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Research Article

Phytochemical Studies, Antioxidant Activity and Protective Effect on DNA Damage and Deoxyribose of *Silene Vulgaris* Extract from Morocco

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ABSTRACT
The aim of this study was to evaluate the antioxidant activity of hydro-ethanolic extract from *Silene vulgaris* (Moench) Garcke leaves. Five methods were used for antioxidant activity, which are 2, 2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and Hydroxyl Radical Scavenging Activity (HRSA) assays and the protective effect of DNA damage was also evaluated. The chemical composition of extract, were evaluated like total polyphenolic contents (TPC), catechins, tannins and O-diphenols. The extract was found to possess a significant antioxidant activity with ABTS, DPPH, FRAP and HRSA assays using Trolox as a standard with a IC50 value 2.05 mg/ml compared to that of the reference standard Trolox for TEAC ABTS assay. The effects on DNA damage provide a partially protective effect against DNA fragmentation.

Keywords: *Silene vulgaris*; Antioxidant activity; Hydroxyl Radical Scavenging Activity; DNA damage; Polyphenolic contents; Catechins, Tannins.

INTRODUCTION

Nowadays, we note an increasing interest in the use of plants which have an antioxidant power either for scientific research as well as for industrial field (dietary, pharmaceutical and cosmetic...). This is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis[1]. The complexity of phytochemical compounds of plant impose the development of many methods to evaluate the antioxidant activity and to estimate the effectiveness of these substances [2, 3]. Thus, extract of *Silene vulgaris* was subjected to three different antioxidant bioassays employing ABTS, DPPH and FRAP assays. Several studies have described the antioxidant properties of medicinal plants, foods which are rich in phenolic compounds because of their high redox power. Such compounds act as reducing agents, hydrogen donors, singlet oxygen catchers, free radical scavengers, and as chelating pro-oxidants metals [4-8]. Yu-Ling et al. showed that phenolic antioxidants in plants are mainly composed of phenolic acids, flavonoids and catechins which have capacities of preventing DNA oxidative damage, and scavenging reactive oxygen species[9]. In the case of protection of DNA from degradation, in vitro studies include tests based on such simple molecules as deoxyribose (the backbone sugar in DNA), as well as lipid peroxidation; in vivo studies may include assessment of antitumour, antiplatelet, antiallergic, antischaemic and anti-inflammatory activities, which will typically resort to animal or human models, in attempts to determine the antioxidant or pro-oxidant potential under live physiological conditions[10-14]. When assaying for antioxidant capacity, hydroxyl radicals are typically generated within a mixture of ascorbic acid, H_2O_2 and Fe^{3+}-ethylenediaminetetracetic acid (EDTA); those radicals that are not scavenged by other components of the reaction mixture will eventually attack deoxyribose, thus degrading it into a series of fragments. Some of the fragments (or even all of them) react upon heating with thiobarbituric acid (TBA), at low pH, thus yielding a pink chromogen: this TBA adduct possesses a three-carbon dialdehyde, malondialdehyde (MDA). If an OH• scavenger is meanwhile added to the reaction mixture, it will compete with deoxyribose for OH• radicals, and consequently inhibit deoxyribose degradation[15, 16]. Furthermore, OH• radicals can also enhance DNA damage, via attack on its phosphate bonds; this type of degradation results in smaller fragments, which can be separated by agarose electrophoresis[16]. The deoxyribose degradation assay has been widely used to evaluate the hydroxyl (-OH) radical-scavenging ability of food, nutrients, and medicines since it was established in 1987. [12] In the deoxyribose degradation assay, -OH is obtained via Fenton reaction[12,16]. Subsequently, -OH attacks deoxyribose and breaks its cyclic furan ring to generate malondialdehyde

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(MDA). MDA combines with 2-thiobarbituric acid (TBA) to produce a chromogen with kmax at 530 nm[17]. Therefore, the A530nm value is proportional to the produced amount of -OH radicals. Higher A530nm values indicate higher levels of -OH radicals. If an antioxidant sample is added, the A530nm value will decrease, suggesting that some -OH radicals are scavenged by the antioxidant. This is the principle of deoxyribose degradation assay. In this study, we aimed to investigate the antioxidant activities such as (i) the antioxidant activities using five in vitro assay models, which are 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay, DPPH radical scavenging assay, ferric reducing antioxidant power (FRAP) method, Hydroxyl Radical Scavenging Activity (HRSA) and protective effect of DNA damage methods (ii) measure the percentage of some chemical components known for their antioxidant power like total polyphenols, tannins, catechins and others compounds.

MATERIALS AND METHODS

Plant material

The plant of *Silene vulgaris* (Moench) Garcke (Caryophyllaceae) leaves was harvested between March and April 2013 from Ait Taleb, Sefrou- Morocco. The plant collected was identified and the voucher specimens have been deposited at the herbarium of National Centre of Forest Research-Rabat-Morocco.

Ultrasound-assisted extraction (US)

Twenty grams of *S. vulgaris* are mixed with 200 mL of n-hexane at 35 kHz frequency. After being extracted, the mixture filtered under vacuum through Whatman paper, and the solvent removed. Then the plant's material re-extracted again with a mixture of ethanol /water (7:3 (v/v)) under the same conditions. The final extract was recuperated from the mixture (ethanol/water) after filtration by Watman paper and evaporation under vacuum at 40°C on a rotary evaporator (Rotavapor R-205 BÜCHI).

Phytochemical screening

Preliminary phytochemical analysis of the extract was performed using the method described previously[18-22]. The polyphenols has been made through the reaction of ferric chloride. Stiasny reaction was used to reveal the presence of catechic and gallic tannins. Furthermore, the flavonoids have been revealed through the reaction to the cyanidin. The saponins have a property to form foam when they are shaken vigorously with water.

Determination of total polyphenols

The total phenolic contents of the extract of *S. vulgaris* were determined with the Folin-Ciocalteu reagent, using gallic acid as standard. The result was expressed as gallic acid equivalents per gram of plant (mg gallic acid/g of plant) [23].

Determination of total tannins

The total tannins content was determined by spectrophotometer by using method described by Sarneckis et al., 2006 [24].

Determination of catechins

Using the method of Swain and Hillis, 1959, based on the ability of catechins to condense with carbonyl compounds in acidic media[25]. The result of the reaction is measured at 500 nm.

Determination of O-diphenols

The O-diphenols content of extract of the *S. vulgaris* is calculated by the method described by (Flanzy & Aubert 1969). The total O-diphenols content is calculated in terms of equivalent D-catequina per g of plant extract[26].

Evaluation of antioxidant activity

Although there is no standardized method to evaluate the antioxidant potential of foods and biological systems, it is recommended to evaluate the antioxidant activity by different methods[27].

ABTS radical cation scavenging activity

This assay is based on decolorization that occurs when the radical cation $ABTS^{\cdot+}$ is reduced to $ABTS^{\cdot}$.The radical was generated by reaction of a 7 mM solution of ABTS in water with 2.45 mM $K_2O_8S_2$ (1:1) [4]. The mixture was held in darkness at room temperature for 16 h, as this is the time needed to obtain stable absorbance values at 734 nm. Once prepared, the reactive mixture may be used over 4 days. The assay was made up with 980 µL of $ABTS^{\cdot+}$ and 20 µL of the sample (at a dilution of 1:50 in water). Optimization studies on the absorbance stability of the mixture indicate that the measurements must be made after 15 min of reaction time. The results were expressed in millimolar Trolox, using the relevant calibration curve.

DPPH radical scavenging activity

This method is based on the reduction of the free radical $DPPH^{\cdot}$ [28] which leads to its decolorization. The presence of the antioxidant leads to a loss of color in the reactive in methanol at a concentration of 60 µM. At this concentration, the solution immediately reaches an absorbance value of around 0.7 at 517 nm. According to the studies in this field, the reaction mixture remains stable for a period of 4 days when kept in darkness at room temperature. The reaction takes place when 980 µL of $DPPH^{\cdot}$ (60 µM) was mixed with 20 µL of the sample (at a dilution of 1:50 in water). A reaction time of 2 h is calculated at room temperature, which is the time needed to arrive at a stable reading of the reactive with the sample. Results were expressed in millimolar Trolox using the dose-response curve described by this substance.

FRAP assay (ferric reducing antioxidant power)

This method is used to measure the reductive power of a sample [29]. It is based on increased absorbance at 593 nm due to formation of tripyridyl-S-triazine complexes with ferric (II) [TPTZFe (II)] in the presence of a reductive agent. The reactive mixture is prepared by mixing 25 mL of 0.3 M sodium acetate buffer solution at pH 3.6, 2.5 mL of TPTZ (10 mM), 2.5 mL of FeCl3 (20 mM), and 3 mL of water. Thirty microliters of the sample (diluted in water at 1:50) was added to 970 µL of the latter reactive mixture and was incubated at 37 °C for 30 min. The results were expressed as millimoles of Fe-(II), using linear calibration obtained with different concentrations of FeSO4.

Hydroxyl radical scavenging activity (HRSA)

Deoxyribose protection was quantified by using the methods described by Halliwell et al 1987[12]. Desoxyribose (2-desoxy-D-ribose) decays when exposed to hydroxyl

Table 1: Phytochemical screening of hydro-ethanolic extract of *Silene vulgaris*.

Chemical constituants	*Silene vulgaris* extract
Tannins	+
Gallic	+
Catechic	
Flavonoids (flavones)	+
Coumarins	+
Quinones	-
Saponins	+

Note: Presence of chemical compounds is: (+) = presence; (-) = absent

radicals generated by the Fenton reaction. The hydroxyl radicals (HO·) were generated through the following system: 10 µL of FeCl3 (0.1 mM), 10 µL of ascorbic acid (0.1 mM), 10 µL of H_2O_2 (1 mM), and 10 µL of EDTA (0.1 mM). Fifteen microliters of the sample (diluted at 1:50 in water) were incubated at 37 °C for 1 h, with 20 µL of desoxyribose (1 mM final concentration) in the presence of FeCl3, ascorbic acid, H_2O_2, and EDTA. As mentioned above, radicals break deoxyribose into fragments which, in the presence of 1mL of 1% TBA in 0.05 M NaOH, under acid conditions (1.5mL of 28% TCA) and at high temperature (100 ◦C for 15 min), give rise to a chromophore (malonaldehyde), and this species was quantified by absorbance at 532 nm. When antioxidants are present, they compete for the hydroxyl radicals, thus decreasing the extent of fragmentation of deoxyribose. The result was expressed as an inhibition % in relation to a control test (without the sample) and triplicate samples were used.

Damage to DNA

Incubation of DNA Calf Thymus

DNA protection was quantified using the method described by Rivero et al. 2005 and Guimaraes et al. 2007. The DNA calf thymus (200 µg) in the absence and the presence of 100 µl of undiluted *S. vulgaris* was exposed to the action of hydroxyl radicals generated by the mixture of 100 µL of ascorbic acid (1 mM final concentration) and 10 µL of copper sulfate (II) (100 µM final concentration) [10,11]. The mixture was incubated at 37 °C for 1 h after which the fragments were separated by electrophoresis.

Agarose Gel Electrophoresis

Electrophoresis was carried out with 1% agarose at room temperature using a Bio-Rad power-Pac 1000 (Hercules, CA) electrophoresis system. Subsequently, the gel containing 15 µL of ethidium bromide (10 mg/mL) was observed under ultraviolet light, using a transilluminator, and photographed.

RESULTS AND DISCUSSION

Phytochemical screening

The result of the phytochemical analysis of *S. vulgaris* leaves revealed the presence of phenolics, flavonoids, saponins, coumarins and tannins (Table 1 and Table 2). The antioxidant activities found in the ethanolic extract can be related to the total polyphenols, flavonoids, tannins,o-diphenols and catechins of the sample. Pharmacological properties of flavonoids related to their antioxidant activity have been reported[30-34].

Table 2: Determination of some chemical compounds of the extract *S. vulgaris*

Compounds contents	Extract *S. vulgaris*
Total polyphenols (mg gallic acid/g of extract)	3.35± 0.12
Catechins (mg D-catechin/g of extract)	0.13± 0,01
Tannins (mg tannic acid/g of extract)	0.28±0.00
O-diphenols (mg D-catechin/g of extract)	0.12± 0,02

It was also reported that tannins may be contributing to a better performance in the antioxidant activity tests. Although tannins in general exhibit antioxidant activity, some authors have noted that several o-diphenol compounds, are considered the most active antioxidants due to their structure–activity relationship[35,36]. In addition, the catechin derivatives are well-known for their antioxidant power towards free radicals and have been found by various other works to have strong antimicrobial properties[37-40].

Determination of total polyphenols

The total of polyphenols content of extract was determined from regression equation of calibration curve (Y= 463.03x -4.7419; R^2= 0.9994) and expressed in gallic acid equivalent. The result was calculated at 3.35± 0.12 mg gallic/g of extract. It is reported that polyphenolic compounds in plants possess strong antioxidant activity and help in protecting cells against oxidative damage caused by free radicals[41-43].

Determination of tannins

The total of tannins content was determined from regression calibrate curve (Y= [Ac280-Am280)+0.0065]/0.0029) and expressed in equivalent of tannic acid. The result was calculated at 0.28 mg tannic acid/g of extract.

Determination of catechins

The total catechins content of the ethanolic extract of *Silene vulgaris* is 0.13± 0,015 mg expressed as D-catechin equivalent in micrograms per g of extract, using a regression calibrate curve (Y= 340.72x -50.56; R^2= 0.998).

Determination of O-diphenols

The o-diphenols content of the ethanolic extract of *S. vulgaris* is 0.12± 0,025 mg D- catechin equivalent per g of plant extract.

ABTS radical cation scavenging activity

In this study, antioxidant activity was expressed as µM Trolox equivalent per g of extract on dry basis as this is a more meaningful and descriptive expression than assays that express antioxidant activity as the percentage decrease in absorbance. As such, the results provide a direct comparison of the antioxidant activity with Trolox[44-47;4] .

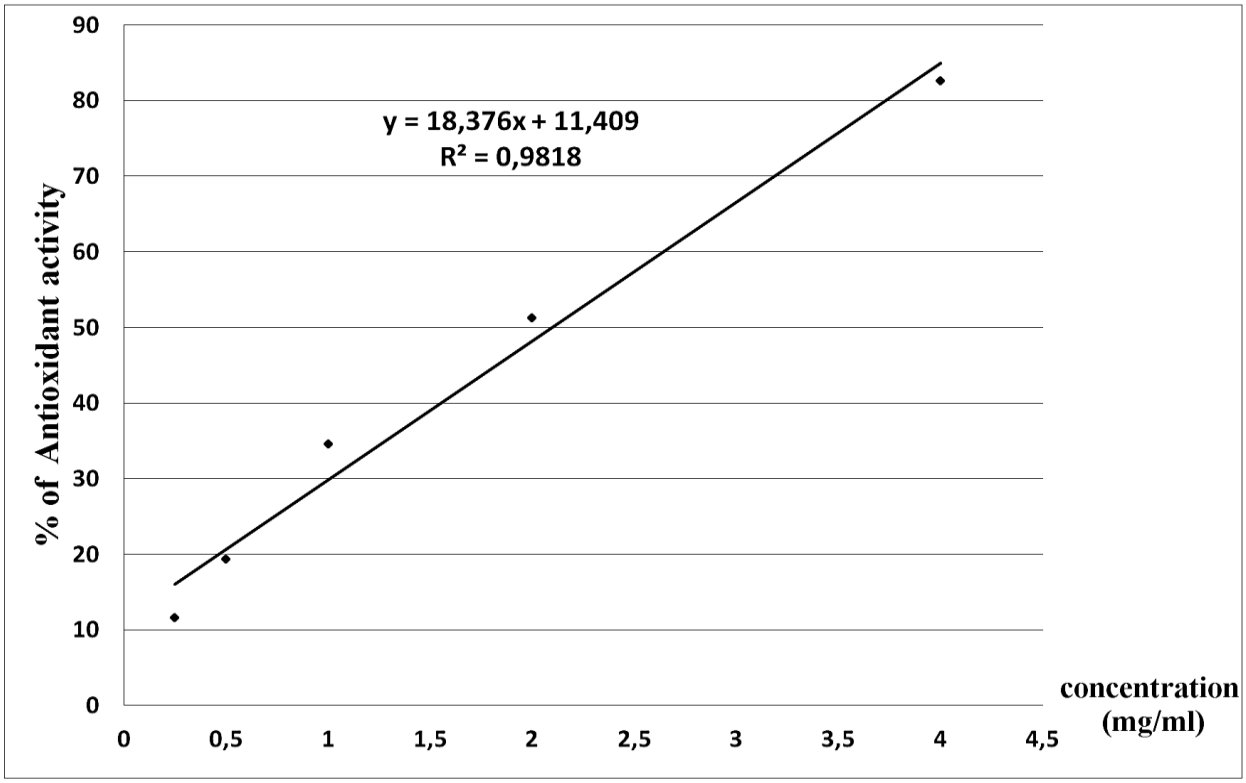

Figure 1: Antioxidant activity of *S. vulgaris* extract evaluated by $ABTS^{\bullet+}$ Assay.

The results of TEAC ABTS by standard antioxidant Trolox show a 30 µM Trolox equivalent per g of extract. The ABTS test measures the relative antioxidant ability of plant to scavenge the radical-cation $ABTS^{\cdot+}$ produced by the oxidation of 2,2'-azinobis-3-thylbenzothiazoline-6-sulphonate [7]. The IC50 value for *S. vulgaris* extract was calculated and defined as the concentration of extract causing 50% inhibition of absorbance, the curve shows stronger scavenging capacity with an IC50 of 2.05 mg/ml wish is equivalent to 0.89 mM of Trolox (Fig. 1).

The extensive investigations on antiradical and antioxidant activities of small phenolics, including flavonoids and phenolic acids have been reported[48]. Apart from these, Hagerman et al., 1998 have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\cdot+}$) and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups[49]. Free radical ($ABTS^{\cdot+}$) scavenging activity of *Silene vulgaris* sample might be due to the presence of high molecular phenolics such as catechins, tannins and flavonoids.

DPPH radical scavenging activity

The free radical scavenging activity of the ethanol extract of *Silene vulgris* leaves were determined by the DPPH method. The DPPH radical has been widely used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods[50,51]. Results were expressed in mM of Trolox. The standard curve of Trolox was performed [(mM Trolox = 1.6903*(A0-AS) - 0.0056) with A0: absorbanse of DPPH (without extract) and AS: absorbance of sample (extract)]. The extract was equivalent to 10 µM of Trolox per g of extract. DPPH assay is simple to use, but has some disadvantages that limit its application[52]. Many antioxidants that react rapidly with peroxide radicals may have a very slow reaction to DPPH or may even be inert to it[53,54].

FRAP assay (ferric reducing antioxidant power)

Antioxidant potential of the ethanol extract of *Silene vulgris* was estimated using ferric reducing antioxidant power method. The extract a concentration of 4mg/ml shows a antioxidant effect wish is equivalent to 10µM Fe (II). This result was determined by the curve of Fe (II): [(mM Fe (II) = 1.4744x + 0.0304) with x: difference between absorbance of sample and control]. FRAP is the only assay that directly measures antioxidants in a sample. The other assays are indirect because they measure the inhibition of reactive species (free radicals) generated in the reaction mixture, and these results also depend strongly on the type of reactive species used.

To better understand the quantitative differences detected among the three methods above (ABTS, DPPH and FRAP), it must be considered that each method works with a different mechanism and the sensibility of each ones. In this sense, it is important to remember that DPPH has been described as more specific for lipophilic antioxidants, FRAP for hydrophilic antioxidants, and ABTS for both classes[55]. Therefore, because ABTS is a good method to evaluate lipophilic as well as hydrophilic antioxidants, it seems logical that this method gave higher values,

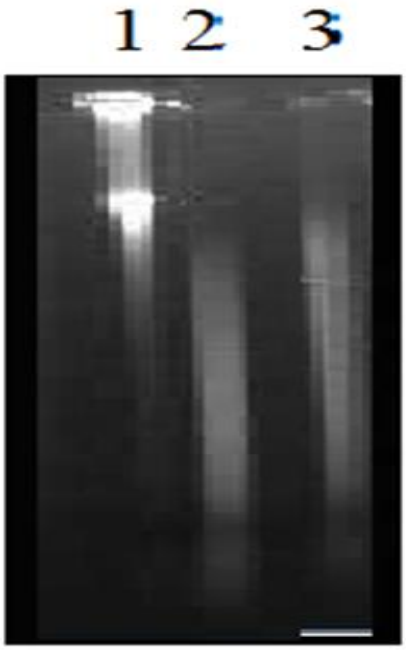

Figure 2: Agarose gel electrophoresis separation of damaged DNA induced by Cu (II), ascorbic acid and the effect of plant extract. The numbered lanes represent DNA alone (lane 1), DNA exposed to Cu (II) and ascorbic acid (lane 2), DNA + Cu(II), ascorbic acid + 100 µl of extract (lane 3).

registering the activity of both types of antioxidant present in the *Silene vulgaris*.

Hydroxyl radical scavenging activity (HRSA)

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[56]. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids.

The plant tested was described in terms of protection of deoxyribose and DNA from degradation. The -OH-radical-generating system containing EDTA, $FeCl_3$, H_2O_2, and ascorbic acid (Fenton reaction model system) was used to generate -OH radicals. Ascorbic acid increases the rate of -OH generation by reducing iron and maintaining a supply of Fe^{2+}-EDTA. The generated -OH radical can degrade deoxyribose into a series of fragments that react with TBA upon heating and at low pH. This reaction forms a pink color that absorbs 532 nm light. When added to the reaction mixture, the test compounds compete with deoxyribose for -OH radicals and inhibit deoxyribose degradation. The extract of *S. vulgaris* presented a strong protection of 84.33 % for scavenging activity for hydroxyl radical (HRSA).

Damage to DNA

The agarose gel electrophoresis was presented in (Fig. 2). The assay of damage to DNA showed a moderate protective action by the *S. vulgaris* under study that, in general, was observable with a dose of 100 µL. The greater mobility-distance from the well to the zone of greatest density is directly proportional to the damage to DNA and inversely proportional to the size of the fragments that are obtained[45]. As shown in (Fig. 2), at the concentration tested (100µl), the DNA was partially protected by the extract of *S. vulgaris* (lane 3). Some authors have shown the usefulness of using DNA scission as an effective method to assess the antioxidant activity against active oxygen species in vitro[57, 10]. Both ascorbic acid and copper may induce damage of DNA, because they produce highly reactive oxygen species which have been implicated in degradation of DNA, thus producing DNA strand breaks that can be adequately visualized by electrophoresis[16].

CONCLUSION

In conclusion, this study demonstrated the presence of bioactive principles in the leaves of Moroccan *Silene vulgaris* **(**Moench) Garcke: such as polyphenolic compounds, tannins, ortho diphenols, catechins and flavonoids which possess antioxidant activity. Hydro-ethanolic extract of *S. vulgaris* seems to present a significant antioxidant activities that were established by five in vitro tests (ABTS, DPPH, FRAP, HRSA and damage to DNA). Further investigations are needed to isolate and identify the active components in the extract, and to develop a new natural drug either for the food and cosmetic industry or for the therapeutic field.

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