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TOTAL PHENOLS CONTENT, FLAVONOID CONCENTRATION AND ANTIOXIDANT ACTIVITIES OF LEAVES EXTRACTS OF *VITEX AGNUS-CASTUS* L. GROWING WILD IN MOROCCO

Fatima EL kamari *, Amal Taroq, Yassine EL atki, Imane Aouam, Badiaa Lyoussi and Abdelfattah Abdellaoui

Laboratory of Physiology Pharmacology and Environmental Health, Department of Biology, Faculty of Sciences Dhar EL Mehraz, University Sidi Mohamed Ben Abdellah, B.P. 1796, Atlas, Fez, Morocco.

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Correspondence to Author:
Fatima El Kamari

Laboratory of Physiology Pharmacology and Environmental Health, Department of Biology, Faculty of Sciences Dhar EL Mehraz, University Sidi Mohamed Ben Abdellah, B.P. 1796, Atlas, Fez, Morocco.

E-mail: kamarisapiens@gmail.com

ABSTRACT: *Vitex agnus-castus* L. (Verbenaceae) commonly known as Chaste tree, is a shrub widely distributed in the Middle East and Southern Europe. Traditionally used for the treatment of several health problems and symptoms, such as premenstrual ones and spasmodic dysmenorrhea, certain menopausal conditions, insufficient lactation, and acne. Several reports have indicated that *Vitex agnus-castus* contained flavonoids, diterpenoids, and essential oils. To our knowledge, no data are available on the antioxidant activities of Moroccan *Vitex agnus-castus*. This work aims to evaluate the antioxidant activity of four extracts from leaves of *Vitex agnus-castus* growing wild in Morocco. Phytochemical tests of the extracts (ethanol, methanol, ethyl acetate, and water) were carried out qualitatively for the presence of Alkaloids, Tannins, Anthraquinones, Terpenes, and saponins. *In-vitro* antioxidant activity, total phenols content and total flavonoid content of different extracts were determined using spectrophotometric methods. The total phenolic content ranged from (10 ± 0.2 to 53.33 ± 1.38 mgEq GAE/g DW), and the total flavonoid concentrations varied from (13.66 ± 0.33 to 95.33 mg RE/gDW). Ethanolic extract of *Vitex agnus-castus* leaves has shown the highest phenols and flavonoid concentrations and strong antioxidant activity. A very strong positive correlation between the total antioxidant activity of the extracts and their content of phenols and flavonoids ($P<0.05$) is observed. Therefore, Moroccan *Vitex agnus-castus* L. can be regarded as promising candidates for natural plant sources of antioxidants with high value.

INTRODUCTION: Free radicals and reactive oxygen species (ROS) are considered to be harmful to human health and play an essential causative role in disease initiation such as neurodegenerative disease, and cancer [1].

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Thus, there is a growing interest in finding natural substances exhibiting antioxidant properties to substitute the synthetic ones, which were restricted due to their side effects. Several previous studies have reported that medicinal plants contain a large variety of free radical scavenging molecules such as phenols, anthocyanins, tannins, alkaloid and saponins which can play a significant role in the prevention and protection against many diseases [2].

Vitex agnus-castus L. (VAC) commonly known as Chaste tree [3] or Chasteberry [4], is a small tree from *Verbenaceae* family, native to the Mediterranean

and Western Asia. It is now cultivated all over the world, including the Southern part of the United States [5]. Traditionally, this plant, particularly its fruit extract, has been used in the treatment of premenstrual syndrome (PMS), abnormal menstrual cycles, amenorrhea, mastodynia, hyperprolactinemia, premenstrual dysphoric disorder, lactation difficulties, and low fertility [6]. The aromatic leaves are used as a spice [7]. Previous studies on the phytochemical analysis of *V. agnus-castus* L. revealed the presence of essential oils [8], flavonoids, iridoids, and diterpenoids [9]. It was reported that VAC was also used as a diuretic, digestive, anxiolytic and antifungal agent [10]. Besides, different extracts of *Vitex agnus-castus* L. fruits have shown a significant cytotoxic, DNA damaging and apoptotic effects in MCF-7 human breast cancer cells [11]. A recent study has demonstrated that the essential oils of fruits and leaves of *Vitex agnus-castus* have strong antibacterial activity against some bacteria causing nosocomial infections in the neonatal and intensive care rooms at the university hospital center of Fez, Morocco [12]. This work aims to evaluate, for the first time, the antioxidant activity, flavonoids and total phenolic content of extracts of *Vitex agnus-castus* leaves from the Moroccan Middle Atlas.

MATERIALS AND METHODS:

Plants Materials: Plant material (leaves of *Vitex agnus-castus)* was collected during June October in 2016 (flowering period) in Khenifra. Identification was confirmed by Professor Amina Bari, botanist (Department of Biological Sciences, Faculty of Science, Sidi Mohammed Ben Abdellah University, Fes, Morocco). The material was dried for 7 to 10 days in the shade temperature and then ground.

Extract preparation: Ethanol (Etoh. E.), methanol (Metoh. E.), water (water E.) and ethyl acetate (Ethy Ac. E.) extractions were performed at the ratio of 10% (w/v) for 48 h under agitation for plant powder. Then the mixture was filtered through a filter paper (Whatman no. 1) and concentrated in vacuo at 45 °C, then stored at 4 °C for further use.

Phytochemical Analysis: Phytochemical tests of the extracts were carried out qualitatively for the presence of alkaloids, saponins, tannins, flavonoids anthraquinones, terpenes, according to the methods described by Edeoga *et al*., with slight modifications [13].

Total Antioxidant Capacity: The total antioxidant capacity of the extract was evaluated by the phosphomolybdenum method as described by Pavithra *et al*., with minor modifications [14]. Briefly, a volume of 25 µL extract was added to 1 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The mixtures were incubated at 95 °C for 90 min and then cooled to room temperature. The absorbance was measured at 695 nm. The total antioxidant activity was expressed as the number of equivalence of ascorbic acid (mg AAE/g DW).

Determination of Total Phenols Content (TPC): Total phenol content of the extract was determined by the Folin-Ciocalteu method described by Jadouali *et al*., with slight modifications [15]. The 0.5 ml of a known dilution of the extract and 2 ml of 7% sodium carbonate solution were added to 2.5 ml of 10% (v/v) Folin - Ciocalteau reagent. The absorbance was read at 760 nm (Jasco v-530) after 2 h of reaction at room temperature in the dark. Gallic acid was used as a standard for the construction of calibration curve **Fig. 1**. Total phenols content was expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/g DW).

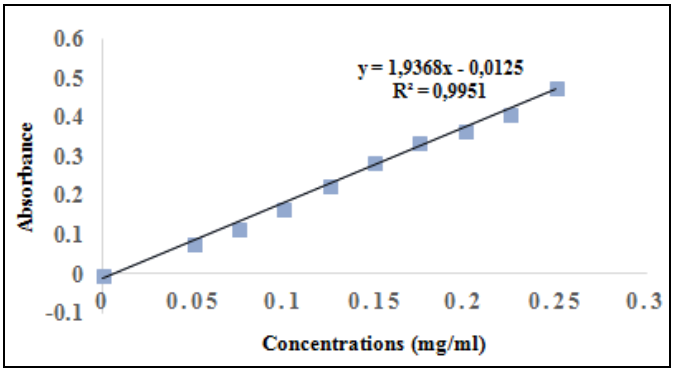

FIG. 1: GALLIC ACID STANDARD CUVE FOR THE CALCULATION OF TOTAL PHENOLS CONTENT

Determination of Total Flavonoid Content: Total flavonoid content of extracts was measured by the aluminum chloride colorimetric assay [16]. 1 ml of sample or rutin standard solution was added to a 10 mL volumetric flask containing 4 ml of distilled water. To the flask 0.30 ml 5% $NaNO_2$ was added, after five minutes 0.3 ml 10% $AlCl_3$ was added to

react for 6 min. After that, 2 ml IM NaOH was added and the total was made up to 10 ml with distilled water. The solution was mixed, and absorbance was measured against the blank at 510 nm (Jasco v-530). Rutin was used as a standard for the construction of calibration curve **Fig. 2**. Total flavonoid content was expressed as mg Rutin equivalents per gram dry weight of each extract (mg RE/g DW).

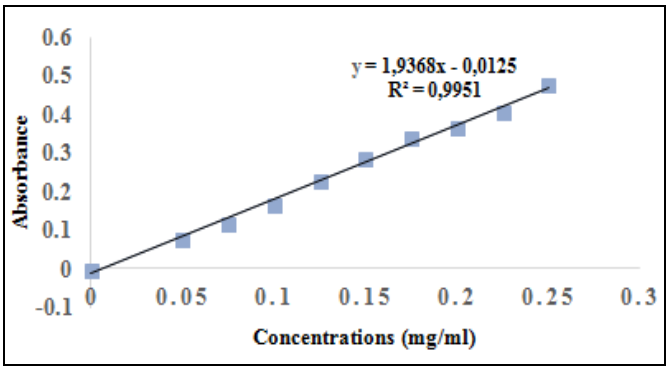

FIG. 2: RUTIN STANDARD CUVE FOR THE CALCULATION OF TOTAL FLAVONOIDS CONTENT

DPPH Radical Scavenging Assay: The radical scavenging activity of the VAC extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was measured using the method of Adebiyi *et al*., with minor modifications [17]. 0.1 ml of various concentrations of the extracts or standard was added to 1.5 ml of an ethanolic solution containing 0.1 mmol of DPPH (2, 2-diphenyl-1picryl-hydroxyl). The absorbance of the mixture was measured at 517 nm with a spectrophotometer (Jasco V-530) after 30 min of incubation time at room temperature in the dark. The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [(A_C - A_S)/A_C] \times 100$$

Where A_C is the absorbance of the control, and A_S is the absorbance of the sample. BHT served as positive control. The IC_{50} values were calculated as the concentration of extract providing 50% inhibition of DPPH radical.

Reducing Power Assay: The reducing capacity of the tested extracts was determined by the procedure of Oyaizu [18]. 200 µl of the extract was mixed with 500µl of phosphate buffer (0.2M, pH 6.6) and 500 µl of potassium ferricyanide $[K_3Fe(CN)_6]$ 1%. The obtained solution was incubated at 50 °C for 20 min. The mixture was acidified with 500 µl of Trichloroacetic (TCA) 10% which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 500 µl of distilled water and 100 µl of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm (Jasco v-530). Quercetin was used as a standard. The results were expressed as IC_{50} (mg/ml). IC_{50} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration.

Statistical Analysis: All tests were performed in triplicate and results were expressed as mean ± SD. The results were compared by one-way ANOVA followed by Tuckey-test, using the GraphPad Prism 5 (Microsoft Software). Differences at $P<0.05$ were considered to be significant. Pearson correlation was performed using the same test.

RESULTS AND DISCUSSION:

Phytochemical Analysis: Results of phytochemical screening of all the extracts are shown in **Table 1**. Flavonoids and phenols are present in all extracts, while saponins were present in water and ethanol extracts and absent in ethyl acetate and methanol extract. Alkaloids and terpenes were not detected in water and ethanol extract. Anthraquinones were observed only in the ethanol extract. These bioactive components have been shown to possess interesting biological activities such as antioxidant, antidiabetic, antibacterial, anti-inflammatory and many others [19, 20, 21].

TABLE 1: PHYTOCHEMICAL SCREENING TEST RESULTS

Tests	Extracts			
	Methanol	Ethyl acetate	Water	Ethanol
Alkaloids	-	+	-	-
Tanins	+	+	-	-
Anthraquinone	-	-	-	+
Terpenes	+	+	-	-
Saponins	-	-	+	+
Flavonoids	+	+	+	+
Phenols	+	+	+	+

+ (present); - (Absent)

Extraction Yield and Total Antioxidant Capacity: The yields of extracts obtained from *Vitex agnus-castus* leaves using various solvents are shown in **Table 2**. Water gives the higher extraction yield with 24%, followed by methanol

(22.44%), ethanol (10.02%) and ethyl acetate (6.12%). Our results are in agreement with those obtained by saglam *et al*., who reported that water gives the highest yield extract (38.06%) when compared with ethanol and n-hexane extracts of *vitex agnus-castus* leaves and fruits [22]. This observation is also supported by results obtained from the extraction from *Limnophila aromatica* where the polar solvents, methanol, and water, gave the best extraction yields [23]. It has been reported that the efficiency of the extraction depends on many parameters, including the extraction time and temperature, the volume and type of the solvents used [24, 25]. The total antioxidant capacity assay showed that the ethanol extract had the most important activity with value 357.66 ± 5.08 mg equivalent to ascorbic acid/g DW. The water extract had a weak antioxidant activity.

TABLE 2: EXTRACTION YIELD AND TOTAL ANTIOXIDANT CAPACITY OF DIFFERENT EXTRACTS OF *VITEX AGNUS-CASTUS* LEAVES

Extract	Yield (%)	CAT (mg Eq A.As/gDW)
Ethanol	10.02	357.66 ± 5.08^{a}
Methanol	22.44	194 ± 1.41^{b}
Ethyl acetate	6.12	138 ± 2.04^{c}
Water	24	33.6 ± 1.16^{d}

Data are expressed as mean ± SEM of tree measurements. Different letters symbolized significant differences (P<0.05) by mean of the non-parametric Turkey-test.

Determination of Total Phenols Content: Most antioxidant activities from plant sources are derived from phenolic compounds [26]. As we can see from **Fig. 3**, the amount of TPC of VAC extracts, measured by Folin-Ciocalteu method varied significantly (P<0.05) from 10 ± 0.2 to 53.33 ± 1.38 mg GAE/g DW. Pure ethanol produced extracts with the highest levels of total phenols content. Our finding is supported by Latoui *et al*., who demonstrated that ethanol behaved as a better solvent than methanol to extract from fruits of VAC the same classes of compounds whose concentrations in the extract reached after only 30 min maximum values (19.2 mg CAE/gDB TP and 5.4 mg CAE/Gdb OD) about 3-5-fold those obtained with methanol [27]. Furthermore, several studies have shown that the amount of polyphenolics in plants depends on biological factors (genotype, organ, and ontogeny), as well as edaphic, and environmental (temperature, salinity, water stress and light intensity) conditions.

Besides, the solubility of phenolic compounds is governed by the type of solvent used, the degree of polymerization of phenolics, and their interaction[28].

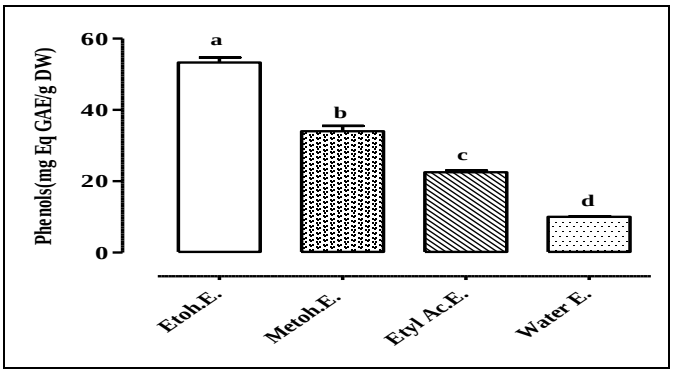

FIG. 3: PHENOLS CONTENT OF DIFFERENT EXTRACTS OF *VITEX AGNUS-CASTUS* LEAVES
Different letters symbolized significant differences (P<0.05) by mean of the nonparametric Turkey-test

Determination of Total Flavonoid Content: The total flavonoid content was expressed as mg Rutin equivalents per gram dry weight of each extract (mg RE/g DW). Results can be seen from **Fig. 4**, the concentration of flavonoids in VAC extracts have ranged from 13.66 ± 0.33 to 95.33 ± 6.16 mg RE/g DW. The ethanol extract contains the highest content of flavonoids. The lowest flavonoids content is obtained in ethyl acetate and water extract. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation [29]. According to work performed by Latoui *et al*., the methanolic extract of VAC leaves from Tunisia contains 19.4 mg Catechin Equivalent/g dry biomass, which is below that found in the present study (48.66 ± 5.33 mg RE/g DW) [27]. Our results have shown the richness of the VAC leaves for these bioactive molecules.

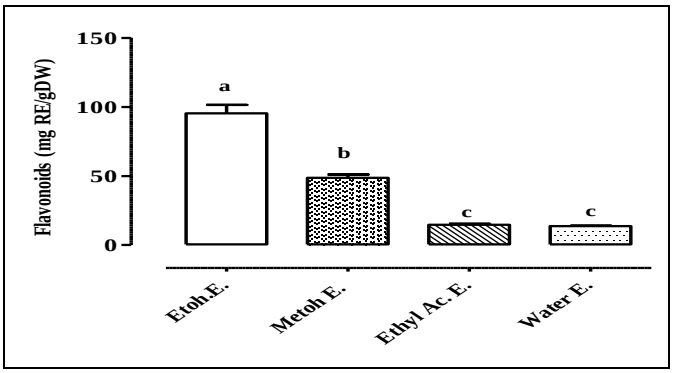

FIG. 4: FLAVONOIDS CONTENT OF DIFFERENT EXTRACTS OF *VITEX AGNUS CASTUS* LEAVES.
Different letters symbolized significant differences (P<0.05) by mean of the nonparametric Turkey-test.

Antioxidant Activities:

DPPH Radical Scavenging Activity: Antioxidant activity of *V. agnus-castus* extracts has been determined by two different test systems namely DPPH and reducing power (FRAP). Results are shown in **Table 3**, from which it can be observed that all the extracts possess radical scavenging potential. Ethanol has proved to be the most active of the extracts with (IC_{50} = 0.53 ± 0.006 mg/ml), while the water extract was the least (IC_{50} = 1.01 ± 0.019 mg/ml). None of the samples evaluated have shown activity as strong as the synthetic antioxidant BHT (0.10 ± 0.001 mg/ml).

An extract is considered to be active against free radicals if IC_{50} < 5 mg/ml [30]. All our extracts have IC_{50} values of less than 5 mg/ml. Therefore, all the extracts for the solvents used are a possible good source of antioxidants. Also, we compared our results with an early report from Turkey, the antioxidant activity of methanolic extract of VAC fruits and leaves collected from Antalya was investigated by DPPH assay, methanol extracts of both leaves and fruits were found to possess strong antioxidant activity [31]. Our data are supported by this result, the methanol extract of Moroccan leaves of VAC exhibit a good antioxidant activity with an IC_{50} value of 0.66 ± 0.012 mg/ml.

TABLE 3: ANTIOXIDANT ACTIVITY OF *VITEX AGNUS CASTUS* EXTRACTS, REDUCING CAPACITY (FRAP TEST) AND RADICAL SCAVENGING ACTIVITY (DPPH TEST) EXPRESSED IN IC_{50} (mg/ml)

	DPPH	FRAP
Ethanol	0.53 ± 0.006^{b}	0.63 ± 0.004^{c}
Methanol	0.66 ± 0.012^{b}	0.44 ± 0.011^{b}
Ethyl Acetate	0.78 ± 0.020^{c}	0.98 ± 0.016^{d}
Water	1.01 ± 0.019^{d}	2.02 ± 0.202^{e}
BHT	0.10 ± 0.001^{a}	0.12 ± 0.004^{a}

Data are the mean of three measurements ± SEM. In the column, Different letters symbolized significant difference (P<0.05) by mean of the nonparametric Turkey-test.

Reducing Power Activity: Regarding the reducing power assay, the strongest activity is exhibited by the methanol extract with (IC_{50} = 0.44 ± 0.011 mg/ml), followed by ethanol and ethyl acetate. However, all extracts are less effective than the synthetic antioxidant BHT.

Correlation of Antioxidant Activities with Flavonoids and Phenols Content: The correlation coefficients between flavonoids, phenols compounds and antioxidant activities of the four extract are shown in **Table 3**. Based on these results, it appears that there is a very strong positive correlation between the total antioxidant activity of the extracts and their content of phenols and flavonoids (P<0.05). Indeed, the high correlation between phenols content and antioxidant activity is well documented [32, 33]. In previous studies, some phenolics were analyzed, and results demonstrated that especially casticin, caffeic acid and chlorogenic acid had been found in high amount in *Vitex agnus-castus* as antioxidant agents [34]. Furthermore, Vitexin was determined in a significant amount in the leaves, as one of the essential antioxidant constituents of *Vitex agnus-castus* [31]. In this study, we have noticed a low correlation in absolute value between phenols and flavonoids in plant extracts and reducing power activity. According to Yen *et al*., the reducing power is associated with some anthraquinones [35]. However, just one of our extract contains anthraquinones.

TABLE 4: PEARSON CORRELATION COEFFICIENTS BETWEEN COMPOUNDS AND ANTIOXIDANT ACTIVITIES

Antioxidant activities	Flavonoids	Phenols
CAT	0.95	0.99
DPPH	-0.87	-0.96
FRAP	-0.62	-0.63

CONCLUSION: In the current work, the total phenols content, total flavonoids content, and antioxidant activity have been investigated in the first time for Moroccan *Vitex agnus-castus*. The highest total phenolic and flavonoid contents are obtained from the ethanol extract of VAC leaves. Ethanol and methanol extracts have exhibited an interesting antioxidant activity. Based on these results, it could be concluded that *Vitex agnus-castus* is a new and inexpensive natural source of antioxidant substances which can combat oxidant damage and prevent pathogenesis of many diseases.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest regarding the publication of this paper.

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