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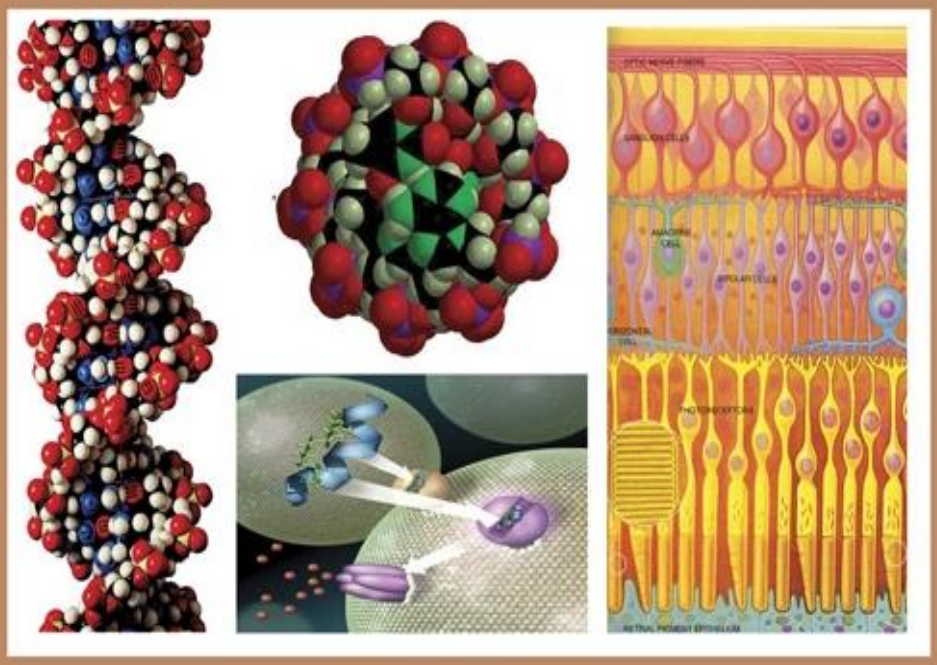

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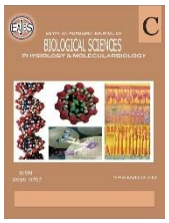

In Vitro Antifungal Activity of The Flavonoid extracts from *Rhamnus alaternus* L. (Rhamnaceae).

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ABSTRACT

Objective: The study was conducted to determine in vitro antifungal activity of flavonoids extracted from leaves and barks from *Rhamnus alaternus* L. (Rhamnaceae) against two fungal strains appertaining to two different classes viz. yeast fungi (*Aspergillus niger* ATCC 16404) and filamentous fungi (*Candida albicans* ATCC 10231). **Methods:** Five flavonoid extracts were obtained from leaves and barks of *Rhamnus alaternus* L. in the mountain of Tessala. Preliminary phytochemical screening was performed by tube staining tests. The inhibition diameters were measured by the solid-state diffusion method. The minimum inhibitory concentrations were determined by the solid dilution method. **Results:** The minimum inhibitory concentration (MIC) varied according to the flavonoid extract, the vegetative organ and the strain fungal type. The diameters of inhibition range from 5 to 24,2 mm for flavonoid bark extracts and from 7,2 to 17 mm for leaf extracts. On the other hand, these fungal strains were almost resistant to the antifungal marketed drugs viz. (Amphotericin B, Fluconazol, Terbinafine and Econazol Nitrate). The minimum inhibitory concentrations (MIC) obtained range between 25 – 50 µg/mL for *Candida albicans* strain and 6,25 µg/mL for *Aspergillus niger*. The phytochemical screening revealed the existence of certain classes of flavonoid such as flavans and flavonols that may be responsible for this antifungal power. **Conclusion:** The antifungal power of flavonoids extracted from leaves and barks of *Rhamnus alaternus* L. varied according to the type of flavonoid extract, its concentrations and resistance or sensitivity of the fungal strains. This plant offers a remarkable therapeutic potential, which reveals its worth in the field of pharmacological industry.

INTRODUCTION

Throughout human history, medicinal plants have been employed as treatments. An estimated 80% of people in developing countries rely almost entirely on traditional medicine and used plants as primary care medicines (Abdala *et al.*, 2021). They are an inexhaustible source of biologically active and natural substances. In fact, secondary metabolites are the subject of many in vivo and in vitro studies, including the search for new natural components such as phenolic compounds (Bhouri *et al.*, 2018).

The hotspot of the Mediterranean basin is a center of endemic species and a region of high biodiversity (Mayers et Cowling, 1999; Médail et Mayers, 2004) with more than 25000 to 30000 species and subspecies according to the authors (Quezel, 1985; Greuter, 1991). In Algeria, we estimated that 3744 taxa listed in the standard flora of Algeria (Quezel et Santa, 1962-1963) are qualified as traditional medicinal plants for therapeutic and aromatic uses. *Rhamnus alaternus* L. (Rhamnaceae) belonging to Rhamnus genus it's a good example of species having a high phytochemical value due to the variety of its active metabolites (Zeouk *et al.,* 2019). It is a perennial dioecious shrub or tree (up to 5 m tall) typical of the Mediterranean area (Davis, 1967) including countries of north Africa (Algeria, Morocco and Tunisia) (Bas et al., 2009). In Algeria, it grows mainly in the north, where it is called "lmliless, l'mlila, Soitfaïr or Safir" (Ait Youssef, 2006). *R. alaternus* has traditionally been used in folk medicine as a digestive, diuretic, laxative, hypotensive (Boukef, 2001), and for the treatment of hepatic and dermatological complications (Ait Youssef, 2012).

In recent years, microbial resistance has been a major challenge for public health. The need of discovering new and novel antibiotics is imperative. However, the natural products represent a potential source of anti-infective agents such as the flavonoids that represent a novel set of leads (Jayshree *et al.,* 2012). Previous studies have demonstrated the tremendous therapeutic potential of plants and secondary metabolites can be sources of biologically active molecules because of the increasing prevalence of the undesirable effects of the antimicrobial drugs and high incidence of infections, but more studies need to be conducted to search for new compounds (Nascimento *et al.,* 2000).

In the present work, we have studied the antifungal activity of the flavonoid extracts from *Rhamnus alaternus* L. against two fungal strains *Candida albicans* and *Aspergillus niger*.

from (American Type Culture Collection): *A. niger* ATCC 16404 et *C. albicans* ATCC 10231.

MATERIALS AND METHODS

Chemicals:

All solvents and reagents used in the experiments were purchased from Merck (Darmstadt, Germany). Oxytetracycline glucose agar (OGA) was purchased from Sigma-Aldrich (Steinheim, Germany). The antifungal discs (Amphotericin B, Fluconazole and Terbinafine) were manufactured by Oxoid (Basingstoe, UK).

Plant Material:

Leaves and barks of *R. alaternus* L. were collected from their natural setting at Tessala mountain in September 2020 (Northwest Algeria, Sidi Bel Abbes City). The plant was taxonomically authenticated by professor Mahroug Samira and certified voucher specimens were deposited at the laboratory. The plant's botanical nomenclature was identified by using the Algerian standard flora (Quezel et Santa, 1963).

Microorganisms:

The fungal strains were purchased

Extraction Protocol:

The crushed leaves and barks were macerated in a mixture of methanol/distilled water (7/3: v/v) at an extraction ratio of 1/10 (w/v) and stirred overnight at room temperature. The hydroalcoholic extract was filtered and methanol was evaporated. 50ml of this extract was frozen and then lyophilized to determine extraction yield (Markham, 1982; Merghem, 1995). The reaming crude extract (Cr. Ex) was extracted several times with hexane (1/1: v/v) until the hexane phase became clear. After evaporation to dryness by using the rotary evaporator, we obtained the hexane extract (Hex. Ex). Then, the aqueous phase was extracted several times with chloroform and then with ethyl acetate to give (Chl. Ex) and (Ac. Ex), respectively. Both extracts were evaporated to dryness. 50 ml of each extract were frozen and lyophilized to determine the

extraction yield. All the lyophilizates were stored at – 20°C until their use. The lyophilized flavonoid extract was weighed to determine the resulting dry weight and the extraction yield was based on 100 g of powder (leaf/bark).

Phytochemical Screening:

The phytochemical screening was made to ensure the presence of flavonoids, and to highlight other polyphenols classes, which may be present in the flavonoids extracts. These tests were performed according to the protocols described by the authors (Dohou *et al.,* 2003; Senhadji *et al.,* 2005). The search for chemical groups was determined in test tubes by precipitation or coloration reactions.

Antifungal Tests:

For the preparation of extract concentrations, 1 mg of each lyophilized flavonoids extracts (Cr. Ex, Hex. Ex, Chl. Ex, Ac. Ex, and Aq. Ex) was introduced into a test tube in which 10 ml of pure DMF were added. These tubes were vortexed until the extract had dissolved completely. For the five extracts, a stock solution at 100 µg/ml was prepared. This solution was then diluted in pure DMF to have for each flavonoid extract, a range of solutions with concentrations of 20, 40, 60, and 80 µg/ml. These five concentrations solutions (100, 20, 40, 60, and 80 µg/ml) were used for the antifungal assay.

The test of the sensitivity of fungal strains was realized by the disc diffusion method, also called the diffusion agar method (Bssaibis et al., 2009): In Petri plates, 20 ml of OGA were poured and left for 20 min to solidify. 1ml suspension of fungi was incubated on the OGA's surface. A 6 mm diameter filter disc (Whatman paper n° 1) that is sterile was impregnated with each concentration (20, 40, 60, 80 and 100 µg/ml). The treated Petri dishes were left for 30 min at room temperature to allow the diffusion of the flavonoid extracts and incubated in an oven at 37°C for 24- 48h. The antifungal activity was assessed by measuring inhibition sizes formed around the disc. The results were expressed as the mean ± deviation of the inhibitory zone diameters in millimeters (mm). In the same conditions, five concentrations solutions (20, 40, 60, 80 and 100 µg/ml) of the standard antifungal drugs: Amphotericin B, Fluconazole and Terbinafine were performed.

The categorization of fungal strains against flavonoids extracts and the antifungal drugs was as follow: sensitive strains + ($10\ mm \leq D \leq Ø$), intermediate strains ± ($d \leq Ø < 10\ mm$), and resistant strains - ($Ø < d$). Where: D is the highest critical diameter (largest diameter reached by the strain), d is the lowest critical diameter (the smallest diameter recorded by the strain), and Ø is the inhibition diameter zone. Each experiment was carried out in triplicate, at the same time, and in the same place.

The Minimum Inhibitory Concentration:

The determination of minimum inhibitory concentrations (MIC) is made by using the broth dilution method (Yahlef *et al.,* 2011; Hammer *et al.,* 1996). It was performed only for flavonoid extract showing good antifungal activity, with an inhibitory zone diameter $\geqslant$ 16 mm. From each flavonoid extract, a stock solution of 100 µg/ml was prepared in pure DMF. From this stock solution, serial dilutions were made to 50, 25, 12.5, 6.25, and 3.12 µg/ml.

The MIC was also defined as the lowest concentration of flavonoid extracts, able to inhibit any visible fungal growth on the culture medium, compared to the control without flavonoid extract.

Statistical Analysis:

The effect of the plant organ, the flavonoid extracts type and its concentrations on antifungal activities summarized by diameters inhibitions zones was highlighted by the analysis of variance (ANOVA) with one, two and several classification criteria at the 5% level. We considered that the difference is not significant for $P < 0,05$, significant when $*P \leq 0,05$, highly significant for $**P \leq 0,01$ and very highly significant $***P \leq 0,001$. These analyses were performed by XLSTAT 2014. Tukey's test was also performed for pair-wise comparisons at 5%.

RESULTS

The flavonoid yield output is determined by the organ plant. The best yield was observed for the crude extract from leaves with 36,6% and that of barks with 31,28%, followed by the Aq. Ex 13,8%, Ac. Ex 7,20%, Chl. Ex 5,68% and Hex. Ex 4,88% from leaf extracts and the Hex. Ex 9,12%, Aq. Ex 7,76%, Chl. Ex 5,68%, Ac. Ex 0,52% from bark extracts. These changes can be explained by the distributions of these pigments in the aerial organs in every stage (Dauguet et Paris, 1974). Furthermore, the solvents utilised have an impact on the yield of flavonoid extraction. The crude extract (Cr. Ex) showed the highest yield. Many studies have confirmed that the flavonoid yield is influenced by the polarity of the organic solvent and its capacity to have a good solubility of flavonoids' hydroxyl groups (Syukriah *et al.,* 2014).

The phytochemical screening (Table 1) showed that polyphenols and flavonoids were present in all extracts reflecting the reliability of the used extraction method. Indeed, stirring in the maceration method accelerates the extraction process, minimizes the contact time with the extracting solvent, and preserves the bioactivity of its constituents. In addition, the development of the extraction at room temperature and the exhaustion of the solvent at a reduced pressure yield the maximum of compounds and prevent any degradation due to the high temperatures used in other extraction methods (Stalikas, 2010). Cr. Ex is the only flavonoid extract, which contains other compounds than flavonoids, namely condensed tannins. This can be explained by the nature of the methanol, which is more polar compared to the other used solvents, and it is characterized by a good solubility for the phenolic compounds (Khoddami *et al.*, 2013).

Table 1. *Phytochemical screening of flavonoid extracts*

Organ	Flavonoid extract	Characterized phenolic compound								
		POL	FLV	CT	HT	FLA	ANT	FLO	PRO	COUM
Leaf	Cr. Ex	+++	++	+	±	++	-	+	-	-
Leaf	Hex. Ex	++	++	-	-	-	-	-	-	-
Leaf	Chl. Ex	++	++	-	-	+	-	-	-	-
Leaf	Ac. Ex	+++	+++	-	-	+	-	+	-	-
Leaf	Aq. Ex	+	++	-	-	-	-	+	-	-
Bark	Cr. Ex	+++	++	-	±	++	-	-	-	-
Bark	Hex. Ex	+++	++	-	-	+	-	-	-	-
Bark	Chl. Ex	++	++	-	-	-	-	-	-	-
Bark	Ac. Ex	+	+++	-	-	-	±	++	-	-
Bark	Aq. Ex	+	++	-		-	-	-	-	-

Ac. Ex: Ethyl Acetate extract**, ANT:** Anthocyanins, **Aq. Ex:** Aqueous extract, **Cr. Ex:** Crute extract, **Chl. Ex :** Chloroform extract**, COUM :** Coumarins, **FLA** : Flavanes, **FLO :** Flavonols**, FLV :** Flavonoids**, Hex. Ex:** Hexane extract**, HT:** Hydrolyzable tannins**, POL:** Polyphenols**, PRO:** Proanthocyanidols**, TC:** Condensed tannins**, +++:** Frankly positive reaction**, ++:** Positive reaction**, -:** Negative reaction**, ±**: Suspicious reaction**.**

The diameters of inhibition zones recorded for the antifungal marketed drugs (Table 2) showed that *A. niger* is most susceptible to Amphotericin B, Fluconazole and Terbinafine, that inhibited completely the growth. However, it was sensitive to Fluconazol with inhibition diameters range 25-35 mm. *C. albicans* was the most resistant strain against all the antifungal marketed drugs.

Table 2. *Diameters of inhibition zones (mm) of antifungigram and sensibility of fungal strains*

Antifungal marketed drugs	µg/ml	*A. niger*		*C. albicans*	
		D	**S**	**D**	**S**
AMB	**100**	TI	++	0	-
	50	TI	++	0	-
	20	TI	++	0	-
	10	TI	++	0	-
FLC	**100**	35	+	0	-
	50	25	+	0	-
	20	0	-	0	-
	10	0	-	0	-
TER	**100**	TI	++	0	-
	50	TI	++	0	-
	20	TI	++	0	-
	10	TI	++	0	-

AMB: l'amphotéricine B, **FLC :** Fluconazole, **TER :** terbinafine, **TI** : total inhibition, ++ : hypersensitive, + : sensitive, - : resistant

The diameters of inhibition achieved by the flavonoid extracts against fungal strains are illustrated in Table 3. Likewise, against *A. niger*, all flavonoid extracts from both organs plant (leaf and bark) record a strong antifungal activity (total inhibition) except Cr. Ex from barks thus giving maximum inhibition diameters of 25,5 mm. For flavonoid extracts from leaves, Ac. Ex and Aq. Ex showed the greatest antifungal activity with inhibition diameters of 17 mm against *C. Albicans*.

The diameters of inhibition obtained by flavonoid extract from barks exhibited the most antifungal effectiveness against *C. albicans* (24,2 mm for Chl. Ex, 22,4 mm for Aq. Ex, 21,8 mm for Hex. Ex, 20,2 mm for Ac. Ex and 17,3 mm for Cr. Ex).

The MIC_s vary widely depending on the flavonoid extract and its concentration, organ plant and fungal strain type (Table 4). The highest MIC of 12,5 µg/ml was recorded against *A. niger* by all flavonoid extracts from leaves and barks (except Cr. Ex from barks). The remaining MIC_s varied between 50-100 µg/ml.

Table 3. *Diameters of inhibition zones in mm (mean ± SD), minimum inhibitory concentration (µg/ml) and fungal strains sensivities*

Flavonoid extracts		Diameters of inhibition zones (mm), minimum inhibitory concentration (µg/ml) and fungal strains sensitivities							
		Leaves				Barks			
		A. niger		*C. albicans*		*A. niger*		*C. albicans*	
	µg/ ml	D	S	D	S	D	S	D	S
Cr. Ex	100	TI	++	14.6 ± 1.30	+	25.5 ± 0.42	+	17.3 ± 1.50	+
	80	TI	++	11.7 ± 1.05	+	25 ± 0.5	+	15.8 ± 1.01	-
	60	TI	++	10 ± 1.73	+	16 ± 1.38	+	15 ± 1.32	+
	40	TI	++	6.6 ± 1.15	±	15 ± 1.5	+	9 ± 0.79	±
	20	TI	++	0	-	8.3 ± 1.44	±	0	-
		MIC = 12,5				**MIC = 50**		**MIC = 100**	
Hex. Ex	100	TI	++	11.7 ± 1.12	+	TI	++	21.8± 0.62	+
	80	TI	++	6.6 ± 1.15	±	TI	++	21.6 ± 0.28	+
	60	TI	++	0	-	TI	++	9 ± 1.55	±
	40	TI	++	0	-	TI	++	5.3 ± 0.92	±
	20	TI	++	0	-	TI	++	0	-
		MIC = 25				**MIC = 12,5**		**MIC = 50**	
Chl. Ex	100	TI	++	15 ± 1.5	+	TI	++	24.2 ± 0.38	+
	80	TI	++	10 ± 1.73	+	TI	++	15.6 ± 1.40	+
	60	TI	++	8.3 ± 1.44	±	TI	++	13.3 ± 1.25	+
	40	TI	++	7.2 ± 1.24	±	TI	++	11.6 ± 1.04	+
	20	TI	++	6.6 ± 1.15	±	TI	++	6.4 ± 1.11	±
		MIC = 12,5				**MIC = 12,5**		**MIC = 50**	
Ac. Ex	100	TI	++	17 ± 1.53	+	TI	++	20.2 ± 0.73	+
	80	TI	++	10 ± 1.73	±	TI	++	16.4 ± 1.42	+
	60	TI	++	0	-	TI	++	12.7 ± 1.11	+
	40	TI	++	0	-	TI	++	8.3± 1.44	±
	20	TI	++	0	-	TI	++	5 ± 0.86	±
		MIC = 12,5		**MIC = 100**		**MIC = 12,5**		**MIC = 50**	
Aq. Ex	100	TI	++	17 ± 1.47	+	TI	++	22.4 ± 0.23	+
	80	TI	++	16.1 ±1.40	+	TI	++	20.4 ± 0.47	+
	60	TI	++	14.3 ± 1.25	+	TI	++	11.9 ± 1.04	+
	40	TI	++	9.2 ± 1.59	±	TI	++	5 ± 0.86	±
	20	TI	++	0	-	TI	++	5 ± 0.86	±
		MIC = 12,5		**MIC = 100**		**MIC = 12,5**		**MIC = 50**	

D: diameters, **S**: sensitivity, **Cr. Ex**: crut extract, **Hex. Ex**: hexan extract, **Chl. Ex**: Chloroform extract, **Ac. Ex**: ethyl acetate extract, **Aq. Ex** : aqueous extract, **TI** : total inhibitory, **MIC** : minimum inhibitory concentration, ++ : hypersensitive, + : sensitive, ± : intermediate, - : resistant

Table. 4 Analysis of the variance at several criteria of classifiaction: effect of flavonoid extract, type of fungal strain and organ plant on antifungal power

Source	DF	SC	MC	F	P > F
Organ plant	1	7,035	7,035	3,052	0,083
Extract	4	15,310	3,828	1,661	0,164
Concentration	4	6,515	1,629	0,707	0,589
Strain	1	378,483	378,483	164,223	< 0,0001***
Organ plant * Extract	4	24,134	6,033	2,618	0,039*
Organ plant * Strain	1	2,413	2,413	1,047	0,308
Organ plant *Extract *Concentration	16	72,089	4,506	1,955	0,022*
Organe * Concentration * Souche	4	22,294	5,573	2,418	0,052*

DF: Degree of freedom; **SS**: sum of squares; **MC**: mean squares; **F**: Fischer; **P**: probability

DISCUSSION

Extraction of phytochemicals from the plant materials is affected by pre-extraction factors (plant part used, its origin and particle size, moisture content, method of drying, degree of processing …etc.) and extraction-related factors (extraction method adopted, solvent chosen, solvent to sample ratio, pH and temperature of the solvent, and length of extraction) (Azwinda *et al.,* 2015; Tiwari *et al.,* 2001).

Some of the important phytochemicals include alkaloids, flavonoids, phenolics, tannins…etc which are distributed in various parts of the plant (Sheel *et al.,* 2014). Nature is a unique supplier of structures of high phytochemical diversity representing phenolics (45%), terpenoids and steroids (27%) and alkaloids (18%) as major groups of phytochemicals (Saxena *et al.,* 2013). The differences in antifungal activity are most likely due to the organic solvents utilised in flavonoid extraction.

The incidence of fungal infections has drastically increased over the past three decades and was simultaneously accompanied by increased acquired and innate resistance to antifungal drugs. However, antifungal resistance occurrence has to be considered independently for each antifungal class and for each fungal genus (Pfaller *et al.,* 2011). Microorganisms develop mechanisms to counteract the fungicidal or fungistatic effects of all antifungals classes that are based on three major mechanisms, namely, reducing the accumulation of the drug within the fungal cell, decreasing the affinity of the drug for its target, and modifications of metabolism to counterbalance the drug effect (Sanglard, 2012). Several research explained that ABC Transporters CDR1 and CDR2 (Candida drug resistance 1 and 2) from *C. albicans* are the two major ABC transporters involved in azole resistance in this species (De Micheli *et al.,* 2002; Gaur *et al.,* 2004).

Fluconazole is suitable for the treatment of superficial candidiasis, disseminated candidiasis and cutaneous candidiasis. Due to its good pharmacokinetic proprieties as well as it's a vast range of activity, fluconazole was the gold-standard treatment of fungal infection. Unfortunately, the over-prescription of these medications by physicians for prophylaxis or treatment led to an increase in resistance to azole drugs. Moreover, fluconazole is almost ineffective against most molds (Vandeputte *et al.,* 2012). Additionally, it is now accepted that filamentous fungi, and particularly those of the *Aspergillus* genus, can grow as biofilms (Ueno *et al.,* 2009). Chandra *et al.* noticed fungal biofilms are resistant to almost all the currently used antifungals, with the exception of echinocandins and lipid formulation of AMB.

The difference in the antifungal activity of flavonoid extracts between each of the organs is likely attributed to the combined effect of several factors. Among them, is the presence of various secondary metabolites, the qualitative and quantitative proportion of phenolic compounds in general and flavonoids in particular, such as flavans and flavonols. Hence, they can also lead to significant differences in terms of inhibition diameters recorded. (Bouterfas *et al.,* 2016).
New therapies are therefore needed against pathogenic fungi. Several approaches were developed during the last several years in order to find new solutions. Researchers aim to discover new antifungal drugs either by testing already existing medical compounds (Marchetti *et al.,* 2000) compounds from natural sources such as plants (Di Santo, 2010), sea (Myers *et al.,* 2011), microorganisms or by systematic screens of chemical compound libraries (Tinge *et al.,* 2011; Walker *et al.,* 2011).

Conclusion:

The antifungal activity of *R. alaternus* flavonoid extracts is significantly influenced by the organ plants and extracted solvents. The extracts from leaves were the most active extracts against the tested stains, followed by those of barks. These differences are likely attributed to the

extraction technique adopted and the organ of the plant used.

In the development of new synthetic drugs, the chemical structures derived from these phytoconstituents can be utilized as models. Identification of phytoconstituents in the plant material helps to predict the potential pharmacological activity of that plant. It is suggested that *Rhamnus alaternus* L. can be used in the treatment of various diseases as it possesses potential pharmacological activities.

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