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EXTRACTION, PURIFICATION AND CHARACTERIZATION OF ROSMATINIC ACID FROM OREGANO (ORIGANUM VULGARE L.) AND USING IT AS AGAINST STREPTOCOCCUS MUTANS CAUSING DENTAL CARIES

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Abstract
Dental caries continues to be one of the most common oral diseases, with a multifactorial and polymicrobial origin. Although Streptococcus mutans has been identified as the primary etiological bacterium in caries research, its effects on the tooth level are still unclear. This study sought to extract rosmarinic acid using an ultrasound-assisted method and methanol as a solvent system for 25 minutes, followed by silica gel chromatography for purification, even though Streptococcus mutans is a significant factor in the initiation and progression of caries. Rosmarinus acid exhibits a maximum absorbance at 330 nm in UV-Vis spectra and a retention period of 2.28 min in HPLC analysis. The presence of carboxyl, hydroxyl, and aromatic ring stretching were detected by FTIR analysis of rosmarinic acid's functional groups, and TLC analysis indicated bright blue spots of rosmarinic acid with an Rf value of 0.5. Streptococcus mutans has a major role in the development and advancement of caries nd the caries-affected patients' tooth plaque contained Streptococcus mutans. These isolates exhibited high antibacterial activity against all tested Streptococcus mutans isolates with 16–128 µg/mL as minimal inhibitory concentrations (MICs), following treatment with pure rosmarinic acid. The acquired results demonstrate the possible uses of Origanum vulgare leaves as a source of rosmarinic acid, which can be used to oral hygiene routines or used therapeutically.

Keywords: Rosmarinus acid, oregano leaves, S. mutans.

Introduction

Many plants, particularly those in the Lamiaceae family, contain the phenolic compound rosmarinic acid. This family includes many species that are used as culinary herbs, such as oregano (*Origanum vulgare* L.) and rosemary (1). Lamiaceae species have been used traditionally in medicine to treat respiratory, Alzheimer's, stress, headache, depression, and asthma (2). These plants also have anti-inflammatory, astringent, antibacterial, anti-allergic, and antioxidant properties. These characteristics are mostly a result of the phenolic compounds, particularly rosmarinic acid (3), that are present in plants. Particularly the phenolic acids provide a significant class of potent natural chemicals having substantial lipid and water solubility that, when used as functional elements in emulsion model systems, can stop oxidative deterioration (4).

Dental plaque is an adhering coating of bacteria and their byproducts that builds up on all tooth surfaces as a white, greenish, or even yellow film. After one to two days without oral care, dental plaque normally gathers at stagnant or retentive sites (5). Dental caries, or tooth decay,

has been a problem for people since the beginning of civilization and is currently one of the most prevalent infectious diseases in the world (6).

It is believed that *S. sobrinus* and members of the *mutans* streptococci group are the primary causes of dental caries. The most frequent pathogens found in human oral illnesses are these bacteria (7). The ability of *Streptococcus mutans* to form dental plaque, or biofilm, on tooth surfaces is one of its primary pathogenicity characteristics, and it has been connected to dental caries in humans (6). By using glucosyltransferases to convert sucrose into adhesive glucan, the bacteria produces glucan that helps its cells attach firmly to dental surfaces (8).

The effectiveness of antibiotic medications is declining, and bacterial resistance to antimicrobial agents has grown and spread; all of these factors have an impact on human health. Thus, since the beginning of humans, traditional alternative medicine has been used as a source to cure infectious disorders (9). Numerous investigations indicate that the incidence of bacterial strains resistant to antibiotics is increasing, particularly in the oral cavity (10). Plants and their extracts have been used as medicines for ages, which is why the emphasis is returning to plant-based alternatives. Therefore, the purpose of this study was to extract and purify rosmarinic acid from oregano leaves and to determine its effectiveness against the dental caries-causing *Streptococcus mutans.*

Materials and methods

Preparation of plant material

The oreganleaves were gathered from grocery stores, then the samples of air-dried leaves were crushed into a fine powder and kept at 5 °C out of the light until analysis.

Ultrasound-assisted extraction of rosmarinic acid

With certain modifications to the procedure outlined by (11), an ultrasonic probe device was used to perform the ultrasound-assisted extraction. 1.5 g of dried powdered leaves were extracted using 50 mL of 100% methanol as the extraction solvent over a range of durations (5, 10, 15, 20, 25, 30, 35, 40, and 45 minutes) at a power of 300 W ultrasonography. A calibration curve containing rosmarinic acid that was built based on 330 nm was used to quantify rosmarinic acid.

Chromatographic purification of rosmarinic acid

Column chromatography in silica gel was utilized to separate the rosmarinic acid from the methanolic extract. For the elution process, the mobile phase was made up of water, methanol, and aqueous trifluoroacetic acid (0.5 % (v/v)). The absorbance was measured for each fraction at 330 nm (12). Prior to HPLC analysis, the material was centrifuged for ten minutes, and before being put straight onto the HPLC column, the resultant supernatant was filtered through Millipore membrane.

Method of analysis for rosmarinic acid

1- Identification by HPLC

The rosmarinic acid extract was submitted to preparative HPLC using an RP-C18 column and an isocratic combination (80:20) of methanol and 0.1% acetic acid for the mobile phase. After

ten minutes of running, the extraction was determined at 320 nm. In contrast to the rosmarinic acid standard's retention period, the peak of rosmarinic acid was extracted from the column (13).

2- Identification by TLC

The elution with mobile phase that consisted of formic acid, acetone, and methylene chloride (0.85:2.5:8.5) was used in TLC characterization, and the separated spots were visible at 254 nm under UV illumination (13).

3- Identification by UV- spectrum

The peak of purity and identity of rosmarinic acid was verified using the UV spectrum. By contrasting the outcome with the reference solution, selectivity was examined.

4- The IR spectrum

The FT-IR spectra of the extracted and standard rosmarinic acid, which ranged from 4000 to 400 cm-1, were recorded using an FTIR spectrophotometer.

Isolation of *Streptococcus mutans*

Patients who were actively experiencing caries had their cervical margin and the surface of their intact enamel swabbed in order to get plaque samples. The samples were brought from the dentistry clinics' brain-heart infusion broth to the microbiological lab in less than ten minutes. They were kept in test tubes in the lab at 37°C for the entire day after being added to 10 ml of brain heart infusion broth. As a selective medium for the cultivation of *S. mutans*, tryptone-yeast-cysteine-sucrose-bacitracin agar was streaked with the growth from the brain heart infusion brothThey were kept in test tubes in the lab at 37°C for the entire day after being added to 10 ml of brain heart infusion broth. As a selective medium for the cultivation of *S. mutans*, tryptone-yeast-cysteine-sucrose-bacitracin agar was streaked with the growth from the brain heart infusion broth. They were kept in test tubes in the lab at 37°C for the entire day after being added to 10 ml of brain heart infusion broth. As a selective medium for the cultivation *of S. mutans*, tryptone-yeast-cysteine-sucrose-bacitracin agar was streaked with the growth from the brain heart infusion broth (14). Every isolated bacterium was identified down to the species and genus levels. It was determined whether bacterial isolates could ferment sorbitol, sucrose, and inulin using biochemical reactions-based techniques like the catalase test, dextran production, and sugar fermentation test. Microscopically, bacterial isolates were also recognized based on characteristics of microorganisms such as Gram responses, morphology, and organization.

Evaluation of antibacterial properties for rosmarinic acid against *Streptococcus mutans*

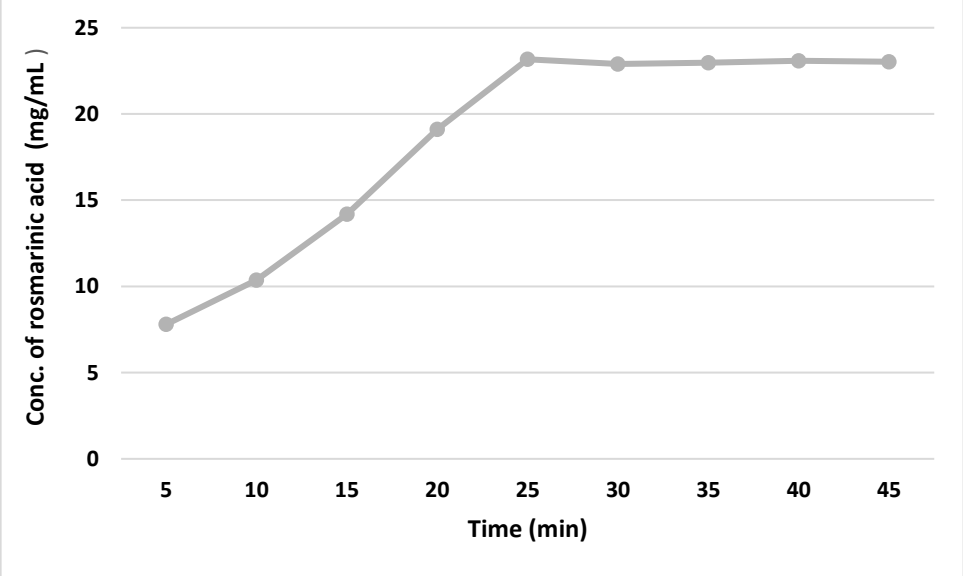
Using the microdilution method, the minimal inhibitory concentration profile of rosmarinic acid was found. A twenty-four-well microtiter plate was utilized; one milliliter of brain heart infusion broth was poured into each well, and one milliliter of BHI broth, which had been serially diluted two times up to 14 wells, was added to the first well along with 50 microliters of rosmarinic acid. One hundred microliters of adjusted inoculum (0.5 McFarland standards) were added to each well. Also included were two control wells: one for as positive control

(without rosmarinic acid) and another for negative control (containing broth only). The plate was covered and incubated anaerobically for an entire day at 37°C. In order to ascertain the minimum inhibitory concentration (MIC), wells were compared with the controls. The minimum inhibitory concentration (MIC) of an antibiotic is its ability to prevent the pathogen from growing. Three duplicates of the experiment were run (15).

Results and discussion

Screening of rosmarinic acid content in oregano leaves extract

The content of rosmarinic acid was measured subsequent to its extraction using methanol and an ultrasonic probe instrument. As the extraction period rose, the concentration of rosmarinic acid grew as well. As shown in figure (1), it reached 23.17 mg/mL after 25 minutes, and there was no further growth after that. Fick's second law, which stipulates that after some time, there is a final equilibrium between the solvent and solid matrix solution concentrations, accounts for this phenomena by causing the extraction yield to decelerate (12).

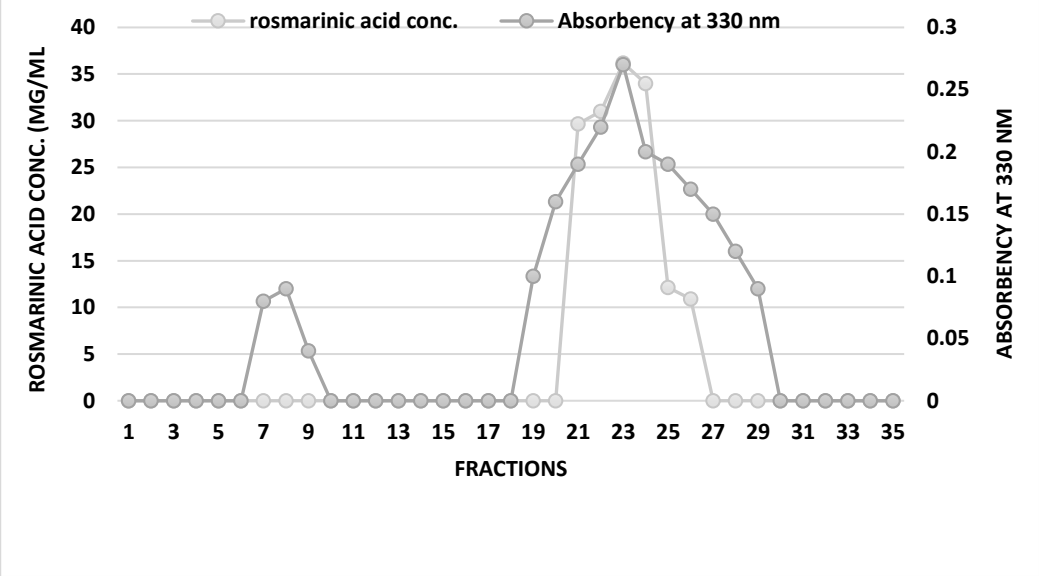


Figure(1);Effect of time on extraction of rosmarinic acid from oregano leaves

An organic solvent with a mild polarity is used to extract rosmarinic acid from plants (16). According to certain research, different quantities of binary solvent combinations, including ethanol, methanol, and water, are utilized to extract rosmarinic acid (17). When creating extracts of plants high in rosmarinic acid, it is preferable to use acidified ethanol or methanol-water mixtures since these solvent systems denature the plant cell membrane and stabilize the rosmarinic acid (18).

Chromatographic purification of rosmarinic acid

After equilibrating with methanol, a chromatographic column filled with silica gel was loaded with the crude extract of rosmarinic acid. As shown in figure (2), the sample was placed at the top of the column, and the adsorbed compounds were placed in the desorbed elution buffer, which included water, 0.25 percent (v/v) aqueous trifluoroacetic acid, and methanol at a final concentration of 38.45 mg/mL.



Figure(2): An adsorption chromatography for rosmarinic acid purification from oregano leaves

Our findings concur with the research by (19) which found that methanol recovers rosmarinic acid more efficiently than ethanol as an extraction solvent. However, (12) shown that the best method for recovering rosmarinic acid was extraction using acidified aqueous ethanol (ethanol -H2O-HCl, 70:29:1, v/v/v) in comparison to other solvent systems.

1- Identification by HPLC

Using the standard rosmarinic acid at 2.49 minutes, the HPLC analysis of the oregano methanolic extract showed that rosmarinic acid was separated as the main component after 2.28 minutes of retention time as shown in figure (3).

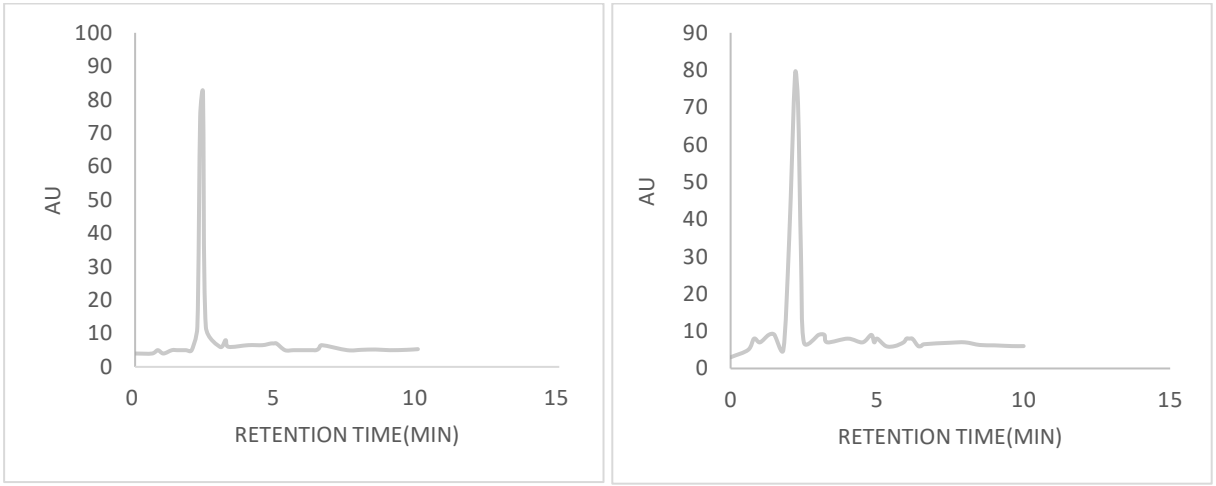


Figure (3): High performance liquid chromatography analysis of a) the standard rosmarinic acid, b) the purified rosmarinic acid

2- Identification by TLC

According to figure (4) the aqueous methanolic extract exhibited the best component separation following adsorption chromatography, with luminous blue spots of rosmarinic acid visible at 366 nm UV light and an Rf value of 0.5 when compared to normal rosmarinic acid.

Figure(4):TLC analysis of standard rosmarinic acid and purified rosmarinic acid

3- Identification by UV- spectrum

The measurement of rosmarinic acid was done at 290 and 330 nm in wavelengths, according to the chromatograms in figure (5), with 330 nm as maximum absorption for rosmarinic acid. In contrast to the normal 329 nm, the maximum UV absorbance of the rosmarinic acid properties derived from *Rosmarinus officinalis* L. herb was 328 nm (13).

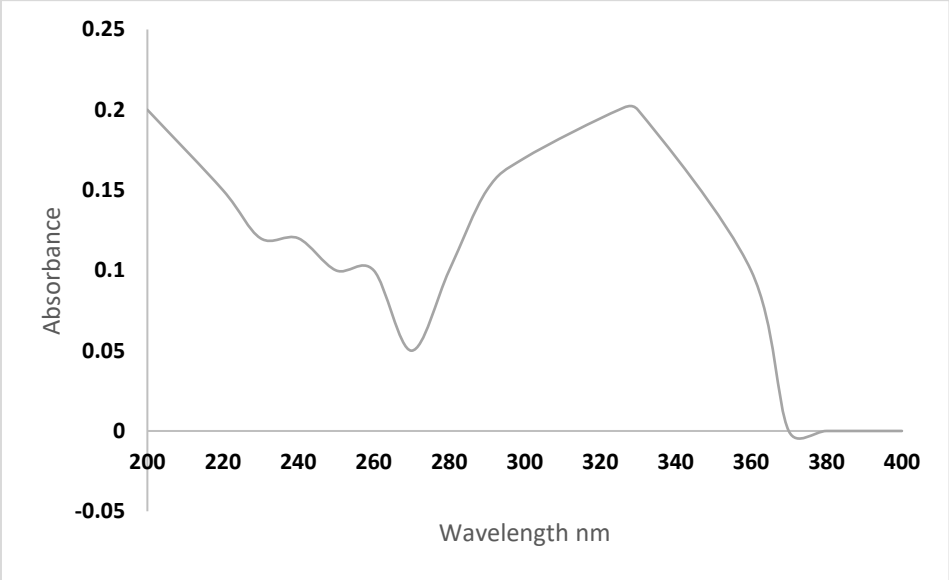


Figure (5): UV- spectrum of purified rosmarinic acid

4- The IR spectrum

The extracted and standard rosmarinic acid FTIR spectra revealed the existence of a wide O-H group absorption band at 3170/cm. Furthermore, there are two absorption bands at 1728 and 1662/cm that are connected to the C=O group. In addition, there were three more peaks at 1520, 1582, and 1492 /cm that were associated with aromatic ring stretching when compared to the typical rosmarinic acid peaks shown in figure (6).

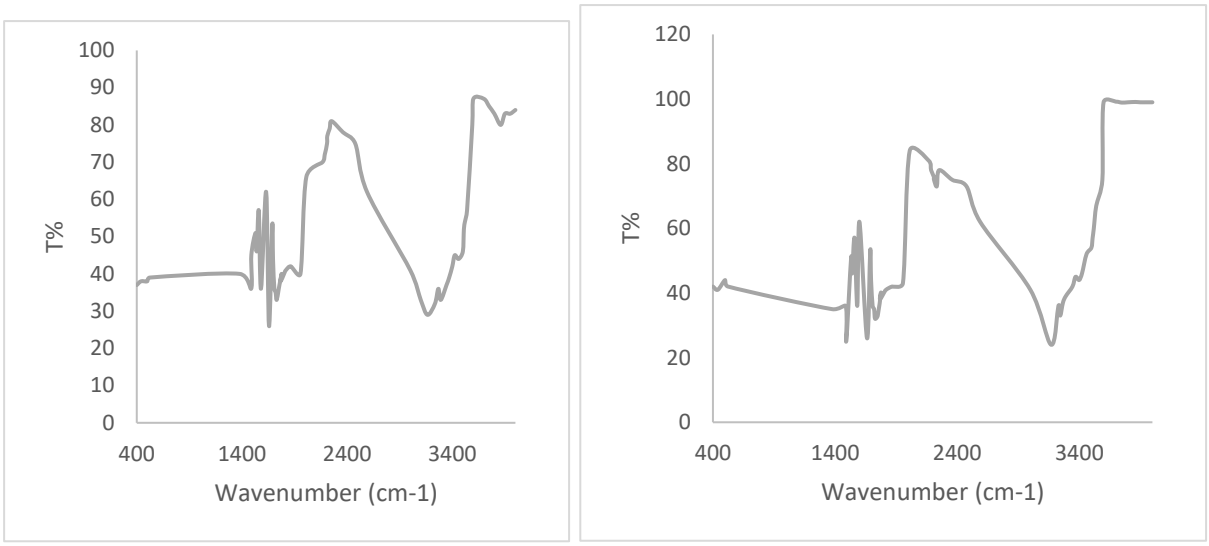
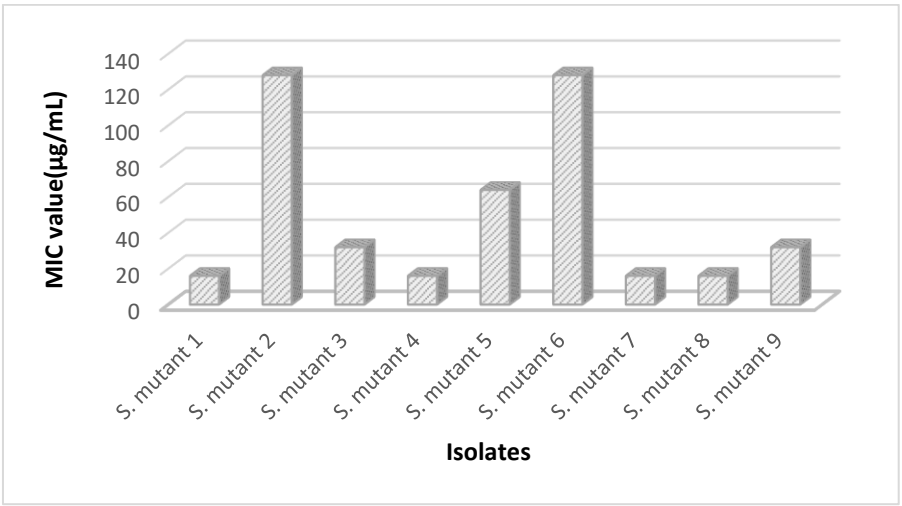


Figure (6): IR spectrum with a) the standard rosmarinic acid, b) purified rosmarinic acid

Evaluation of antibacterial properties for rosmarinic acid against *Streptococcus mutans*

From the dental plaque of patients with active caries, nine isolates of *Streptococcus mutans* were isolated. Following treatment of the isolates with pure rosmarinic acid, the isolates showed significant antibacterial activity of this phenolic compound against all tested isolates of *Streptococcus mutans*, with MIC values ranging from 16 to 128 µg/ml (figure-7). Because of this, it has a promising usage in the dental industry for products like toothpaste and mouthwashes that aim to stop the growth of *Streptococcus mutans.*



Figure(7): Detection minimium inhibitory concentrations of purified rosmarinic acid toward *Streptococcus mutans* isolates

Dental caries, the most common oral illness, is thought to be mostly caused by *S. mutans*. In addition to poor oral hygiene and insufficient fluoride exposure, the most accurate description of dental caries is as a complex biofilm-mediated illness that is primarily caused by the frequent consumption of fermentable carbohydrates such glucose, fructose, sucrose, and maltose (14).In addition to directly contributing to the demineralization of tooth surfaces, *S. mutans* has the ability to produce acidic end products that may have an impact on the microbial flora associated with caries during the cariogenic process (20).

According to (21), rosmarinic acid possesses antibacterial activity against *Salmonella, E. coli, B. subtilis*, and *Staphylococcus aureus.* Furthermore, by generating morphological changes in cheese and meat samples, such as cell shrinkage and the creation of burr-like structures on the cell surface, rosmarinic acid has inhibitory effects on the S. aureus cocktail, as well as by reducing and decreasing all viable cells (22). It's possible that cellular enzyme inactivation and modifications to membrane permeability are part of RA's method of action (23, 24).

Conclusion

An excellent source of rosmarinic acid is thought to be an oregano (*Origanum vulgare* L.). Dental caries is mostly caused by *Streptococcus mutans*, which is resistant to the antibacterial effects of rosmarinic acid. This promotes the use of rosmarinic acid as a herbal adjuvant to synthetic drugs for the treatment of periodontitis and caries through growth and biofilm management.

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