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Phytochemical Composition and Multifaceted Bioactivities of *Rhamnus alaternus* Extracts

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ABSTRACT

Rhamnus alaternus, a plant species widely recognized for its medicinal potential, has been extensively researched regarding the extraction of bioactive compounds and their wide-ranging applications. This review focuses on the methods of extraction, phytochemical analysis, and the diverse biological effects of compounds sourced from *R. alaternus*. Two principal extraction methodologies, namely Soxhlet extraction and aqueous extraction, have been employed to extract bioactive compounds from *R. alaternus*. Soxhlet extraction utilizes organic solvents to efficiently extract a broad spectrum of compounds, whereas aqueous extraction offers an eco-friendly option, especially for water-soluble compounds. The analysis of phytochemicals in *R. alaternus* extracts unveils a rich array of bioactive components including alkaloids, sugars, flavonoids, phenolics, tannins, saponins, and terpenoids. Quantitative assessment and investigation of these constituents have underscored their substantial antioxidant, antidiabetic, anti-inflammatory, antimicrobial, and antimutagenic properties. These characteristics position *R. alaternus* extracts as compelling contenders for applications in pharmaceuticals and nutraceuticals. In summary, *R. alaternus* presents itself as a valuable reservoir of bioactive compounds exhibiting a wide range of pharmacological activities, suggesting potential for forthcoming therapeutic and preventive uses within the healthcare and pharmaceutical sectors.

1. Introduction

Italian buckthorn, also known as *Rhamnus alaternus*, is a plant species that grows naturally in rocky hillsides, scrublands, and woodlands in the Mediterranean regions of Southern Europe, North Africa, and Southwest Asia (Ben Ammar et al., 2007). It belongs to the Rhamnaceae family and has smooth, grayish-brown bark that becomes rougher with age. Its leathery, dark green leaves are lustrous, and it has a dense, convex crown with a shallow, spreading root system (Kubitzki., 2004).

The plant's leaf and bark parts can be extracted for flavonoids, alkaloids, tannins, carbohydrates, quinone, oxalate, and saponin using different solvent systems such as methanol and water. (Zeouk et al., 2019).

The leaf extracts of Italian buckthorn possess flavonoids and tannins, which have antimutagenic properties (Carranza et al., 2015). The plant's bioactive components have various pharmacological properties, including antidiabetic, antibacterial, antifungal, antimutagenic, and antioxidant properties in its leaf extract, making it suitable for therapeutic purposes. Its antioxidant qualities help fight diseases caused by oxidative stress, while its antimicrobial and anti-diabetic qualities make it promising for dietary supplements or functional meals (Bhouri et al., 2011). *Rhamnus alaternus* extracts are known for their antioxidant properties, which make them useful in anti-aging lotions and serums for skin treatment.

In addition, due to their antimutagenic properties, these extracts can be taken as a dietary supplement to prevent cancer and induce apoptosis (Jemal et al., 2003; Kim et al., 2003; Al-Dabbas et al., 2006). The leaf extract of *R. alaternus* may have Geno protective properties, which can help protect the human genome against the harmful effects of chemical treatment. Furthermore, it may also prove to be a valuable source for the development of new cancer treatments (Leila Gadouche et al., 2022).

Extracting bioactive components from Italian buckthorn is a sustainable and natural way to create health-promoting products. The nutraceutical, cosmetic, and pharmaceutical sectors can

benefit from the bioactive components extracted from Italian buckthorn, making it a promising area of research. This research has the potential to significantly contribute to the scientific community's understanding of plant-based medicine.

2. Materials and Methods

Extraction and Phytochemical Screening

Rhamnus alaternus was obtained from a botanical garden. The leaves were carefully picked, cleaned, and dried in a well-ventilated area. Once fully dry, they were ground into a fine powder for further analysis. Two extraction methods were used - the Soxhlet and aqueous extraction methods. Methanol was used as the solvent for the Soxhlet extraction method to prepare a methanol extract, while water was used for the aqueous extract.

The Methanol extract of *Rhamnus alaternus* was screened for the presence of various compounds through phytochemical analysis. The results show that it contains alkaloids, carbohydrates, flavonoids, saponins, tannins, quinones, oxalate, fat, and fixed oils. These compounds are known to have diverse bioactive compounds with various pharmacological activities. For instance, alkaloids are known for their analgesic and anti-inflammatory effects, while flavonoids and tannins are recognized for their antioxidant properties. Quinones have antimicrobial and antiviral properties. Interestingly, all the compounds were detected in the aqueous extract except for tannin.

Quantification process

Estimation of alkaloids

For the quantification of alkaloids, 10 mg of the extract residue was solubilized in 1 ml of Dimethyl Sulfoxide (DMSO). Subsequently, 100 µL of the resultant sample was aliquoted into individual test tubes. The pH of phosphate buffer was adjusted to 4.7 using 0.1N sodium hydroxide (NaOH), 5 ml of Bromocresol Green (BCG) solution, and 5 ml of Phosphate buffer(2M) was added, and the mixture was homogenized. The resulting complex was then extracted sequentially using 1, 2, 3, and 4 ml of chloroform, respectively, through vigorous agitation. Finally, the extract was quantified via spectrophotometry by recording its absorbance at a wavelength of 470 nm.

Estimation of flavonoids

To estimate the flavonoid concentration in plant extracts,10 mg of the extract residue was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Prepare a separate stock solution by dissolving 25 mg of Rutin in 25 ml of methanol and diluting it with 1 ml of methanol. Subsequently, dilute the aliquot with distilled water and add 300 µL of 5% sodium nitrite. Incubate the solution at room temperature for 5 minutes. Then, introduce 300 µL of Aluminum Chloride and allow the reaction to proceed for 6 minutes. The absorbance of the sample solution was detected at the wavelength of 510 nm using spectrophotometry.

Estimation of carbohydrates

To estimate the carbohydrate concentration in plant extracts, a 10 mg residue extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Increasing from 0.1 ml to 1 ml aliquots, each test tube was filled with distilled water up to 1 ml. After that, 3 ml of 3,5-dinitro salicylic acid (DNS) reagent was added. Then the samples were heated in a boiling water bath for 15 minutes. Subsequently, 1 ml of 40% potassium sodium tartrate (PST) solution was added and the absorbance was measured at a wavelength of 540 nm using a spectrophotometer.

Estimation of tannins

For the estimation of tannin, dissolve 10 mg of the extract residue in 1 ml of dimethyl sulfoxide (DMSO) to prepare the sample solution. Different tubes were employed, with aliquots of the solution gradually increasing from 0.2 ml to 1 ml. Subsequently, distilled water was added and made up to 7.5 ml. Following this, 0.5 ml of Folin-Ciocalteu (FC) reagent was added followed by the addition of a 1 ml solution of 35% sodium carbonate. Then subjected to incubation for 30 minutes at room temperature, and the absorbance of the sample was measured by using a spectrophotometer at a wavelength of 700 nm.

Estimation of fats and fixed oils

The quantification of fats and fixed oils was employed using the titration method. Employing a neutral solvent mixture comprising 50 mL of petroleum ether, 50 mL of 95% ethanol, and 2 mL of 1% phenolphthalein, the pH of the solution was adjusted to 7 using 0.1N potassium hydroxide (KOH). Sample preparation involved the addition of 1 mL of the sample (10 mg in 1 mL methanol) to 10 mL of the neutral solvent. Titration was performed until the solution reached a pale pink color, indicating the neutralization of the free fatty acid content within the sample.

Analysis of Biological Activity

Antioxidant activity

In the DPPH assay, the samples were prepared by dissolving 10 mg of the extract in 1 ml of DMSO and combined with different concentrations of Gallic acid solutions and methanol made up to 100 µL. Subsequently, DPPH solution (3 ml) was introduced followed by agitation and incubation in darkness at room temperature for 15 minutes. Then the absorbance was measured at 517 nm using a spectrophotometer. A calibration curve of absorbance versus Gallic acid concentration was then plotted to quantify the antioxidant activity based on their radical scavenging ability.

In the ABTS assay, the samples were prepared by dissolving 10 mg of the extract in 1 ml of DMSO. Gallic acid and standard solutions were dispensed into test tubes with different volumes ranging from 10-50 µL, followed by the addition of methanol and made up to 100 µL. Subsequently, 3 ml of ABTS solution was added, mixed, and incubated for 15 minutes. The absorbance of the samples was measured at 517 nm using a spectrophotometer, and a calibration curve of absorbance versus Gallic acid concentration was plotted to quantify the antioxidant activity.

In the FRAP method, test samples and Gallic acid standards were introduced into separate test tubes with methanol to achieve a volume of 100 µL. 2.5 ml of Phosphate buffer (0.2M) and Potassium Ferrocyanide were then added, followed by incubation at 50°C for 20 minutes. The reaction was halted, and interfering substances were removed by adding 2.5 ml of 10% Trichloroacetic acid (TCA) solution. After centrifugation, distilled water and ferric chloride were added to the supernatant,

and the absorbance was measured at 700 nm using a spectrophotometer to assess the antioxidant capacity of the samples.

Activity Units/ml = (absorbance of test – absorbance of blank)

(18.1 x 5 x 0.5)

Where 18.1 is the molar extension coefficient of PNPG,5 is the incubation time after adding an enzyme, and 0.5 is the volume of an enzyme.

Antimicrobial activity

For the antifungal evaluation, Minimum Inhibitory Concentration (MIC) assays were performed utilizing Potato Dextrose Broth (PDB) medium against *Aspergillus niger* and *Candida albicans*. The methanol and aqueous extracts of *Rhamnus alaternus* were prepared at concentrations of 10 µL (1 mg), 20 µL (2 mg), 30 µL (3 mg), and 40 µL (4 mg), respectively. Each concentration of the extract was introduced into distinct wells of a microtiter plate containing PDB medium that had been inoculated with a standardized suspension of *Aspergillus niger* and *Candida albicans*. Subsequently, the plates were incubated at room temperature to facilitate fungal growth. The MIC, defined as the lowest extract concentration that completely inhibited visible fungal growth relative to the control, was determined following a specified incubation period. MIC values were measured in millimeters. The antibacterial efficacy of methanol and aqueous extracts derived from *Rhamnus alaternus* against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* was assessed utilizing the Minimum Inhibitory Concentration (MIC) assay. Initially, standardized bacterial suspensions of each strain were prepared in Luria-Bertani (LB) broth. Subsequently, different concentrations of the methanol and aqueous extracts of *Rhamnus alaternus* (10 µL [1 mg], 20 µL [2 mg], 30 µL [3 mg], and 40 µL [4 mg]) were introduced into microtiter plate wells containing the bacterial suspensions. Control wells comprising solely LB broth and bacterial suspension devoid of extracts were incorporated for comparison. The plates underwent incubation at 37°C to facilitate bacterial growth, following which MIC values were ascertained as the minimum extract concentration inhibiting discernible bacterial growth post-incubation. MIC values were quantified in millimeters.

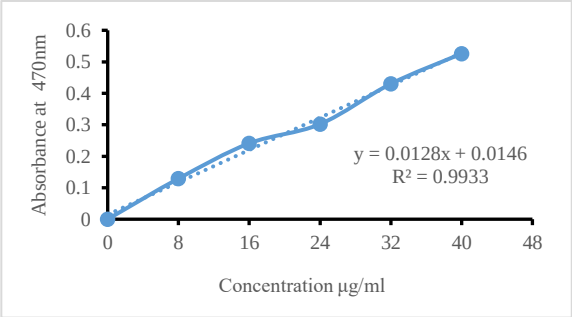
Antimutagenic activity

To prepare the cell culture for evaluating the antimutagenic effects, the DMEM medium was dispensed into each well of a 96-well plate. Subsequently, the cell line was seeded into individual wells, followed by the addition of aqueous and methanol samples along with dimethyl sulfoxide (DMSO) as the solvent. The plates were then transferred to a CO2 incubator for 24 hours to facilitate the interaction and uptake of the test substance by the cells. For comparison, control wells containing only DMEM medium and DMSO were included. Following the incubation period, the samples were assessed by a spectrophotometer measuring their absorbance at 575 nm to determine their antimutagenic activity.

High-performance liquid chromatography

The analysis was made on the C18 column (symmetry, 4·6mm×250mm) in an isocratic mode with the mobile phase acetonitrile and water in the ratio 4:6 with the RP-HPLC at a flow rate of 1mL/min. The standard saponin with the concentration of 2 mg/mL and samples (2.5mg/mL) were dissolved in the mobile phase 20µL was injected and the elution was monitored at 203nm. The amount of saponin present in the samples was estimated using the formula,

Sample area x Standard amount x Dilution of sample x Mean weight



Standard area x Dilution of standard x Sample amount

3. Results and Discussions

Extraction and Phytochemical screening

Both extracts of *Rhamnus alaternus* contain similar phytochemicals, except for tannins which are only present in the methanol extract. The phytochemical screening of *Rhamnus alaternus* extracts, conducted using both aqueous and methanol solvents, provides valuable insights into the chemical composition and potential bioactivity of the plant material. The presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, tannins, saponins, fats, and fixed oils in the extracts elucidates their pharmacological potential and therapeutic efficacy. (Nekka A et al., 2015)

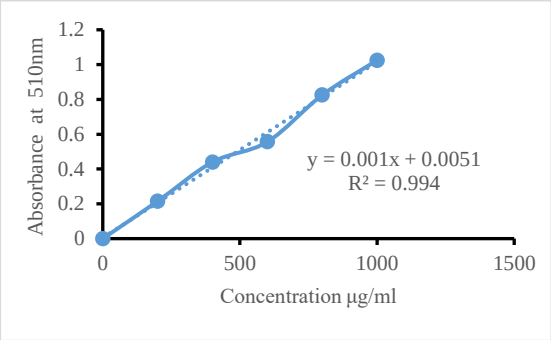
This implies the possibility of *Rhamnus alaternus* having therapeutic use. Its nutritional value is further enhanced by the presence of carbohydrates, which are essential for energy metabolism. A different component that comprises *Rhamnus alaternus* is flavonoids, which have anti-inflammatory and antioxidant characteristics suggesting their possible application in conventional medicine as a natural antioxidant agent. Because saponins have been shown to have antifungal and cholesterol-lowering properties, they are a valuable ingredient in pharmaceutical formulations. Additionally, present and possessing antibacterial and anti-inflammatory qualities, tannins have a long history of use in traditional medicine. Although oxalates are linked to kidney stones, they might also be part of the phytochemical profile.

Table 1: The aqueous and methanol extracts of *Rhamnus alaternus* contain various phytochemicals.

Type of phytochemical	Aqueous Extract	Methanol Extract
Alkaloids	+	+
Carbohydrates	+	+
Cardiac glycosides	-	-
Flavonoids	+	+
Phlobatannins	-	-
Amino acids and proteins	-	-
Saponins	+	+
Sterols	-	-

Tannins	-	+
Terpenoids	-	-
Quinones	+	+
Oxalate	+	+
Fats and fixed oils	+	+

+: Presence of phytochemical; -: Absence of phytochemical



Quantification process

Estimation of alkaloid

The estimation of alkaloids from the plant extract indicated significant pharmacological potential, shedding light on *Rhamnus alaterns'* medicinal properties. The alkaloid content for aqueous and methanol extracts of *Rhamnus alaternus* was estimated by a spectrophotometric method. A calibration curve of absorbance versus caffeine concentration was then plotted for the standard graph. (Amabye 2015)

Fig 1: Standard graph for the estimation of alkaloids

From the above graph, the estimated amount of alkaloid present in the *Rhamnus alaternus* methanol extract was found to be 0.309 mg/ml, for the aqueous extract it was found to be 0.313 mg/ml. In the methanol extract, alkaloids were identified as plant metabolites exhibiting alkali-like chemical reactivity and pharmacologic activity.

Estimation of Carbohydrate

Estimating the amount of carbohydrates in a plant is important for determining its nutritional value and understanding its energy content. The carbohydrate content for aqueous and methanol extracts of *Rhamnus alaternus* was estimated by the spectrophotometric method. A calibration curve of absorbance versus glucose concentration was then plotted for the standard graph.

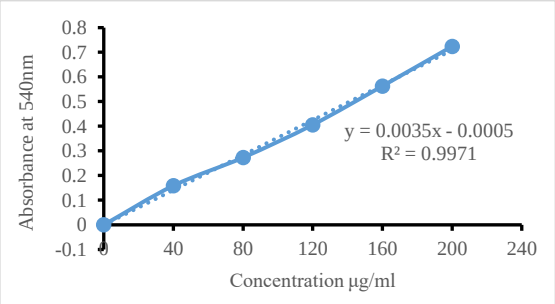


Fig 2: Standard graph for the estimation of carbohydrate

From the above graph, the estimated amount of carbohydrates present in the *Rhamnus alaternus* methanol extract was found to be 0.886 mg/ml, for the aqueous extract it was found to be 0.545 mg/ml. The methanol extract showed a significant presence of carbohydrates, indicating its potential in pharmacology. (Amabye 2015)

Estimation of Flavonoids

The estimation of flavonoids in a plant is important to determine its potential as a natural antioxidant and to understand its health benefits. The flavonoid content for aqueous and methanol extracts of *Rhamnus alaternus* was estimated by a spectrophotometric method. A calibration curve of absorbance versus Rutin concentration was then plotted for the standard graph. (Amine Nekka et al., 2021)

Fig 3: Standard graph for the estimation of Flavonoids

From the above graph, the estimated amount of flavonoids present in the *Rhamnus alaternus* methanol extract was found to be 1.181 mg/ml, for the aqueous extract it was found to be 0.734 mg/ml. The significant amount found in the aqueous extract suggests that it has anti-inflammatory and anticancer activities.

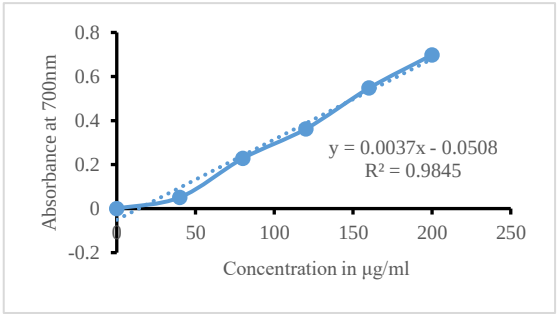
Estimation of Tannin

Tannin estimation of *Rhamnus alaternus* plant extract revealed its pharmacological potential for traditional medicinal applications. The tannin content for aqueous and methanol extracts of *Rhamnus alaternus* was estimated by a spectrophotometric method. A calibration curve of absorbance versus tannic acid concentration was then plotted for the standard graph. (Fouzia Trea et al.,2021)

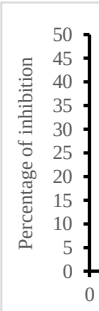
Fig 4: Standard graph for the estimation of Tannin

From the above graph, the estimated amount of tannin present in the *Rhamnus alaternus* methanol extract was found to be 0.687 mg/ml, for the aqueous extract it was found to be 2.120 mg/ml. The estimation of Tannin from the *Rhamnus alaternus* plant extract revealed a significant presence, indicative of its therapeutic properties.

Estimation of fats and fixed oils

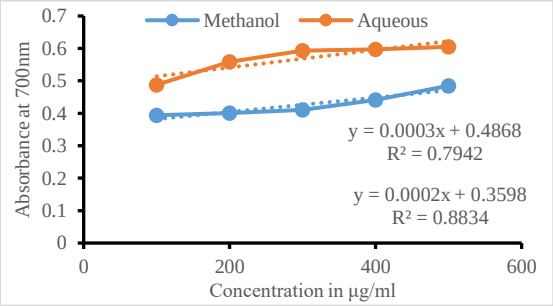


The amount of fats and fixed oils present in a plant sample can reveal its nutritional value and potential uses in medicinal preparations. This information can enrich the plant's utility, making it a valuable source of dietary fats or a base for herbal preparations. The presence of fats and fixed oils in a sample can



be identified by the appearance of a pale pink color. (Amabye 2015)

We have found that the standard has 0.469%, the methanol sample has 0.588% and the aqueous sample has 0.513 %. This



infers the amount of fats and fixed oils presented in *Rhamnus alaternus.*

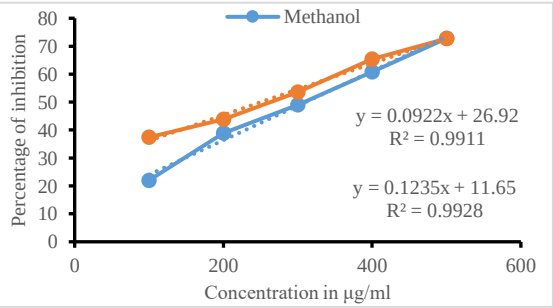
Analysis of Biological Activity

Antioxidant activity

In the DPPH assay, a reduction in absorbance indicates strong antioxidant activity by effectively scavenging DPPH radicals. This suggests that the *Rhamnus alaternus* has the potential to counteract oxidative stress and protect against cellular damage. In the ABTS assay, the absorbance decrease was indicative of the *Rhamnus alaternus* ability to neutralize the ABTS•+ radical, reflecting its antioxidant activity by scavenging free radicals. In the FRAP method, a higher value indicates stronger antioxidant activity. This illustrates the *Rhamnus alaternus* ability to reduce oxidative stress and protect cells from damage. (Amine Nekka et al., 2021)

Fig 5: DPPH assay

In the DPPH assay, the percentage inhibition of the *Rhamnus alaternus* methanol extract was found to be 0.530 mg/ml, while



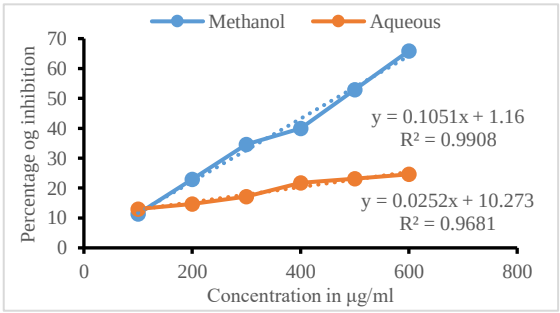
for the aqueous extract, it was 1.268 mg/ml. The methanol extract was found to be more effective than the aqueous extract.

Fig 6: ABTS assay

In the ABTS assay, the percentage inhibition of *the Rhamnus alaternus* methanol extract was found to be 0.310 mg/ml, while for the aqueous extract, it was 0.250 mg/ml. The methanol extract was found to be more effective than the aqueous extract.

Fig 7: FRAP method

In the FRAP method, the antioxidant activity of the *Rhamnus alaternus* methanol extract was found to be 248.201 mg/ml, while for the aqueous extract, it was 165.044 mg/ml. The methanol extract was found to be more effective than the aqueous extract.



Antidiabetic activity

Rhamnus alaternus was subjected to alpha-glycosidase inhibition assays to assess its ability to regulate postprandial glucose levels. Inhibition of alpha-glycosidase enzyme delayed carbohydrate breakdown in the intestine, leading to a gradual release of glucose into the bloodstream. Therefore, a potent inhibitory effect observed in *Rhamnus alaternus* suggested its potential to control blood glucose, which is crucial for diabetic management.

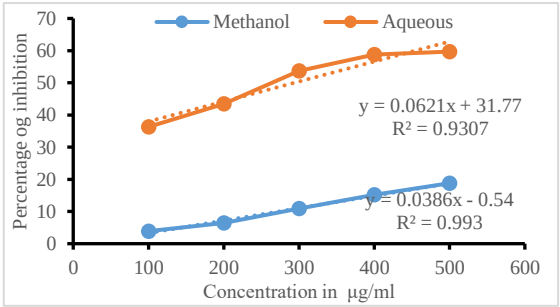


Fig 8: Antidiabetic Assay

Antimicrobial activity

MIC tests were conducted on *Rhamnus alaternus* to evaluate its antimicrobial effects against *Candida albicans* and *E.coli* by leveraging its bioactive compounds. The investigation aimed to determine the minimum inhibitory concentration necessary to inhibit microbial growth, highlighting its potential therapeutic applications. *Rhamnus alaternus* aqueous extracts were active against bacterial and fungal strains. The zone of inhibition was formed against *Candida albicans* and *E.coli*. The concentration of plant extract shows high antimicrobial activity (Amabye., 2015)

Table 2: MIC of Aqueous sample against *Rhamnus alaternus*

Organism	MIC of Aqueous sample in mm			
	10mg	20mg	30mg	40mg
C.albicans	10	11	14	15
E.coli	16	14	14	16

Antimutagenic activity

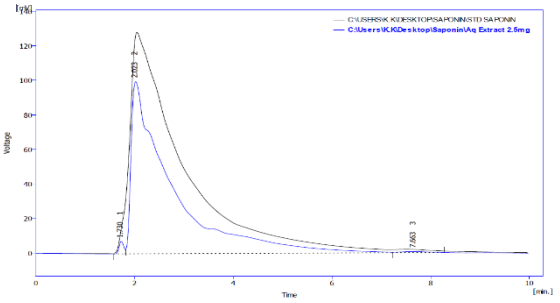
The assessment of antimutagenic activity on *Rhamnus alaternus* was crucial due to its potential role in mitigating genetic damage caused by mutagens. To determine the ability of *Rhamnus alaternus* extracts to inhibit the mutagenic effects we assessed the plant extract on a cell line and observed significant activity in inhibiting mutagenesis. (Amine Nekkaa et al. 2021)

Fig 11: Antimutagenic Assay

The plant extract demonstrated substantial antimutagenic effects on the cell line, with the highest inhibition percentage reaching 65.9% at a concentration of 100 mg/ml for the methanol extract.

High-performance liquid chromatography

The estimation of saponins in *Rhamnus alaternus* plant extract has applications in medicinal products. The saponin content for



aqueous and methanol extracts of *Rhamnus alaternus* was estimated by an RP-HPLC method. The amount of saponin found in HPLC analysis was 0.479 mg/ml.

Fig 12: Saponin estimation- HPLC

4. Conclusion

Subsequent investigation revealed that, in comparison to the water extraction approach, the Soxhlet extraction method produced greater concentrations of several bioactive chemicals. This implies that the concentration and composition of phytochemicals extracted from *Rhamnus alaternus* can be influenced by the extraction process selected. Additionally, the phytochemical screening identified a variety of bioactive chemicals, suggesting that *Rhamnus alaternus* may have a broad spectrum of pharmacological activities. The identification of alkaloids, carbohydrates, tannins, saponins, flavonoids, quinones, lipids, and fixed oils highlights the chemical composition's complexity and points to a diverse range of potential therapeutic uses. The bioactive chemicals isolated from *Rhamnus alaternus* appear to have free radical-scavenging characteristics, which could be a contributing factor to their prospective health benefits, especially in terms of reducing disorders connected to oxidative stress, based on the observed antioxidant activity. Furthermore, the bioactive compounds' antidiabetic activity indicates their potential usefulness in the treatment of diabetes mellitus and associated metabolic diseases. This discovery emphasizes how

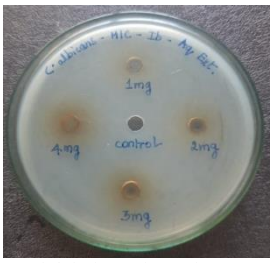


Fig 9: *C. albicans*-MIC-Ib-Aqueous extract

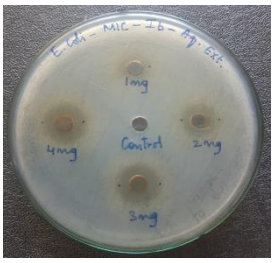


Fig 10: *C. albicans*-MIC-Ib-Methanol extract

crucial it is to investigate natural antimicrobial and DNA damage prevention agents, respectively. The historic use of *Rhamnus alaternus* in folk medicine to treat a variety of infectious diseases and disorders is supported by these findings. Given the circumstances, the thorough examination of *Rhamnus alaterns'* bioactive substances and their antimicrobial, antidiabetic, antioxidant, and antimutagenic properties offers important scientific proof in favor of the plant's possible medical uses in the nutraceutical and pharmaceutical sectors. To clarify the precise mechanisms of action and therapeutic efficacy of these bioactive chemicals for a range of medical disorders, more research is necessary.

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