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Valorization of *Anagyris foetida*; determination of phenols, antioxidants and antifungal activity

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Abstract

This study aims to valorize *Anagyris foetida* through the evaluation of the antioxidant capacity, the content of total polyphenols as well as the antifungal activity of its various extracts. The work was carried out on the leaves, seeds and stems of *Anagyris foetida* collected from two different Tunisian sites Nefza and Zaghouan. Three type of solvent were used for extracts preparation; water, ethanol and hexane. To evaluate the antioxidant activity, we used the DPPH (2.2-diphenyl-1-picrylhydrazyl) method. Total phenols content was conducted using Folin–Ciocalteu reagent. The antifungal activity was tested against three strains; *Aspergillus oryzae, Aspergillus nidullans* and *Aspergillus calavatus.* Results showed that the most significant antioxidant activity was recorded for the ethanolic extract of the leaves from Zaghouan with an IC50 of 6.68 µg/ml. The results showed that leaf extracts are richer in polyphenols compared to other organs. The results of antifungal activity showed that that there is a highly significant difference between the two sites where the best responses were recorded for Nefza extracts.

1. Introduction

Among the remedies with which Man has surrounded himself to treat his health problems, medicinal and aromatic plants have all occupied an important place. The therapeutic virtues of plants have been experienced since then and their precious characteristics have been transmitted from generation to generation or recorded in old writings [1]. Reputable remedies have prevailed despite the development of modern medicine which has marginalized the use of natural medical techniques. Currently, medicinal and aromatic plants are of great importance thanks to the progressive discovery of the applications of their active principles. Indeed, the pharmaceutical industry relies heavily on the diversity of secondary plant metabolites to find new molecules with novel biological properties.

Plants are extremely complex in terms of their chemical composition. They contain several thousand different constituents, of which only a few (or sometimes only one) are responsible for the therapeutic effect or the toxic effect [2]. Man has known and used aromatic and medicinal plants (MAP) since ancient times. All the great ancient civilizations have used PAM for their medicinal and perfuming properties as well as ritual uses. The Sumerian, Akkadian and Babylonian civilizations already produced preparations based on aromatic plants. Similarly, China, the cradle of phytotherapy, India, Egypt, Greece and the Romans have capitalized on knowledge in this area which they have even

recorded in works dedicated to MAP. Tunisia has in its flora a large number of plants that could have medicinal properties and belonging to various families. Our choice fell on the fetid whipworm (*Anagyris foetida*). This plant has been very little studied and requires a better knowledge of its biological properties. Almost studies were focused on its seed germination and multiplication [3,4,5]. Fiew studies reported the nutritional and therapeutic values of this plant [6,7].
The main objective of this study is the valorization of *Anagyris foetida* through the evaluation of the antioxidant capacity, the content of total polyphenols as well as the antifungal activity of its various extracts.

2. Methodology

2.1 Plant material

The work was carried out on the leaves, seeds and stems of Anagyris foetida collected from two different sites Nefza and Zaghouan. The different parts were dried to dry weight, then ground using an electric grinder.

2.2 Preparation of extracts

The water, ethanol and hexane extracts were prepared by macerating 10 g of plant material powder in 100 ml of solvents for 24 h. The solution was then filtered and left at room temperature until complete evaporation of the solvent. The dry extracts were collected and weighed then resuspended again in the solvents [8]. The percentage of dry crude extract of water, hexane and ethanol was calculated according to the following formula:

$$R(\%) = M / M0 * 100$$

Where:
R (%): Yield expressed in %.
M: Mass in grams of the resulting dry extract.
M0: Mass in grams of plant material to be treated.

2.3 Antioxidant activity

To evaluate the antioxidant activity, we used the DPPH (2.2-diphenyl-1-picrylhydrazyl) method. Briefly, 15 µl of each of the different concentrations of the extracts were incubated with 1.5 ml of the solution of DPPH in 0.004% ethanol. In parallel, a negative control is prepared by mixing 15 µl of solvent (water, ethanol or hexane) with 1.5 ml of the DPPH solution. After an incubation period of 30 minutes, absorbances were determined at 517 nm **[9].** The percentage inhibition (I%) of the DPPH radical was calculated as follows:

$$IR = [(DOc - DOe)/DOc] * 100$$

Where:
Doc: control absorbance
DOe: absorbance of the DPPH containing the samples to be tested.
For each extract we determined the IC50 value which is the concentration of the substrate which causes the loss of 50% of the activity of DPPH [10].

It was expressed in g/ml and compared with that of BHT [11].

2.4 Total phenols content

A quantity of 500 µl of the extracts of each sample was mixed with 100 µl of the freshly prepared Folin–Ciocalteu reagent (10 times diluted) and 2 ml of sodium carbonate Na2CO3. The whole is incubated at room temperature for 30 minutes and the reading is carried out against a blank using a spectrophotometer at 755 nm.

From an aqueous stock solution of gallic acid, with a mass concentration of 0.5 g/l, a standard range of solutions in an aqueous medium was prepared.

100 µl of 10% folin-Ciocalteu reagent (10 times diluted in distilled water) is added. After two minutes of incubation, 2 ml of 2% Na2CO3 sodium carbonate are added. The tubes are then shaken and placed in the dark for 30 minutes at room temperature.

The reading of the absorbance of each solution prepared using a UV-Visible spectrophotometer of the Schimadzu 1601 type, at a wavelength of 755 nm against a blank prepared in the same way except that it does not contain gallic acid but distilled water instead of the test substance. The absorbance values of each concentration allowed us to plot the calibration curve for gallic acid [12].

2.5 Antifungal activity

To test the antifungal activity of extracts of *Anagyris foetida*, 3 phytopathogenic fungal strains were used: *Aspergillus oryzae, Aspergillus nidullans* and *Aspergillus calavatus.*

The aqueous extracts, the ethanol extracts and the hexane extracts from the two Nefza and Zaghouan sites were incorporated separately into tubes containing 20ml of PDA medium maintained in superfusion (the extract concentration is 0.013g/20ml of PDA). Each tube was instantaneously homogenized by manual shaking and then its content was poured into a Petri dish.

After cooling the medium, a mycelial disc with a diameter of 6 mm taken from the young culture of each fungal strain was placed in the center of the Petri dish containing the appropriate extract. The plates were then incubated at 25°C for five days. The fungicidal effect was determined by calculating the growth diameter of the strain and comparing it to that of a negative control, i.e. a PDA medium without extract, each extract has its control, for the ethanol the same dose of ethanol was added with the PDA medium and the same for the hexane extract [13].

For each antifungal test, the extract was tested in its pure state, three tests were carried out and it is the average value of the three measurements of the growth zone which was taken into consideration.

The results were calculated according to the method of Singh et al. (1993) while calculating the percentage inhibition I according to the following formula:

$$\mathbf{I(\%) = [(dC-dE) /dC] \times 100}$$

Where:

dC: diameter of control (mm)

dE: diameter in the presence of the tested extract (mm)

2.6 Statistical analyzes

The statistical processing of the data was carried out using the SAS GLM (General Linear Models) procedure. An analysis of variance relative to the parameters studied was carried out. The most significant correlations between them are also noted. Results are presented as the mean of three replicates ± standard deviation.

3. Results and Discussion

3.1 Extract yield

The values determined for the calculation of the yields of the aqueous, ethanolic and hexane extracts of the leaves, stems and seeds of *Anagyris foetida* are shown in Table 1.

Table1. Yield of aqueous, ethanolic and hexane extracts of the leaves, stems and seeds of *Anagyris foetida*

Site	Plant material	Solvent	Yield (%)
Nefza	Leaves	Water	$17,9^{b} \pm 1,97$
		Ethanol	$9,59^{c} \pm 3,74$
		Hexane	$5,26^{e} \pm 1,08$
	seeds	Water	$7,06^{d} \pm 0,54$
		Ethanol	$7,97^{d} \pm 2,03$
		Hexane	$6,78^{d} \pm 1,37$
	Stems	Water	$7,02^{d} \pm 0,48$
		Ethanol	$3,67^{f} \pm 1,27$
		Hexane	$2,07^{f} \pm 1,17$
Zaghouan	Leaves	Water	$24,21^{a} \pm 4,50$
		Ethanol	$5,12^{e} \pm 2,20$
		Hexane	$2,11^{f} \pm 1,54$
	seeds	Water	$8,15^{d} \pm 0,34$
		Ethanol	$8,94^{c} \pm 3,39$
		Hexane	$3,26 \pm 1,02$
	Stems	Water	$5,51^{e} \pm 1,67$
		Ethanol	$2,74^{f} \pm 0,47$
		Hexane	$1,89^{f} \pm 0,36$

Values with different letters are significantly different

According to the statistical analyses, the best yields were observed in all the aqueous extracts for the two sites. The highest yield was obtained by the leaves with a percentage of around 24.21% (Zaghouan) and 17.9% (Nefza), followed by that of the seeds (8.15% (Zaghouan) and 7 .06% (Nefza) and stems (7.02% (Nefza) and 5.51% (Zaghouan)).

The extracts obtained by using ethanol and hexane gave very similar yields without significant differences between the two. The solvents used during this experiment are of different polarity; water and ethanol are polar solvents, while hexane is rather apolar. The significant differences recorded between the yields can be explained by the differential solubility of the different compounds in the solvents used, which is mainly related to the polarity [14]. Reading the extraction yields shows that the greatest yields were obtained with the polar solvent (water). In this case, it is possible to conclude that *A. foetida* is probably richer in polar compounds than in apolar compounds.

3.2 Antioxidant activity

The anti-radical activity was carried out by the method of 2,2-diphenyl-1 picrylhydrazyl (DPPH) which is a method frequently used for its simplicity. This method is based on the reduction of an alcoholic solution of DPPH in the presence of an antioxidant which donates a hydrogen or an electron, the non-radical form DPPH-H is formed [15]. The antioxidant activity of the extracts is expressed in IC50

which defines the effective concentration of the substrate which causes the loss of 50% of the activity of the DPPH radical. The IC50 values are shown in Table 2. The use of the DPPH test made it possible to demonstrate that the tested extracts of *Anagyris foetida* have a significant antioxidant power. The lowest concentration of inhibition results in the most powerful antioxidant power.

Table2. IC50 values (in µg/ml) of crude extracts and fixed oil of *Anagyris foetida*

Site	Plant material	Solvent	IC_{50} (µg/ml)
Nefza	Leaves	Water	$167,8^{f} \pm 4,73$
		Ethanol	$69,6^{d} \pm 4,25$
		Hexane	$32,04^{b} \pm 0,10$
	Seeds	Water	$40,71^{c} \pm 2,61$
		Ethanol	$71,09^{d} \pm 6,41$
		Hexane	$69,83^{d} \pm 4,13$
	Stems	Water	$170,06^{f} \pm 0,57$
		Ethanol	$79,65^{d} \pm 1,77$
		Hexane	$151,51^{f} \pm 18,54$
Zaghouan	Leaves	Water	$119,54^{e} \pm 5,13$
		Ethanol	$6,68^{a} \pm 0,02$
		Hexane	$326,42^{g} \pm 43,94$
	Seeds	Water	$305,81^{g} \pm 9,96$
		Ethanol	$336,14^{g} \pm 77,08$
		Hexane	$47,64^{c} \pm 10,28$
	Stems	Water	$29,5^{b} \pm 0,62$
		Ethanol	$24,95^{b} \pm 0,47$
		Hexane	$61,83^{d} \pm 6,65$

Values with different letters are significantly different

The statistical results showed the existence of an organ effect per harvest site. Thus there is a significant difference between the studied extracts. The most significant activity was recorded for the ethanolic extract of the leaves harvested from Zaghouan with an IC50 of 6.68 µg/ml. The lowest activity was achieved by the ethanolic extract of Zaghouan seeds with the highest IC50 value which was estimated at 336.14 µg/ml. These results showed that the different parts of the *Anagyris foetida* plant have significant antioxidant power. Indeed, antioxidants have a very important nutritional value given their ability to fight against several diseases such as cancer, atherosclerosis and coronary and cardiovascular diseases [16]. Antioxidants are nutrients found in large amounts in many plants, especially in berries, carrots, spinach and tomatoes, but also in cereals, coffee and tea. The most common antioxidants are ß-carotene (provitamin A), vitamin C (ascorbic acid), vitamin E (tocopherol), polyphenols and lycopene. Their activity opposes that of free radicals, chemicals produced in living cells that damage DNA. Vitamins C and E are, for example, molecules that help fight against states of fatigue [17].

In this study, the antioxidant activity was determined using the DPPH test. Indeed, the oxidation processes are complex and the antioxidants are diverse, there are hydrophilic and hydrophobic components, so there is not a universal method by which the antioxidant activity can be measured quantitatively in a very precise way. It is necessary to combine the responses of different and

complementary tests to have an indication of the antioxidant capacity of the sample to be tested [18,19,20].

3.3 Total phenols content

The values of the total polyphenol content are summarized in Table 3 and are expressed in g of gallic acid equivalent per ml of extract (g GAE/ml).

Table3. Values of polyphenol content of crude extracts and fixed oil of *Anagyris foetida*

Site	Plant material	Solvent	Tptal phenols content
Nefza	Leaves	Water	$1,11^{b} \pm 0,08$
		Ethanol	$1,23^{a} \pm 0,04$
		Hexane	$0,22^{f} \pm 0,05$
	Seeds	Water	$0,75^{c} \pm 0,05$
		Ethanol	$0,19^{f} \pm 0,05$
		Hexane	$1,18^{b} \pm 0,03$
	Stems	Water	$0,85^{c} \pm 1,25$
		Ethanol	$0,52^{e} \pm 0,02$
		Hexane	$0,74^{c} \pm 0,05$
Zaghouan	Leaves	Water	$0,80^{c} \pm 0,04$
		Ethanol	$0,49^{e} \pm 0,03$
		Hexane	$0,6^{d} \pm 0,2$
	Seeds	Water	$0,47^{e} \pm 0,07$
		Ethanol	$0,56^{d} \pm 0,02$
		Hexane	$0,51^{e} \pm 0,2$
	Stems	Water	$0,68^{d} \pm 0,03$
		Ethanol	$0,6^{d} \pm 0,05$
		Hexane	$0,78^{c} \pm 0,09$

Values with different letters are significantly different

The results showed that leaf extracts are richer in polyphenols compared to other organs.
A significant difference was recorded between the solvents used for the extraction, the highest content was observed for the ethanolic extracts of Nefza leaves with about 1.23 g GAE/ml.
The results presented in Table 3 clearly show that these phenolic compounds are present in the different parts of *Anagyris foetida* with levels that differ according to the organ of this plant and the solvent used in the extraction. Teresta et al. [21] demonstrated that the solvent used in the extraction has an importance in the variation of the concentration of total phenolic acids for the same plant. Kiselova et al. [22] found that there is a correlation between antioxidant activity and total polyphenol content for a given compound. The benefits that polyphenols could bring to human health are of particular interest to 2 areas: herbal medicine, since the explanation of the supposed effectiveness of many medicinal plants is based in whole or in part on the presence of phenolic compounds in these plants and hygiene eating. Indeed, several studies indicate that polyphenols could reduce the risk of occurrence of a certain number of pathologies, in particular those related to aging and oxidative lesions (cancers, cardiovascular or neurodegenerative diseases, etc.) [23].

Numerous *in vitro* studies have shown that certain polyphenols (flavonoids) could affect their biological targets by modulating certain enzymatic activities, gene expression or cell signaling, by interacting with membrane or cell receptors, or via epigenetic regulations [24].

3.4 Antifungal activity

The percentages of inhibition of the three fungal strains are illustrated by the histograms in figures 1, 2 and 3. The results obtained during this study showed that the extracts exert a significant inhibitory activity on all the fungal strains studied. Statistical analyzes showed that there is a highly significant difference between the two sites where the best responses were recorded for Nefza. The difference between the two sites may be the cause of the chemical composition which is probably different between the extracts. Statistical analyzes have also shown that there is a fungal strain effect. *Aspergillus nidulans* shows the greatest sensitivity. *Aspergillus clavatus* is, on the other hand, the most resistant strain.

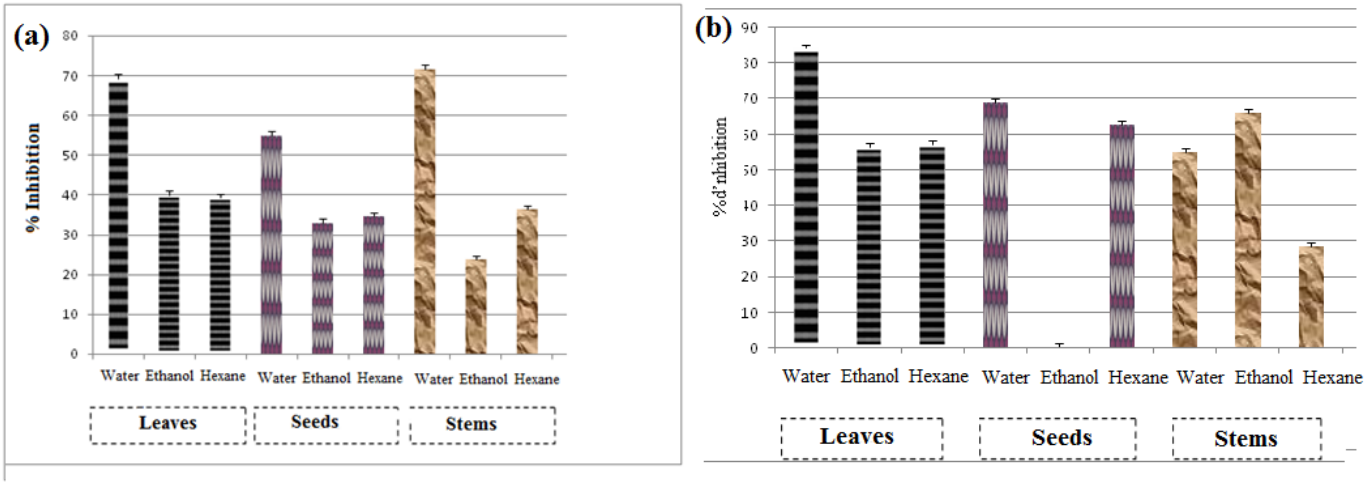

Figure 1. Inhibition effect of crude extracts of *Anagyris foetida* on *Aspergillus nudilans* (a: extracts from Nefza, b: extracts from Zaghouan)

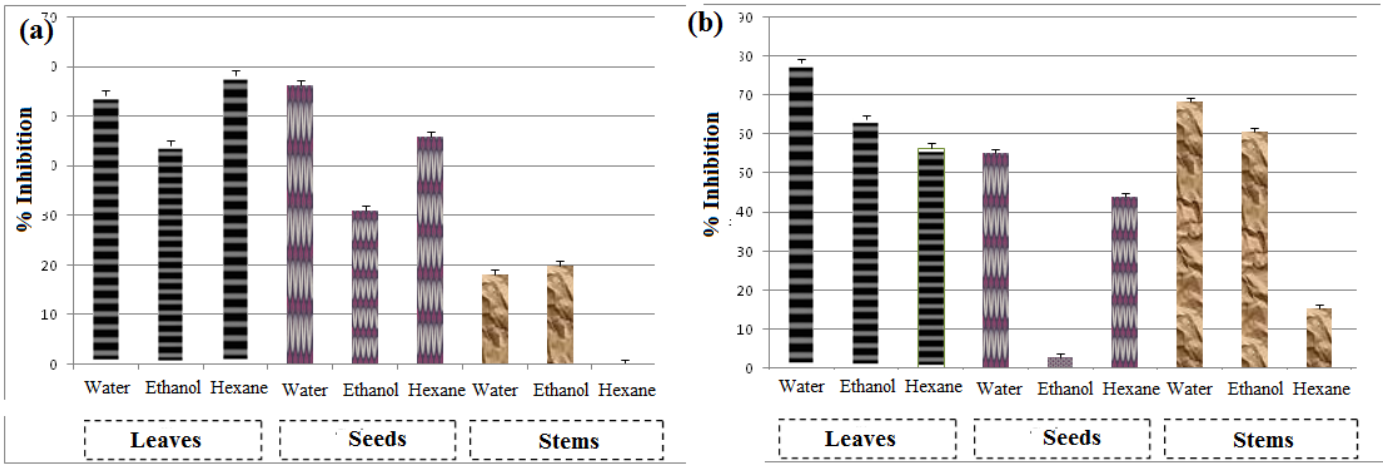

Figure 2. Inhibitory effect of crude extracts of *Anagyris foetida* on *Aspergillus oryzae* (a: from Nefza, b: from Zaghouan)

The different Nefza extracts tested on the *Aspergillus nudilans* strain showed powerful antifungal activity, particularly for the aqueous extracts which marked the highest percentages of inhibition, followed by that of ethanol and hexane. The best results were obtained for the leaf extracts with inhibition percentages of 69.33% for the aqueous extract, 40% for the ethanol extract and 39.36% for the hexane extract. The stems showed a percentage of inhibition of about 61.66% for the aqueous extract, it is the extract which has the most important fungicidal effect on *Aspergillus nidulans*. For Zaghouan, the extracts showed a significant antifungal power, indeed all the extracts of the leaves have percentages of inhibition which exceed 50% (80% for the aqueous extract). On the other hand, the ethanolic extract of the seeds showed a low percentage of inhibition.

For extracts from Nefza, the fungal strain *Aspergillus oryzae* was more sensitive in the presence of leaf and seed extracts. These aqueous and hexane extracts allowed the inhibition of mycelial growth of this strain with a percentage which exceeds half of the latter, the hexane extract of the leaves recorded a percentage of inhibition of the order of 58.33%. The weakest antifungal power was reached in the presence of stem extracts with a percentage inhibition of around 18% for the aqueous extract and 20% for the ethanol extract, while the hexane extract showed a weak effect.

For extracts from Zaghouan, the aqueous extracts showed the highest inhibition effect resulting in a percentage inhibition of around 78.33% for the leaves. On the other hand, the ethanolic extract of the seeds showed the lowest percentage inhibition (2.66%).

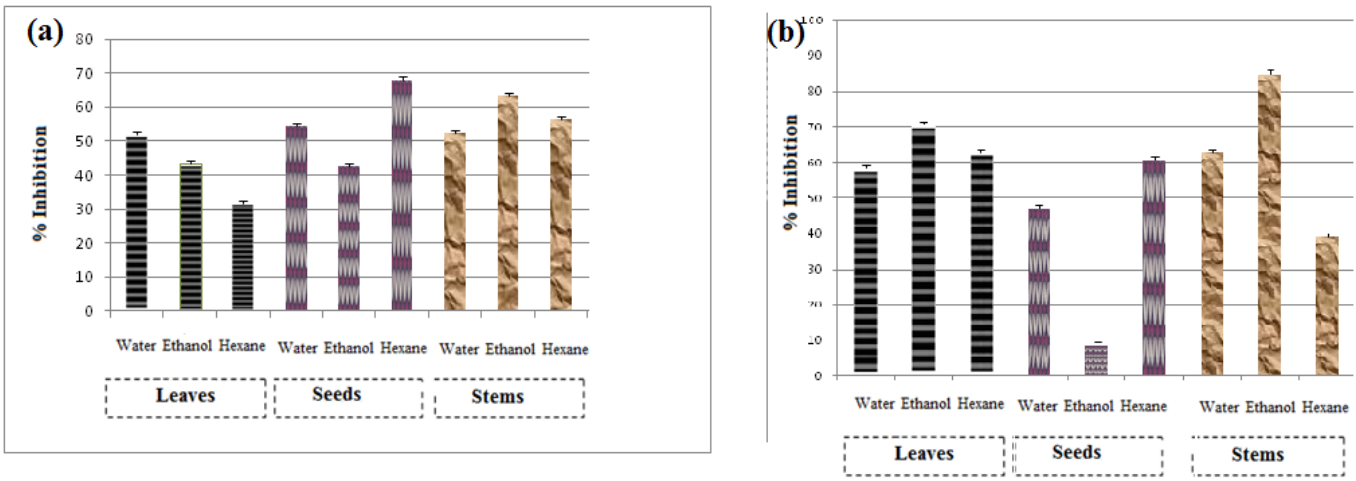

Figure 3. Inhibitory effect of crude extracts of *Anagyris foetida* on *Aspergillus clavatus* (a: from Nefza, b: from Zaghouan)

For *Aspergillus clavatus* stem extracts from Nefza location showed the best fungicidal effect. These extracts inhibited more than half of the growth of this fungal strain (63.33% for the ethanolic extract) followed by that of the seeds and leaves. The inhibition effect revealed for extracts from Zaghouan was clearly higher for the ethanolic extract of the stems (85%). However, the ethanolic extract of the seeds showed the weakest antifungal potency (8.33%).

All the raw extracts of *Anagyris foetida* showed a variation in effectiveness on the three fungi tested, the difference in antifungal effect between the different extracts may be the cause of the difference in chemical composition. Phenolic compounds are produced in response to fungal infection by plants. Indeed, the effectiveness of these substances evaluated *in vitro* showed an inhibitory action on microorganisms [25]. Many researchers have demonstrated the relationship between the chemical structure of phenolic compounds and their antifungal activity.

Conclusion

In this study, we were interested in studying the content of total polypenols and the antioxidant and antifungal power of the various extracts of *Anagyris foetida* harvested from the regions of Nefza. and Zaghouan.

Despite its richness in polyphenols and natural antioxidants as well as its response against microorganisms, *Anagyris foetida* represents a plant that has been little studied and exploited.

It is necessary to develop forest plants that can have medicinal and food properties to allow their rational exploitation.

In order to complete and open many interesting perspectives to our study, we propose, in a future work, to carry out detailed research on the secondary metabolites of *Anagyris foetida*, to analyze its chemical composition, evaluate its antioxidant power with other methods, test its effect on other microorganisms such as bacteria and determine its insecticidal power.

Given its richness in antioxidants, it is necessary to enhance this plant, more precisely its different parts, and increase the chance of its use in different fields such as medicine and pharmacy.

Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest. *Compliance with Ethical Standards:* This article does not contain any studies involving human or animal subjects.

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