen et al anti-hemolysis, DPPH radical-scavenging, and lipid peroxidation experiments did not show a link between antioxidant capabilities and total crocin (chemical responsible for colour of saffron) levels, indicating that crocins likely did not play a significant role in the antioxidant capacities of fractions and extracts[17]. The level of RA (Rosamarinic acid), the most common phenolic component found in basil, was significantly and favorably linked with the antiradical actions. RA may influence various biological functions, such as anti-inflammatory or anticancer characteristics, in addition to its antioxidant properties[18]. Jasmonic acid (JA) and arachidonic acid (AA), chemicals employed as elicitors (that produce resistance in plants), did not significantly alter its capacity to scavenge free radicals in tulsi in contrast to the control[19]. PDG (Powdered Dry Ginger) displayed the lowest levels of antioxidants, which is consistent with its lower levels of phenolics and flavonoids[20]. The DPPH free radical scavenging activity of methanolic extract of dry ginger was higher than expected at 39.6, 64.7, 77.6, and 84.4% in 0.25, 0.5, 0.75, and 1.0 mg of sample,

respectively[21,22]. Our study revealed the highest zone of inhibition of 90.22, 84.3,88.93% at a concentration of 50 microgram per millilitre which is near to that of standard value, but p value is not significant in all the three assays.

In conclusion, in this formulation 4 herbal components like Saffron, Withania somnifera, Tulsi are Dry ginger included to determine the antioxidant property in treatment of recurrent aphthous stomatitis. This study gives a clear understanding that the naturally occurring constituents of medicinal herbs will be able to resolve oral ulcers irrespective of their etiologyHence, herbal medicines can be used as an adjunct to synthetic drugs available commercially.

This study has performed the antioxidant efficacy of withania somnifera, tulsi, dry ginger and saffron , further studies like Anti-Inflammatory, cytotoxic properties are required to proceed with gel formulation in treatment of recurrent aphthous stomatitis. Further invitro evaluation of the anti-inflammatory, cytotoxic and antimicorbial properties followed by a cinical trial is necessary to evaluate and validate the clinical efficacy of the herbal components in the management of RAS

Author Contributions: Concept/Design : MM, DSP, RS; Data acquisition: MM, DSP, RS; Data analysis and interpretation: MM, DSP, RS; Drafting manuscript: MM, DSP; Critical revision of manuscript: MM, DSP, RS; Final approval and accountability: MM, DSP, RS; Technical or material support: -; Supervision: MM, DSP, RS; Securing funding (if available): n/a.
Ethical Approval: Scientific Review Board-The date of the study is SRB/SDC/OMED-2204/23/136 from the Ethics committee of Saweetha Faculty of Dentistry dated 27.11.2023 and the number of the study is.
Peer-review: Externally peer-reviewed.
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