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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *Vitex agnus castus* L.

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ABSTRACT

Antioxidant and antimicrobial activities of methanolic extract of *Vitex agnus castus* were studied. The antioxidant properties of the extracts were evaluated using different antioxidant tests, including ferric chelating, scavenging activity of hydrogen peroxide and cupric reducing antioxidant capacity. The highest antioxidant activity was observed to be 93.5±0.8% by scavenging activity of hydrogen peroxide. However, total flavonoid and phenolic content of the methanolic extract were determined. Antimicrobial activity tests were carried out using disc diffusion method and broth microdilution method with 7 bacteria and 1 yeast. Results suggested that *Vitex agnus castus* may be important in variety improvement, nutraceuticals, bio-pharmaceuticals and food additives as possible cost-effective natural antioxidant.

KEYWORDS: Vitex agnus castus, Phenolics, Antioxidant activity, Flavonoid, Antimicrobial activity

INTRODUCTION

The use of herbal drugs, forming a major part of complementary and alternative medicine or traditional medicine, is on the rise world-wide [1]. The global trend leads to an increased demand of medicinal plants for pharmaceuticals, phytochemicals, nutraceuticals, cosmetics, and other products and is an opportunity sector for commerce over the world. Medicinal effects of the plants, such as vegetables, fruits, seeds and roots are attributed to their phytochemical constituents. Studies have indicated that the phytochemicals as flavanoids, carotenoids and other phenolic compounds provide significant antioxidant activity and health benefit [2, 3]. Antioxidants may exert their effects on biological systems through different mechanisms including metal chelation and electron donation as reducing agent by reducing the oxidative damage associated with many diseases like cardiovascular disease, cataracts, atherosclerosis, diabetes mellitus, immune deficiency diseases and aging [4-6]. Several methods are available to identify antioxidant activities of natural compounds of the plants and biological systems [7-9].

Vitex agnus-castus L. (Verbenaceae) is a medicinal plant [10], 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 works have been reported that *Vitex agnus castus* contained flavonoids, diterpenoids, ecdysteroids as well as essential and fatty oils in the fruits, flowers and leaves [11-15].

In this study, we evaluated the antioxidant activity of methanolic extract of *Vitex agnus castus* grown in the south of Turkey by ferric chelating method, scavenging activity of hydrogen peroxide assay and cupric reducing antioxidant capacity (CUPRAC) assay. In addition to antioxidant analysis, total flavonoid and phenolic content of the methanolic extract were determined. Antimicrobial properties of the extract were studied by using disc diffusion method and broth microdilution method with 7 bacteria and 1 yeast.

MATERIALS AND METHODS

Chemicals

Gallic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), aluminium chloride, hydrogen peroxide solution, sodium carbonate, neocuprine, ammonium acetate, standard copper solution and methanol were supplied by Merck. Folin & Ciocalteu's phenol reagent, rutin, trolox and quercetin were obtained from Sigma Aldrich. All chemicals and reagents were of analytical grade. Double distilled water was used in the experiments.

Plants

The leaves of *Vitex agnus castus* were collected from a garden of Antalya (southern Turkey) in August 2008. The plant collected was identified by a senior scientist, Dr. Suleyman Gokturk at Akdeniz University, Department of Biology, Antalya, Turkey. The plant materials were cleaned, washed, dried and carefully powdered. All samples were kept in tightened light-protected containers.

Preparation of Extracts

Vitex agnus castus leaves powdered by grinding were extracted with methanol for 6 h at 35 °C using a magnetic

stirrer [16]. The mixture was filtered through a filter paper (Whatman No. 1). Resulting solution was evaporated under vacuum to 10 ml, then dried at –50 °C in a lyophiliser (yield, 11% w/w). Extract was kept in a freezer for further experiments.

Determination of Total Phenolic Content

The concentration of total phenolics was measured by the method of Gulcin et al. (2007) [17]. In this method, an aliquot of diluted extracts and standard solutions of gallic acid with different concentrations ranging between 0.01-0.5 mg ml^{-1} were added to a 25 ml volumetric flask containing 9 ml of ddH_2O. 2.5 ml of 10% (v/v) Folin & Ciocalteu's phenol reagent and 7.5 ml of 20% (w/v) Na_2CO_3 were added to the mixture and shaken vigorously. After incubation for 120 min at room temperature, the absorbance was recorded at 750 nm. The total phenolic content of *Vitex agnus castus* extract was expressed as mg gallic acid equivalents (GAE mg/g) for the dry extract by using standard curve equation ($y = 4.214x + 1.031$, $R^2 = 0.999$). All samples were analysed in three replications.

Determination of Total Flavonoid Content

Total flavonoid content of the methanolic extract of *Vitex agnus castus* was measured according to Ebrahimzadeh et al. (2008) [18]. In this method, 0.5 ml of appropriately diluted sample or standard solution was added to a 10-ml volumetric flask containing 1.5 ml methanol. 2.8 ml of double-distilled water, 0.1 ml of 1 M sodium acetate and 0.1 ml of 10% (w/v) $AlCl_3$. After incubation for 30 min at room temperature, absorbance of the reaction mixture was measured at 415 nm with a double-beam Shimadzu UV-Vis spectrophotometer (Shimadzu, Japan). The calibration curve was prepared by quercetin at concentrations between 0.01-0.5 mg ml^{-1}. The total flavonoid content of the extract was expressed as mg quercetin equivalents (QE mg g^{-1} dry extract) by using standard curve equation, $y = 5.219x + 0.054$, ($R^2 = 0.998$). All tests were carried out in triplicate.

Cupric Reducing Antioxidant Capacity (CUPRAC)

The cupric reducing antioxidant capacity of the methanolic extract from *Vitex agnus castus* was determined according to Gulcin et al. (2008) [19]. 1 ml of 10 mM Cu(II), 7.5 mM neocuprine, and NH_4Ac buffer (1 M, pH 7.0) solutions were mixed in a test tube to prepare the reaction mixture. Extracts at different concentration (0.01-0.5 mg ml^{-1}) were added to the reaction mixture until 4.1 ml of final volume. After 1 h of incubation, the absorbance was recorded at 450 nm. CUPRAC procedure was repeatedly applied to trolox solutions at different concentrations, and calibration curve of trolox (0.2-2 mM) was drawn. The results were expressed as mM trolox equivalents to antioxidant capacity (TEAC) per g dry weight of the extract by using standard curve equation, $y = 4.923x + 0.075$, $R^2 = 0.998$. The samples were run in triplicate.

Ferrous Ion Chelating Capacity

The method described by Que et al. [20] was used to determine the ferrous ion chelating activity of the methanolic extract of *Vitex agnus castus*. 0.1 ml of 2 mM $FeCl_2.4H_2O$, 0.2 ml of 5 mM ferrozine (3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine) and 3.7 ml of methanol were mixed with 0.1 ml of methanolic extract at different concentrations (0.05-0.5 mg ml^{-1}) in a test tube and incubated for 10 min. The absorbance at 562 nm was recorded, and ferrous ion chelating capacity was calculated as follows:

$$I\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is absorbance of the blank, A_{sample} is absorbance of extract or standard. Rutin was used as comparative standard. The samples were run in triplicate.

Scavenging Activity of Hydrogen Peroxide

The ability of the plant extract to scavenge hydrogen peroxide (H_2O_2) was described by the method of Benkeblia et al. [21]. 2 mM of H_2O_2 solution was prepared in phosphate-buffered saline (PBS, pH 7.4). H_2O_2 concentration was determined spectrophotometrically at 230 nm. 0.1 ml of the extract was added to H_2O_2 solution (0.6 ml) and absorbance of the hydrogen peroxide at 230 nm was read after 10 min against a blank solution containing extract in PBS without H_2O_2. The scavenging of H_2O_2 was determined by using the following equation by Gülçin et al. [22]:

$$I\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is absorbance of blank (extract in PBS without H_2O_2), A_{sample} is absorbance of extract or standard with H_2O_2. Solutions of quercetin, rutin and ascorbic acid were used as comparative standards. The samples were run in triplicate.

Antibacterial and Antifungal activity

Biological activity of the methanolic extract of *Vitex agnus castus* was determined by using 7 bacteria and 1 yeast including *Escherichia coli* ATCC 25922*, Escherichia coli* ATCC 3166 :09 K:35 K:99, *Pseudomonas aeruginosa* ATCC 29853, *Klebsiella pneumoniae* Type B *, Staphylococcus aureus* ATCC 6538, *Streptococcus salivarius* RSHE 606*, Bacillus cereus* ATCC 11778 and *Candida albicans* as test organisms. Bacterial strains and yeast were cultured overnight at 37 °C in Nutrient Broth. Two different methods, disc diffusion method and the broth microdilution method were employed for the determination of antimicrobial activity.

Disc Diffusion Assay

The extract was dissolved in DMSO:PBS (1:1) to a final concentration of 100 mg ml^{-1} and sterilized by filtration (0.45 µm, Millipore filters). 100 µl of suspension containing 10^8 CFU ml^{-1} of bacteria (0.5 Mc Farland Standard turbidity) and 10^6 CFU ml^{-1} of yeast were spread on

Mueller-Hinton Agar (MHA) medium, respectively [23]. The discs (6 mm in diameter) were impregnated with 20 µl of the extracts (2000 µg/disc) at the concentration of 100 mg ml^{-1} and placed on inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extract. Ampicillin (10 µg/disc) and Penicillin G (10 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 °C for 24 h for clinical bacterial strains, but 48 h for the yeast. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms [24]. Each assay in this experiment was repeated twice.

Broth Microdilution Test

The minimal inhibition concentration (MIC) values of the extract were also studied for the microorganisms. The inocula of microorganisms were prepared from 12-h broth cultures and suspensions adjusted to 0.5 McFarland standard turbidity. The 96-well plates were prepared by dispensing into each well 100 µl of nutrient broth. A 100-µl from *Vitex agnus castus* extract, initially prepared at 100000 µg ml^{-1} level, was added into the first wells. Then, 100 µl from first well was transferred into 10 consecutive wells and diluted and then, 100 µl inoculum was distributed to each well. Ampicillin solution was used as positive control. Then, plates were incubated at appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., Highland Park, VT, USA) [25, 26].

Statistical analysis

The statistical analysis was carried out by using OriginPro 7.5 software. One-way ANOVA was applied to data and results were compared by using Tukey`s test. A difference was considered to be statistically significant when the *p*-value is lower than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Antioxidant Activity

Flavonoids and phenolic compounds are secondary metabolites of the plants. They have the ability to scavenge reactive oxygen species which are harmful to tissue and organs [2, 3]. They exert their action scavenging or chelating process in antioxidant mechanism.

In this study, the total flavonoid and phenolic contents of the methanolic extract of *Vitex agnus castus* were determined. The total flavonoid content of the extract was defined as quercetin equivalent. And also, the total phenolic content of the extract was expressed as gallic acid equivalent. The total flavonoid and phenolic content of *Vitex agnus castus* extract were 27.45±1.36 mg QE and 48.05±1.02 mg GAE per dry extract, respectively ($p<0.5$). The results revealed that the methanolic extract of *Vitex agnus castus* contained significantly more phenolics and flavonoids than several other plants [27, 28].

The trolox equivalent antioxidant capacity (TEAC) was defined as equvailent to mg trolox per mg extract in the study. The TEAC value of the methanolic extract of *Vitex agnus castus* was determined according to the CUPRAC method. The TEAC value was measured from the absorbance of solution allowed to stand for 30 min at room temperature at 450 nm after addition of the extract. $TEAC_{CUPRAC}$ values were simply calculated by standard curve equation obtained from calibration curve of trolox at different concentrations of the extract. The results of CUPRAC antioxidant assay of *Vitex agnus castus* extract at different dilutions are depicted in Figure 1 showing good agreement (R^2= 0.998) between measured $TEAC_{CUPRAC}$ values and solid extract. Hovewer, $TEAC_{CUPRAC}$ value of the extract was significantly related to the total phenolic and flavonoid contents. Figures 2 and 3 show the correlation between the total phenolic and flavonoid content and CUPRAC values. The linear curves had higher corre-

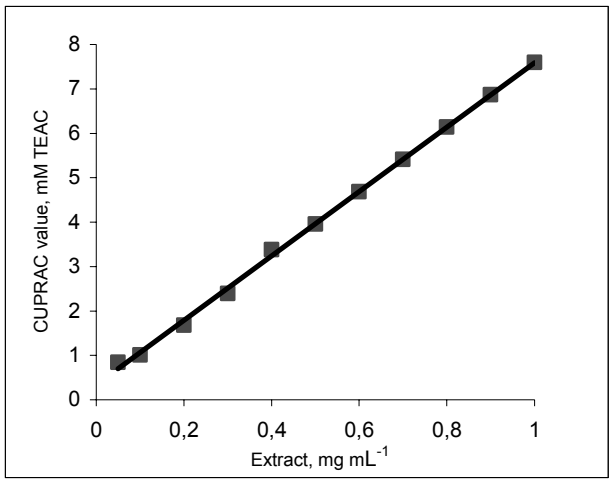

FIGURE 1 - CUPRAC values of the methanolic extract of *Vitex agnus castus* at different concentrations (R^2 = 0.998)

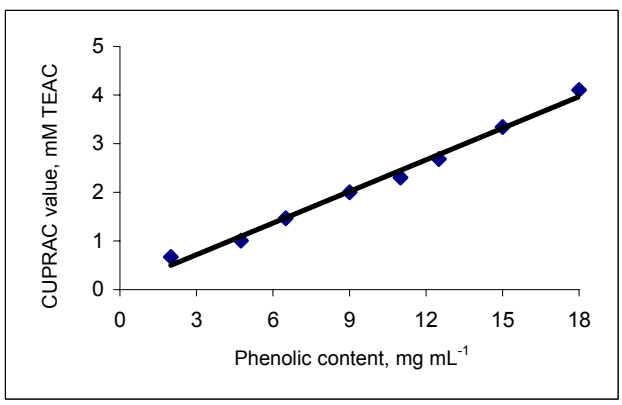

FIGURE 2 - CUPRAC values against total flavonoid content of the methanolic extract of *Vitex agnus castus* ((R^2 = 0.998)

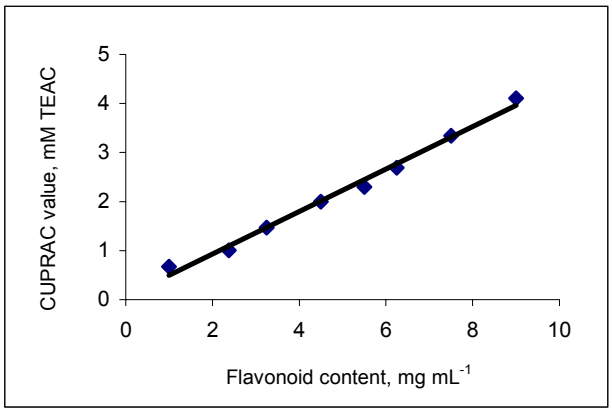

FIGURE 3 - CUPRAC values against total flavonoid content of the methanolic extract of *Vitex agnus castus* (R^2 = 0.998).

lation coefficients (R^2) of CUPRAC versus the total phenolic and flavonoid contents, 0.998 and 0.998, respectively. The results showed that CUPRAC values were associated with the total flavonoid and phenolic contents of the extract. However, the major antioxidant capacity of the extract is a direct outcome of their phenolic and flavonoid contents [29, 30].

Flavonoids, such as flavones and flavonols, exist abundantly in plants, vegetables and fruits. Especially, flavonols are known to act as antioxidants, both as radical scavenger and metal chelator [29]. The ability of the plant extracts to chelate transition metal ions can be followed spectroscopically. In addition to their ability to donate hydrogen atoms, phenolics and flavonoids in the plant extracts resulted in acting as chain-breaking antioxidants. And they also inhibit free radical formation by chelating transition metal ions [31]. The chelating ability of the methanolic extract from *Vitex agnus castus* on ferrous ions was determined spectroscopically at 562 nm. As shown in Figure 4, the methanolic extract had significantly stronger ferric chelating antioxidant capacity (p<0.05). However, chelating ability of rutin

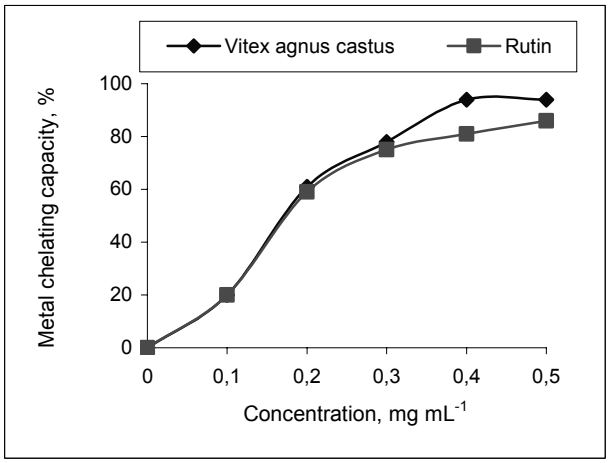

FIGURE 4 - Metal chelating capacity at different concentrations of the methanolic extract of *Vitex agnus castus* and rutin on ferrous ion

(92.1±1.1%), used as comparative standard antioxidant, was approximately higher than that of the methanolic extract (86.2±0.8%). High chelating ability of the extract is attributed to high flavonoid content.

Hydrogen peroxide is a product of normal metabolic pathway in human metabolism. Hydrogen peroxide causes oxidative damage by production of hydroxyl radicals in the cell [31]. Phenolic compounds, such as phenolic acids, flavonols, flavonoids and anthocyanidins, inhibit these hydroxyl radicals from hydrogen peroxide. In our experiments, absorbance change of H_2O_2 by addition of the methanolic extract of *Vitex agnus castus* is shown in Figure 5 and antioxidant activity of the extract was compared with those of rutin and quercetin as standards. The scavenging activity of the extract and standards on H_2O_2 was in the order of the methanolic extract (93.5±0.8%) > quercetin (89.4±0.5%) > rutin (86.1±0.7), at concentration of 0.5 mg ml^{-1}. There were significant differences (p<0.05) among the extract and standards.

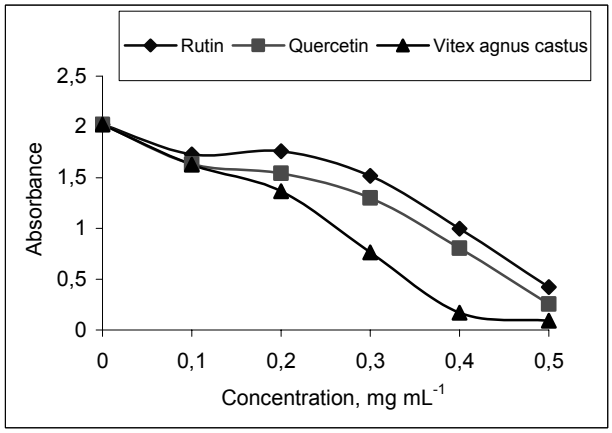

FIGURE 5 - Absorbance change against increasing concentration of the methanolic extract of *Vitex agnus castus* and standards, rutin and quercetin.

The main role of the phenolics and flavonoids is scavenging of the free radicals [29, 30]. Antioxidative properties of various extracts from the plants are of great interest in both academic researches and food industry, since their possible use as natural additives emerged from a growing interest to replace synthetic antioxidants by natural ones.

Antibacterial and antifungal activity

In this study, seven different bacteria and one yeast species were used to screen the possible antimicrobial activities of the methanolic extract of *Vitex agnus castus*. Disc diffusion assay and broth microdilution methods were performed. The extract did not exhibit antibacterial activity against all bacteria in the disc diffusion method. Inhibition zone was measured only for *Candida albicans* as 11 mm at 100 mg ml^{-1}. *Candida albicans* is the microbe responsible for most clinical yeast infections, e.g. in mouth infections. In the broth microdilution method, we confirmed

that all bacterial strains used in the experiments were resistant to the methanolic extract of *Vitex agnus castus*. However, the extract exhibited antifungal activity against *Candida albicans*. MIC value was determined as 0.39 mg ml^{-1}. In previous studies, some *Vitex* species manifested good growth inhibition against Gram-positive bacteria (0.02–8.00 mg ml^{-1}) but lower activity against the Gram-negative bacteria and yeast (0.50–8.00 mg ml^{-1}) [32]. Antimicrobial activity of *Vitex trifolia* leaves has been investigated before. All extracts inhibited the growth of gram-positive and gram-negative species. Only DCM leaf extract caused inhibition to *Candida albicans* at 5 mg ml^{-1} dose [33]. Disc diffusion assay was performed with petrol extract and ethanolic extract of *Vitex trifolia*. Petroleum ether fraction was found to be more active than ethanolic one. [34]. In our study, the methanolic extract of *Vitex agnus castus* exhibited only antifungal activity.

CONCLUSION

The methanolic extract of *Vitex agnus castus* exhibited stronger antioxidant activity by cuprac reducing antioxidant capacity, ferric chelating ability and scavenging ability of H_2O_2 as compared with activities of standard antioxidant compounds, rutin and quercetin. The results indicated that there was a good relation between antioxidant activity and the total phenolic and flavonoid contents of the extract. The main reason of high antioxidant activity of *Vitex agnus castus* is due to variation of different phenolic compounds. The methanolic extract of *Vitex agnus castus* is a complex mixture of the total phenolic and flavonoid compounds. So, *Vitex agnus castus*, rich in phenolics, is a new naturally potential antioxidant source. However, strains used in the experiment were resistant to activity of the methanolic extract. It possessed noticeable antifungal activity against *Candida albicans* when compared with standard and strong antimicrobial compounds, such as ampicillin and penicillin.

The results of this study present that the methanolic extract of *Vitex agnus castus* can be used as a source of natural antioxidants and a possible food supplement, or also in pharmaceutical industry. However, the components responsible for the antioxidant and antimicrobial activities of the extract are currently unclear. Therefore, it is suggested that further works should be carried out on the isolation and identification of the antioxidant components in *Vitex agnus castus*.

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