Phytochemical screening, quantitative analysis and antioxidant activity of *Vitex agnus castus* L. (Lamiaceae) from South-East of Morocco

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This study was designed to assess the phytochemical screening of leaves and seeds of *Vitex agnus castus* harvested south-East of Morocco and to evaluate their potential antioxydante activity properties using 2, 2 diphenyl-1-picrylehydrazil radical scavenging. Methanol 80% extracts of leaves and seeds were obtained by maceration. Phytochemical screening, quantitative determination of total polyphenols, total flavonoids, Aglycones and anthocyanins contents were assessed. Antioxidant activity was tested using 2, 2 diphenyl-1-picrylehydrazil radical scavenging. Phytochemical screening revealed the presence of alkaloids, terpenes and catechic tannins in leaves and seeds, anthraquinones in seeds and saponins in leaves. Leaves and seeds showed a high content of total polyphenols (574.895 ± 4.600 mg GAE/g dried extract and 551.895 ± 2.300 mg GAE/g dried extract respectively); Total flavonoids content was high in leaves. Quantification of aglycones revealed an important amount of aglycones in leaves (0.118 ± 0.032 mg QE/g dried plant material). Thin layer chromatography revealed a wide variety of flavonoids and the presence of kaempferol in seeds. Results of the present work suggest that there is strong pharmacological potential in developing *V. agnus castus* as a drug to be used in antioxidant activity.

Keywords: Phytochemical screening, polyphenols, flavonoids, aglycones, antioxidant activity, *Vitex agnus castus.*

Introduction:

The world is fertile with natural and medicinal plants. Medicinal plants are now more focused than ever because they have the capability of producing many benefits to society indeed to mankind, especially in the line of medicine and pharmacological. The medicinal power of these plants lies in phytochemical constituents that cause definite pharmacological actions on the human body (Akinmoladun et *al.*, 2007). Chaste tree (*V. agnus castus L.*) native from arid and semi arid Mediterranean and Western Asia is an aromatic, ornamental and deciduous shrub (Dogan et *al.*, 2011). *V. agnus castus L.* was formerly

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classified within the family of Verbenaceae (Meena et al., 2010) but now the phylogenetic classification located it in the Lamiaceae family.

Traditionnally, *V. agnus castus* has been used in the treatment of menstrual disorders (amenorrhoea, dysmenorrhoea), premenstrual syndrome (PMS), corpus luteum insufficiency, hyperprolactinaemia, infertility, acne, menopause and disrupted lactation, cyclic breast pain cyclical mastalgia and inflammatory conditions, diarrhea and flatulence (Ono et *al.*, 2008; Costa et *al.*, 2007; Azarnia et *al.*, 2007; Carmichael et *al.*, 2008; Dugoua et *al.*, 2008; Cossuta et *al.*, 2008). In Morocco powdered seeds, mixed with honey, are used as warming in the treatment of cooling, or mixed with honey against kidney stones, urinary ailments and as warming in colds. The roasted seeds, mixed with those of fenugreek are swallowed by women to take overweight (Bellakhdar, 1997).

As part of our continuing investigation of medicinal and aromatic plants of south of Morocco (Asdadi et *al.*, 2015a, b) we report in this research the phytochemical screening, a quantitative determination of total polyphenols content, total flavonoids contents and aglycones content as well as an evaluation of antioxidant activity of leaves and seeds of *V. agnus castus.*

Material and methods:

Studied plant

The species *V. agnus castus* was collected in the oasis of Tata (South East of Morocco) during the month of May 2013. The plant was identified and deposited in the herbarium of laboratory of Biotechnology, Planta Sud unity, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Leaves and seeds were separated, dried in the shade and conserved at the laboratory temperature.

Extraction using MeOH 80%

Dried plant material of leaves and seeds of *V. agnus castus* was ground and submitted to ultrasound and maceration with agitation in methanol/water (4:1) during 24 hrs. After filtration, the solvent of extraction was removed by evaporation to dryness under reduced pressure at 40°C until the sample weight was constant.

Alkaloids

The presence of alkaloids was demonstrated by two different tests: the tests of iodoplatinate's and of the dragendorff's reagent. The methanol 80% extracts of the leaves and seeds of *V. agnus castus* were submitted to thin layer chromatography (TLC) Plates (GF 254 60; Merck). Silica plates prepared were eluted in the solvent ethyl acetate/methanol/water (100/13.5/10, v/v/v). The chromatograms were dried after migration. The reagents of iodoplatinate and dragendorff were applied. *Peganum harmala* methanol 80% extract react to the two tests and was used as positive control.

Saponins

Presence/absence of saponins was determined by the calculation of the index of foam (European pharmacopoeia, 1997). For this, a decoction was prepared of 2 g of finely ground plant material in 100 mL of water. Boiling takes 30 min. The extract was then cooled and filtered. The volume was adjusted to

100 mL. 1 to 10 mL of each decoction was placed in 10 tubes (same length and same diameter) and the volume was completed in each tube to 10 mL with distilled water. The tubes were capped with the thumb and violently stirred in a horizontal position for 15 sec. The length of the residual foam in each tube was measured in centimeter. The foam index (I) is calculated using the following formula.

$$I = \text{Length of the residual foam in the } n^{th} \text{ tube} * 100/0.0n$$

The presence of saponins is confirmed with the foam index greater than 100.

Terpens

TLC on silica gel of methanol 80% extract of different samples was carried out in benzene. After drying, plates were sprayed with antimony chloride, and then placed in an oven at 110°C for 10 min. Any detected fluorescence under UV at 365 nm indicates the presence of terpens (Randerath, 1971).

Free quinones

Dried methanol 80% extract is dissolved in ether-ethanol (1:1). To the ethero alcoholic solution were added few drops of 5% lead acetate. The appearance of a red color reveals the presence of free quinones (Ribéreau-Gayon et *al.*, 1968).

Anthraquinones

Dried methanol 80% extract was dissolved in chloroform. After filtration, 1 mL of 10% potassium hydroxide KOH was added. If after the agitation, the aqueous phase turns red, the presence of anthraquinones is confirmed (Rizk, 1982).

Tannins

Dried methanol 80% extract was dissolved in methanol. A few drops of 1% $FeCl_3$ were added. The appearance of a dark blue color indicates the presence of gallic tannins while the appearance of a greenish brown color indicates the presence of catechic tannins (Bate-Smith, 1962).

Total polyphenols content

Total polyphenols were determined by Folin-Ciocalteu method (Aguilar-Garcia et *al.*, 2007). 50 µL of a stock solution prepared from dissolved dry methanol extract 80% of different samples and brought to appropriate dilutions was mixed with 1.25 mL of 10 fold diluted Folin-Ciocalteu phenol reagent. After 2 min of incubation at room temperature, 1 mL of 7.5% sodium carbonate was added and the mixture was incubated for 15 min at 50°C. A calibration curve of gallic acid was established and the measurement was done at 760 nm. Total polyphenols values were expressed as milligram gallic acid equivalent per gram of dried extract (mg GAE/g DE).

Total flavonoids contents

Two ml of 80% methanol extract brought to the suitable dilution were mixed with 100 µL of 2-aminoethyl-diphenylborate (NEU) (1% in methanol). The absorption was determined at 409 nm by visible UV spectrophotometer and compared with that of standard quercetol (0.05 mg/mL) treated with the same reagent and under the same conditions as the extract. Total flavonoids content was calculated quercetol equivalent using the following formula (El Hariri et *al.*, 1991).

$$F\,(\%) = A_{ext} \times 0.05 \times 100/A_q \times C_{ext}$$

Where A_{ext} is the absorption of the sample, A_q is the absorption of quercetol and C_{ext} is the concentration of the extract in mg/mL.

Acid hydrolysis

Aglycones were extracted using the protocol of Lebreton et *al*. (1967) modified by Jay et *al.* (1975). To 2 g of dry plant material powder 160 mL of cold 2N HCl was added in glass bottles. The mixture was heated in a water bath (100°C for 40 min) with air insufflations. Allowed to cool and filtered. The extraction by acid hydrolysis was done again 3 times for each sample.

Extraction of aglycones

Acid solutions obtained after acid hydrolysis were transferred to separation funnels and aglycones extraction was realized with ethyl ether (3 × 20 mL). Extracts obtained, yellow-colored, were made to evaporate in a fume cupboard, and then taken up in 5 mL of 95° and stored at 4°C until use.

Extraction of anthocyanins

After the extraction of aglycones flavonoids, the same acid solution used to extract aglycones were used to extract the anthocyanins using n-butanol (3 × 20 mL).

Aglycones content

Aglycones flavonoids content were done based on chelating properties of $AlCl_3$. Measuring the optical density was carried out by UV visible spectrophotometer (UV Spectrophotometer, type HP Vectra Chemstations software program) between 380 and 460 nm. The absorbance of the differential peak against a blank containing no $AlCl_3$ was proportional to aglycones flavonoids content in samples. Aglycones flavonoids content expressed in equivalent quercetol per gram of dried plant material was calculated using the formula:

$$T \text{ aglycones} = (A / \varepsilon) \times M \times V \times d / p \text{ (mg QE/g DP)}$$

Where A is the differential absorbance peak, ε is the molar absorption coefficient of quercetol (=23000), M is the quercetol molar mass (= 302), V is the volume of the ethanolic solution of aglycones flavonoids, d is the dilution factor and p is the dry weight of hydrolyzed plant material.

Content of anthocyanins

Anthocyanins content was obtained by scanning the spectrum between 480 and 600 nm (Porter et *al.*, 1985; Lebreton et *al.*, 1967) and retaining the maximum absorbance. The content was calculated using the following formula:

$$T \text{ anthocyanes} = (\gamma A / \varepsilon) \times M \times V \times d / p \text{ (mg/g DP)}$$

γ is the correction factor (= 6) of the performance processing proanthocyanes (about 17%); A is the absorbance at the maximum absorption wavelength; ε is the molar absorption coefficient of cyanidol (=34 700); M is leucocyanidol molar mass (=306); V is the volume of the ethanol solution; d is the dilution factor; p is the weight of dry matter of the hydrolysed vegetable material.

Identification procedure

Methanol 80% extracts previously prepared and stored at 4°C were subsequently subjected to TLC on silica gel plates silica gel 60 F254 (10 × 20 cm, 0.2 mm layer) using Ethyl acetate/Methanol/Water

(100:13.5:10, V/V/V) as mobile. Detection with UV light at 365 nm was performed before and after spraying with 1% (in methanol) of NEU reagent.

Antioxidant activity

In vitro antioxidant activity was assessed by measuring the scavenging power of free radical 2, 2-diphenyl-1-picryhydrazyl (DPPH), according to Chen et *al.* (2004) and Leitao et *al.* (2002) with some modifications. Dried methanol 80% extracts of leaves and seeds of *V. agnus castus* were dissolved in pure methanol to prepare a stock solution. From stock solution were prepared different concentrations (0.1 mg/mL, 0.05 mg/mL, 0.025 mg/mL and 0.012 mg/mL). 500 µL of every concentration were mixed with 500 µL of methanol solution of DPPH (0.004 %). After an incubation period of 30 min in the dark at ambient temperature, the absorbance was read at 517 nm wavelength. The inhibition of free radical DPPH by BHT (butylhydroxytoluene) and Covi-ox T50 were also analyzed with the same concentrations and the same conditions for comparison. The inhibition of free radical DPPH percentage (I) was calculated as follows:

$$I\,(\%) = 100 \times (A_{control} - A_{test}) / A_{control}$$

Where $A_{control}$ is the absorbance of the control (containing all reagents without the test product) and A_{test} is the absorbance of the test compound (containing all reagents and the test product). The value IC_{50} was calculated from the graph of the DPPH scavenging effect percentage against the sample concentration and it was used to characterize the antioxidant activity. All tests were performed in triplicate for each concentration.

Statistical analysis

The graphical representation of the data was performed using the Excel software. The mean value is accompanied by the standard error of the mean (Mean ± SEM). Statistical analysis of the results was performed by analysis of variance using STATISTICA software, version 6 (Stat-Soft, 2001). The significance of the difference tested was determined by comparing the probability *p* associated with the Newman-Keuls statistical theoretical threshold of $\alpha = 0.05$.

Results and discussions

Phytochemical screening

The present study carried out on leaves and seeds of *V. agnus castus* harvested in the oasis of Tata located in South-Easth of Morocco revealed the presence of medicinally active constituents. Results of phytochemical screening and quantitative analysis are summarized in table 1. Alkaloids, tannins and terpens were present in both leaves and seeds of *V. agnus castus* while saponins were present in leaves and absent in seeds and anthraquinones were present in seeds but absent in leaves. Free quinones and anthocyanins were not detected in both examined samples. These bioactive components are naturally occurring in most plant materials and known to possess interesting biological activities such as antioxidant, anticarcinogenic, antiviral, antibacterial, antidiabetic and anti-inflammatory (Adebayo et *al.*, 2010; Jaime et *al.*, 2013; Teugwa et *al.*, 2013; Gupta et *al.*, 2012).

The obtained results of table 1 and figure 1 showed that total polyphenols were present in important amount in both leaves and seeds of *V. agnus castus.* In fact polyphenols are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants. This plant can be successfully used as an inexpensive source of phenolic compounds, especially in the food and pharmaceutical technology (Latoui et *al.,* 2012). Percentage of flavonoids in leaves was more important than those in seeds.

Antioxydant activity

The antioxidant activity of leaves and seeds of *V. agnus castus* was assessed by antioxidant DPPH assay. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity (Sánchez-Moreno et *al.*, 1998). To a lower IC_{50} value corresponds a higher antioxidant power. Results of table 2 showed clearly that leaves extract was more effective than seeds extract. Covi-ox T50 exhibited the highest scavenging effect with 0.003 ± 0.002 mg/mL. leaves and seeds extracts exhibited a scavenging effect more than BHT. Figure 2 shows that the DPPH scvanging effect of different samples increases with the concentration increase.

The coefficients of correlation between IC_{50} of leaves and seeds of *V. agnus-castus*, total polyphenols and total flavonoids were determined. A negative correlation was found between IC_{50} and total polyphenols content and between IC_{50} and total flavonoids content. That means that DPPH scavenging activity increases significantly with the presence of high concentrations of total polyphenols and total flavonoids. In fact polyphenols have been reported and provided to be potent hydrogen donators to the DPPH radical (Von Gadow et *al.,* 1997) because of their excellent structural chemistry (Rice-Evans et *al.,* 1997). Polyphenols are represented in majority by tannins and flavonoids (Baxter et *al.,* 1998) and positive correlation between total polyphenols and total flavonoids was found (Table 3).

TLC is usually performed in one dimension by gravity flow ascending development with a single mobile phase in a solvent vapor saturated, paper lined glass N-chamber. The reagent of NEU has been used to reveal flavonoids. This reagent, indeed, reveals them as colorful stains in blue, orange, green, red and yellow fluorescent (Wagner and Bladt, 1996). The seeds showed low intensity fluorescence in comparison with leaves (figure 3). And this coincides with the total flavonoids assay results where the seeds had a low content against other leaves. According to the witness, kaempferol was present in the seeds and absent in the leaves. Diagrams of leaves and seeds TLC was dominated with bright yellow coloration and according to Mabry et *al.* (1970) and to Wagner and Bladt (1996) it's corresponds to chalcones and to aurones.

Conclusion

The phytochemical screening of this investigation attested the presence of several secondary metabolites in leaves and seeds of *V. agnus castus* harvested in a marginalized oasis of south east of Morocco; Dosages done at the end of the phytochemical screening, permitted to quantify total polphenols, total flavonoids and aglycones contents. Leaves were very rich of flavonoids and polyphenols, these

components are responsible of the important scavanging effect of free radical DPPH showed by leaves and seeds of *V. agnus castus*. The results of this study can be a rational scientific explanation to the large use of *V. agnus castus* in non conventional medicine by the populations.

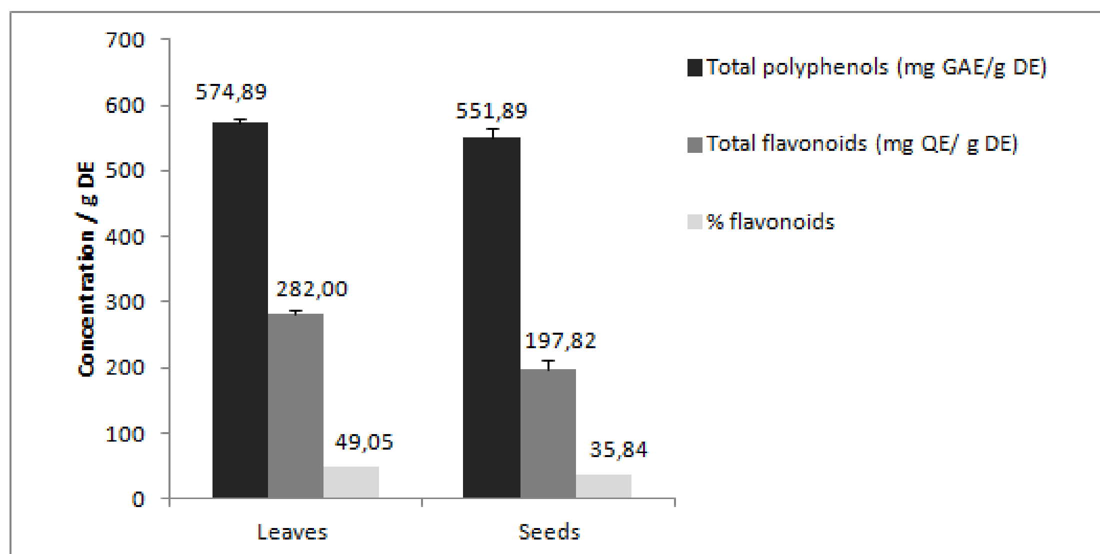

Figure 1: Total polyphenols content (mg GAE/g dried extract), total flavonoids content (mg QE/g dried extract), and flavonoids percentage (%) of leaves and seeds of *V. Agnus castus. L.* Each value represents a mean ± SD (n=3), p <0.05.

Table 1: Phytochemical screening and quantitative analysis of methanol 80% extract of leaves and seeds of *V. agnus castus*.

Secondary metabolites		Samples	
		Leaves	Seeds
Alkaloids	Dragendorff	nd	nd
	Iodoplatinate	+++	+++
Tannins	Condensed tannins	+++	++
	Hydrolysable tannins	nd	nd
Terpens		+++	+++
Saponins		+++	nd
Anthraquinons		nd	+++
Free quinones		nd	nd
Total polyphenols content		574.895 ± 4.600 ^{a} (mg GAE/g DE)	551.895 ± 2.300 ^{a} (mg GAE/g DE)
Total flavonoids content		282.004 ± 4.911^{b} (mg QE/g DE)	197.826 ± 14.251^{c}(mg QE/g DE)
Aglycones contents		0.118 ± 0.032 ^{d} (mg QE/g DP)	0.049 ± 0.054 ^{e} (mg QE/g DP)
Anthocyanins content		Not detected	Nod detected

Each value represents a mean ± SD (n=3), p <0.05. Different letters a, b, c, d and e are significantly different according to Newman & Keuls tests at $p < 0.05$.

Table 2: IC_{50} values (in [mg/ml] ± standard deviation) of examined extracts of *Vitex agnus castus* in comparison with synthetic antioxidant references (BHT and Covi-ox T50).

Sample	IC_{50}
Leaves	0.034±0.009 [b]
Seeds	0.079± 0.009 [c]
BHT	0.0252 ± 0.011[d]
Covi-ox T50	0.003 ± 0.002 [a]

Each value is presented as mean ± standard deviation (n = 3). Different letters a, b and c are significantly different according to Newman & Keuls tests at $p < 0.05$.

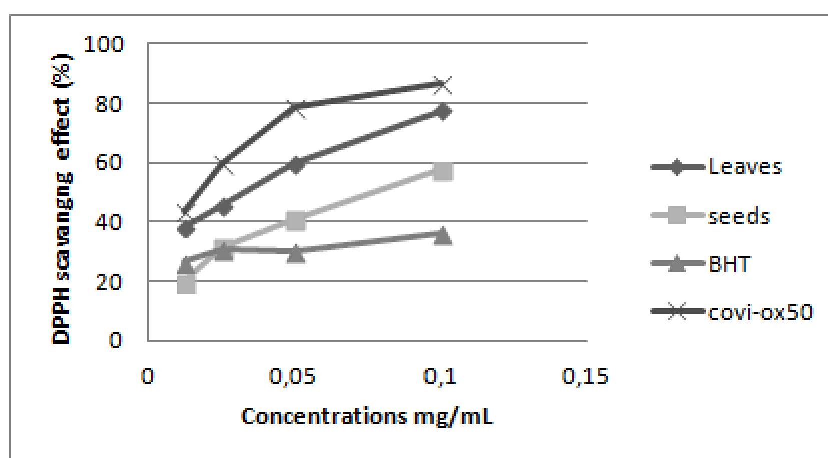

Figure 2: DPPH scavenging effect (%) of leaves and seeds of *V. agnus castus* and standards.

Disclosure of interest

The authors declare that they have no competing interest.

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