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Phytochemistry, HPLC profile and antioxidant activity of aqueous extracts of fenugreek (*Trigonella foenum graecum* L.) seeds grown in arid zones of Algeria

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Abstract: Fenugreek is an herb that has the ability to resist multiple environmental insults, pests, diseases and many pathogens through the synthesis of substances allowing this plant protection, these substances are called phytochemical compounds or secondary metabolites. The aim of this study is to characterize these secondary metabolites in the extracts of fenugreek seeds grown in Algeria and compare the phytochemical profile and antioxidant activity of the aqueous extracts obtained by maceration and decoction. We carried out a "phytochemical screening" on both types of extracts in order to highlight the different classes of secondary metabolites and assaying total polyphenols by the method of Follin ciocalteu, total alkaloids and tannins contained in these aqueous extracts and appreciate their antioxidant activity using the FRAP technique. A characterization of phytochemical molecules of the two types of Fenugreek extracts was done by chromatographic method with HPLC. Several phytochemical groups were identified in the extracts of fenugreek: tannins, saponins, flavonoids, terpenes and reducing nitrogen compounds. Chromatographic analysis of the samples show that 4 phytochemical molecules were identified in the aqueous maceration extract: kaempferol, genistein, vanillin and myrecitine and 3 molecules for the

aqueous extract of decoction are: rutin, kaempferol and vanillin. The aqueous extract of decoction was characterized by the total polyphenols content (31.7 ± 0.031 mg GAE / 100 g of extract), total alkaloids ($2.12 \pm 0.015\%$) and tannins ($9.75 \pm 0.06\%$) that are higher than those obtained in the aqueous extract of maceration: total polyphenols (18.9 ± 0.12 mg GAE / 100g of extract), total alkaloids ($1.71 \pm 0.02\%$) and $8.69 \pm 0.02\%$ for the tannins. A non-significant difference was marked for antioxidant activity found in both types of extracts (56.90 ± 0.013 mg AAE / 100g decoction extract against 56.37 ± 0.24 mg AAE / 100g maceration extract). In the end we conclude that fenugreek may be a promising plant genetic resource responsible for an important biological activity, except its nutritional and therapeutic properties due to their phytochemical compounds.

Keywords: *Trigonella foenum graecum* L., aqueous extract, phytochemistry, antioxidant activity, HPLC, Algeria.

Introduction

Fenugreek (*Trigonella foenum graecum* L.) is a legume from *fabaceae* family exploited for culinary and medicinal purposes, widespread in the Mediterranean, North Africa, India and China [1]. Besides its therapeutic properties, it has other characteristics such as the ability to resist multiple environmental constraints, pests, and many pathogenic microorganisms through the synthesis of substances called secondary metabolites [2]. These metabolites provide the antioxidant effect that represents the biological mechanism responsible for not only antibacterial and antifungal activities but also for fighting against various attacks on the plant [3]. Chemical control against diseases affecting vegetable crops and stored food has a significant impact on the environment in addition to the emergence of pathogenic agents and pests' resistant to insecticides and fungicides. To overcome this issue, alternative solutions were investigated, such as the use of substances of plant origin as biopesticide rich in secondary metabolites and capable of providing a protective effect due to their antioxidant and biocidal properties [4]. A study conducted by Madhava-Naidou et al. [5] on phytochemical composition of the powder of Fenugreek seeds demonstrated biological activities including antioxidant effect. This phytochemical composition fluctuates depending on the geographic origin of the plant, climate, cultural practices and extraction techniques [6]. The purpose of this study was to identify groups of phytochemical molecules with chromatographic method (HPLC) and to evaluate the antioxidant activity of aqueous extracts of fenugreek seeds grown in Algeria.

Material and Methods

Plant material

The plant material used to make the aqueous extraction is represented by the seeds of fenugreek (*Trigonella foenum graecum* L.) grown in the region of Biskra -Algeria, identified by the laboratory of Scientific Research and Technical Center on Arid Regions (CRSTRA) -Biskra.

Aqueous extracts

Fenugreek seeds are cleaned, sorted then passed to extraction process, we have prepared two types of aqueous extracts from seeds of fenugreek and potable water to 25%; the aqueous extract of maceration (EAM) is obtained by immersing the seeds in water and let stand for 24 hours, for the aqueous extract of decoction (EAD) is prepared after 5 minutes of boiling [7].

Photochemical screening

Active molecules (tannins, polyterpenes, flavonoids, free anthraquinones, and nitrogen compounds, cardiogenic glycosides, reducing sugars, coumarins and saponins) were identified according the methods previously described [8, 9, 10, 11, 12].

Sterols and polyterpenes

5ml of the extract were evaporated, the residue was dissolved hot in 1ml of acetic anhydride and 0.5ml sulfuric acid (96%) was added to triturate. A blue to blood red color indicates a positive reaction.

Free anthraquinones

5 ml of the extract were added to 2.5ml of ammonium hydroxide (20%) stirring the mixture; a red coloration indicates a presence of free anthraquinones.

Flavonoids

By the reaction of cyaniding, few drops of concentrated hydrochloric acid (96 %) were added to 5 ml of each extract and then, 2- 3 Magnesium chips and let for a few minutes. The appearance of intense orange-pink color indicates a positive reaction.

Tannins

2-3 drops of ferric chloride (2 %) were added to 2 ml of each extract. The reaction is considered positive when a blue-black coloration with a precipitate appears after a few minutes.

Saponines

5ml of extract were added to 10ml of distilled water. Two minutes later, the formation of foam (height greater to 1cm), persistent during 1 hour indicates the presence of saponins.

Reducing sugars

5ml of Fehling liquor were added to 5 ml of the extract and incubated for 8 minutes at 70° C. Formation of a brick red precipitate indicates a positive reaction.

Coumarins

1 ml of the sample (extract) was introduced in a test tube with a filter paper impregnated with Sodium hydroxide (20 %) and heated until boiling. A clear solution indicates a positive reaction (Lactone cycle reaction).

Nitrogen compounds

1ml of extract was mixed with 2 ml of aqueous sodium hydroxide (20%) and then 2 to 3 drops of aqueous solution of copper sulfate (2%) were added. A violet coloration indicates a positive reaction (reaction of Biuret).

Cardiotonic glycosides

1ml of each extract was added to 0.5ml acetic acid (0.1 N) containing traces of ferric chloride (2%) and thereafter 5 ml sulfuric acid (98 %). Formation of two phases, the first red-brown and the second one blue-green confirms their presence (Killer-Kiliani reaction).

Carotenoides

After evaporating 5 ml of aqueous extract to dryness, 2 to 3 drops of antimony trichloride were added to chloroform (99%). Final red coloration turning to blue means a positive reaction.

Chromatographic analysis

The analytical method used is high-performance liquid chromatography (HPLC), it allows the identification of substances according to their polarity in the solvents, the model of HPLC used for analytical control was: YL 9100- South Korea, this system is provided with a PERKIN-ELMER type of pump set concentration gradient mode, an injection valve 20µl, column C18 with a cross linked silica: 125mm × 4mm,

a double UV detector and a computer integrator for synthesis graphs. The operating conditions are as follows: - Mobile phase in binary gradient: 2% acetic acid and pure acetonitrile -an injection rate of 1 ml / min - Temperature 38 ° C - Detection at 280nm (channel 1) and 340 nm (Channel 2). The resulting chromatographic profile is compared to standards (standard pure of phytochemical molecules) injected into the same operating conditions as that of the sample. Retention time R_t of each component is determined by the integrator gives a peak on the chromatogram (AFNOR, 1996).

Phytochemical assay

Total polyphenols

The content of total phenolic compounds is determined by the method of Singleron and Rossi [13] by the use of the reagent *Folin cio-caltea*, introduce 2.5 ml of Folin-Cio-Caltea (diluted ten times) in a test tube and add 0.5 ml of each extract, let for 3 minutes and then add 1ml of sodium carbonate 1ml (20%). The mixture was incubated at room temperature in the dark for 15 minutes. The absorbance was measured at 760nm using the spectrophotometer (Shimadzu UV-1202). The results were expressed as milligrams of gallic acid equivalent (GAE mg / 100g of extract) using the calibration curve of gallic acid as standard.

Tannins

30 g of seed powder were added to 100 ml of petroleum ether and let to act for 24 hours. After filtration, the marc was recovered while chlorophyll and lipids removed. The recovered marc was diluted in 50 ml of di-ethyl ether then filtered to remove phenols and catechins. The marc was thereafter dissolved in 100 ml of methanol at 60°, filtered in a pre-weighed flask. The methanol filtrate was evaporated until a constant weight is achieved [14]. The tannin content (T) was calculated using the following formula:

W : capsule weight plus tannins.

$$T = (W - W_0) / W_e \times 100 (\%)$$

W₀: empty capsule weight.

W_e: weight of the sample.

Total alkaloides

Shake 3 g of the sample with 25 ml of sulfuric acid (10%) and 5ml of distilled water. Complete to 50 ml with distilled water. After filtration, ammonium hydroxide (20%) was added until reaching pH = 8-9 then extraction was done with 50 ml of chloroform. The filtrate was evaporated in water bath to obtain the dry residue [15]. Alkaloid content (A) is calculated by the following formula [16]:

$$A = (W - W_0) / W_e \times 100 (\%)$$

W : capsule weight plus alkaloids.

W₀ : empty capsule weight.

W_e : weight of the sample.

Antioxidant activity

The evaluation of the antioxidant activity was performed by the technique *FRAP* because phenolic compounds provide the reduction of ferric iron Fe³⁺ to Fe²⁺ [17], 0.5ml of the different dilutions of the sample is mixed with 1.25 ml of a phosphate buffer solution 0.2M (pH = 6.6) and 1.25 ml of a solution of potassium hexacyanoferrate 1%, the whole is incubated at 50 ° C for 20 minutes, then cooled at room temperature, 2.5 ml of trichloroacetic acid at 10 % are added to stop the reaction, then the tubes are centrifuged at 3000 turns / min for 10 minutes, 1.25 ml of the supernatant are added to 1.25ml of distilled water and 250µl of an iron chloride solution at 0.1%, the reading absorbance is against a white 700nm using a spectrophotometer [18]. The calibration curve is established from the absorbances for the read range of ascorbic acid solutions. The antioxidant activity of the sample is expressed as mg Ascorbic Acid Equivalent (mg AAE) per 100 gram of sample by the formula [19]:

$$\text{Antioxidant activity (mg AAE/100 of sample)} = (C/C_0) \times 100$$

C=Concentration (mg/l ascorbic acid); C₀= Concentration of the extract (g/l).

Results

Phytochemical screening

Table 1 shows the results of phytochemical screening of aqueous extracts from fenugreek seeds.

Table 1. Phytochemical screening of aqueous extracts from fenugreek seeds.

Chemical groups	Mediator reactions	Types of extracts	
		Maceration	Decoction
Sterols and polyterpenes	Anhydrous acetic	++	+++

Saponins	–	+++	+++
Tannins	FeCl ₃	+++	+++
Free anthraquinone	NH ₄ OH	++	+++
Coumarins	NaOH	–	–
Cardiotonic glycosides	FeCl ₃	+	++
Nitrogen compound	Cu ⁺⁺	+	++
Reducing sugars	Fehling	–	–
Carotenoids	–	–	–
Flavonoids	Mg ⁺⁺	+++	+++

(-): absent ;(+): low; (++): medium; (+++); high

Qualitative analyses of the chemical groups (table 1) showed in one hand the absence of coumarins, carotenoids and reducing sugars for both types of extracts, and in the other hand presence of tannins, saponins, flavonoids, sterols, free anthraquinones, cardiotonic glycosides, and nitrogen compounds.

Chromatographic analysis

The molecular separation of aqueous extracts from fenugreek seeds by HPLC was realized in two different wave lengths 280nm and 340nm, the results obtained are shown in the chromatograms with peaks and retention time of each molecule (Figures 1 and 2).

Fig.1.Chromatogram of the aqueous extract of macerated fenugreek seeds at 280nm and 340nm.

Fig.2. Chromatogram of aqueous extract of decocted fenugreek seeds at 280nm and 340nm.

The results of HPLC revealed the presence of 23 components for aqueous maceration extract (figure1) and 39 components to that of the decoction (figure2), the identification of molecules contained in the samples is based on comparison between their retention times (Rt) with that of pure standards under the same experimental conditions, compounds identified in the aqueous extracts of Fenugreek seeds are mentioned in table 2.

Table 2: Phytochemical molecules of aqueous extracts of fenugreek seeds detected by HPLC.

Retention time (min)	EAM	EAD
3.4	/	Rutin
4.3	Kaempferol	Kaempferol
5.2	Genistein	/
14.6	Vanillin	Vanillin
19.5	Myricitine	/

/ : Not determined

Chromatographic analysis of the samples identified 4 phytochemical molecules for aqueous maceration extract (EAM) namely: Kaempferol, Genistein, Vanillin and Myricitine and 3 molecules for aqueous decoction extract (EAD) which are: Rutin, Kaempferol and Vanillin, the rest of the analytes that appear on the chromatograms could not be identified.

Phytochemical assay

Total polyphenols

Figure 3 represents the average content of total polyphenols of aqueous extracts from fenugreek seeds.

Fig.3. .Average total polyphenols content of aqueous extracts of fenugreek seeds.

Total polyphenols content from the aqueous decoction extract estimated to 31 ± 0.031 mg GAE/100g, was higher to that from maceration extract (18.9 ± 0.12 mg GAE/100g).

Tannins

Figure 4 gives the average content of tannins aqueous extracts from fenugreek seeds.

Fig.4.Average tannins content of aqueous extracts of fenugreek seeds.

A slight elevation of the tannin content was observed in the aqueous extract of decoction compared to the aqueous extract of maceration: $9.75\% \pm 0.06$ against $8.69 \pm 0.02\%$.

Total alkaloids

The average rate of total alkaloids is shown in figure 5. Alkaloids content was $1.71 \pm 0.02\%$ in aqueous extract of maceration and $2.12 \pm 0.015\%$ for decoction.

Fig.5. Average total alkaloids content of aqueous extracts of fenugreek seeds.

Antioxidant activity

Figure 6 shows the evaluation of the antioxidant activity expressed in mg equivalent of ascorbic acid per 100 g of each extract.

Fig.6. Average antioxidant activity of aqueous extracts of fenugreek seeds.

Antioxidant activity values determined by the test of FRAP were 56.37 ± 0.24 mg AAE/100g for maceration extract and 56.90 ± 0.013 mg AAE/100g for the decoction one. The test of reducing iron (FRAP) is a colorimetric, fast, and universal test applied to the organic and aqueous extracts of plants measured at 700nm.

Discussion

According to the work of Rodolfo et al. [16] performed on the same plant, the phytochemical screening showed the presence of flavonoids, nitrogen compounds, carotenoids and saponins with no tannins. While another research of Rahmani et al. [20] has used the powder of fenugreek seeds, that found the presence of saponins, steroids, alkaloids, and mucilage. Fluctuations and variations found in the phytochemical composition can be attributed not only to the extraction technique but also to the interaction of many factors, the most important of them are cultural practices such as weeding that has a positive effect on phytochemical molecules yields, several studies have confirmed this trend [6], drying time can also intervene extraction quality as reported by Gomes et al. [21] in a comparative study where dry plants and yellow foliage give the best of quantitative and qualitative extracts. The phytochemical composition of the extracts obtained depends on climatic conditions, geographic origin of seeds and cultural practices, it is even more complex to guarantee a constant quality of the extracts [22]. Other studies had shown the influence of extraction technique and vegetative cycle on the yield and quality of the extract [21]. In the case of our study, two extraction techniques have been used: maceration of fenugreek seeds with water was kept at 24 h, and the decoction which requires a solvent boiling temperature for 5 min to solubilize a significant content of phenolic compounds. According to Laws and Buchanan [23], the phenolic compounds involved in the protection against plant's environment (pathogens) or physical (radiation, drought). The phytochemical molecules are involved in defense mechanisms of plants against different attacks because their accumulation is generally greater in resistant plants [24]. They act in the reinforcement of plant cell walls, lubrication and tissue lignifications [25]. These secondary metabolites also have an antimicrobial effect related to membrane alterations [26] and inhibition of the synthesis of the RNA and DNA synthesis [27]. Studies that have been made by Benhamou and Patrice [2] found that fenugreek seed's extract (*Stifenia*) is primarily intended for fight against vine mildew; however, it seems effective against peach blister (*Taphrina deformans*) and mildew in various plants due to its richness in polyphenols.

The chromatographic profile provided precious data on secondary metabolites of aqueous extracts of fenugreek seeds grown in arid areas of Algeria, the results showed the richness of samples in phenolic compounds known by their biological activities. Chromatographic analysis of the samples identified 4 phytochemical molecules for aqueous maceration extract namely: kaempferol, genistein, vanillin and

myrecitine and 3 molecules were identified for aqueous extract of decoction (EAD): Rutin, kaempferol and vanillin.

Kaempferol is a pigment which belongs to the family of flavonol glycosides synthesized by hydrolysis of heteroside found in the epidermis to protect the plants against harmful radiation. Myrecitine is a glycoside family of flavonols present in many plants and is a natural antioxidant. Rutin is a diglycoside able to trap free radicals, several studies have shown that rutin has less antioxidant capacity than the myrecitine and higher than kaempferol [28], other studies have been done on the phytochemical composition of the methanol extract of fenugreek seed powder by chromatographic method where the results revealed the presence of several phenolic compounds belonging to the class of flavonoid glycosides which the major constituent was kaempferol with a retention time of 53 min [29]. The results of the characterization by HPLC depend on the separating power of the column, the flow velocity and the composition of the mobile phase and column temperature [30].

The polyphenols assay was performed by the method of Follin cio-caltchu for both types of extracts including that the extract of decoction gave the best content, polyphenols are effective on insect's pests by decreasing their reproduction rate, biological activity of polyphenols concerns not only insects, it has been demonstrated that polyphenols constitute a physicochemical barrier allowing the plant more resistant towards plant pathogenic fungi [31]. The diversity of the activities of these molecules in resistant plants may well be a biocontrol strategy against pests [32]. Polyphenols are the most abundant antioxidants in medicinal and aromatic plants [33], they have in vitro an antioxidant potential allowing them to act as oxygen extinguishers reducing agents [34].

The tannins content recorded for aqueous extracts of fenugreek seeds was better, compared to that recorded in the works of Al-Maamari et al. [35] for the same species that have found a tannin content of 0.78%, this difference is probably due to the soil composition. Tannins are substances of organic origin that are found in all parts of the plant [36], those are proton donors to free radicals produced during peroxidation allowing cessation of auto-oxidation reaction [37]. They have the property of precipitating fungal proteins and heavy metals and enter the tissue regeneration [38].

The dosing of alkaloids in both types of extracts of fenugreek seeds brought low levels compared to those reported by Khireddine [16] who found a rate of 25.98% in the fine powder of fenugreek seeds. Alkaloids are extracted by a nonpolar solvent in an alkaline solution or with a polar solvent in an acid solution [39]. These substances are endowed with high biological activity even in small quantities, alkaloids are involved in the mechanisms of plant defense against herbivorous, they act as natural defenders and toxic to insects [40].

The presence of antioxidant molecules in aqueous extracts of fenugreek seeds was confirmed by the technique of reduction of iron, phytochemicals molecules increase the intensity of blue color in the reactive group ($\text{Fe}^{3+} / \text{Fe}^{2+}$). The results are recorded on the antioxidant activity at 700 nm are higher than those found by Cheurfa [41] in the hydro-alcoholic extract of fenugreek namely $39.77 \pm 0.04 \text{ mg AAE} / 100 \text{ g}$ of extract.

The biomolecules antioxidant activity is exercised not only by inhibiting free radicals; it also manifests itself by neutralizing oxidative enzymes and chelation trace metal ions responsible for the production of reactive oxygen species [42]. The phenolic compounds and other secondary metabolites in the plant are good natural antioxidants [43] that intervene in a natural way in protecting plants against various predatory insects, herbivorous [40]. They have the ability to degrade proteins of some plant pathogenic fungi [38].

Conclusion

The analytical study of aqueous extracts from fenugreek seeds showed that this plant develops a particular metabolism allowing it to synthesize bioactive molecules mainly flavonoids known by their protective effect against biotic and abiotic stress threaten the plant. Phytochemical screening revealed presence of several secondary metabolites from both types of extracts (tannins, saponins, flavonoids, terpenes, and nitrogen compounds). Moreover, the quantitative analysis showed high concentration in some bioactive molecules from aqueous extracts: total polyphenols ($31.7 \pm 0.031 \text{ mg GAE} / 100 \text{ g}$ extract of decoction versus $18.9 \pm 0.12 \text{ mg GAE} / 100 \text{ g}$ extract of maceration), tannins ($8.69 \pm 0.02\%$ in the extract of maceration while $9.75 \pm 0.06\%$ for decoction extract) and $1.71 \pm 0.02\%$ of total alkaloids in the maceration extract against $2.12 \pm 0.015\%$ for that of the decoction. The antioxidant profile revealed by the iron reduction method showed significant activity estimated at $56.90 \pm 0.013 \text{ mg AAE} / 100 \text{ g}$ of aqueous decoction extract which was slightly higher than that of the aqueous maceration extract ($56.37 \pm 0.24 \text{ mg AAE} / 100 \text{ g}$). The phytochemical and antioxidant studies of aqueous extracts from fenugreek seeds have shown that they could be a promising active material in crop protection.

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