Antioxidant and antibacterial properties of a lichen species *Diploschistes scruposus* (Schreb.) Norman

Bahar Bilgin Sökmen[1]*, Sinem Aydın[2], Kadir Kınalıoğlu[2]

[*1]*Department of Chemistry, Faculty of Science and Arts, Giresun University, 28100 Giresun, Turkey*
[2]*Department of Biology, Faculty of Science and Arts, Giresun University, 28100 Giresun, Turkey*

Abstract

In this study, antioxidant and antibacterial activities of ethanol extract of *Diploschistes scruposus* (Schreb.) Norman (Graphidaceae) were investigated. Antioxidant activity of ethanol extract of *D. Scruposus* was investigated by five different methods: DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging activity, $ABTS^{\cdot+}$ (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity, reducing power and determination of total phenolic contents. Different antioxidant activities of the extract were studied in comparison to known antioxidants. 50% inhibition concentration (IC_{50}) values for lichen extract was observed 54.37±0.71 and 45.45±0.80 in DPPH assay and $ABTS^{\cdot+}$ assay, respectively. There were 161.4±0.01 µg/mL gallic acid equivalent of total phenolic compounds in the 1 mg of ethanol extract of *D. scruposus*. Antibacterial activity of lichen extract was determined by agar well diffusion method. The maximum antibacterial activity was observed against *Bacillus subtilis* and the minimum antibacterial activity was observed against *Escherichia coli.* This study shows that ethanol extract of *D. scruposus* can be used as an accessible source of natural antioxidant and antibacterial agent.

Keywords: Lichen, Free radical scavenging activity, total phenolic content, antioxidant activity, antibacterial activity.
***Corresponding Author**: Bahar Bilgin Sökmen (e-mail: baharsokmen@yahoo.com)
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Diploschistes scruposus (Schreb.) Norman likeninin antioksidan ve antibakteriyal özellikleri

Özet

Bu çalışmada, *Diploschistes scruposus* (Schreb.) Norman *(*Graphidaceae*)* likeninin antibakteriyal ve antioksidan özellikleri araştırılmıştır. *D. scruposus* likeninin etanol ekstresinin antioksidan aktivitesi beş farklı metodla çalışılmıştır: DPPH (2,2-difenil-1-pikrilhidrazil) radikal süpürme aktivitesi, $ABTS^{\cdot+}$ (2,2'-azino-bis (3-etilbenzotiyazolin-6-sülfonik asit) radikali süpürme aktivitesi, indirgeme gücü ve toplam fenolik içeriğinin belirlenmesi. Test ekstresinin değişik antioksidan aktiviteleri bilinen antioksidanlarla kıyaslanmıştır. Liken ekstresinin %50 inhibisyon konsantrasyon değerleri (IC_{50}), DPPH radikali süpürme aktivitesi için 54.37±0.71; $ABTS^{\cdot+}$ radikali süpürme aktivitesi için 45.45±0.80 olarak gözlenmiştir. *D. scruposus* etanol ekstresinin 1 mg'ında 161.4±0.01 µg/mL gallik asit eşdeğeri toplam fenolik bileşikleri bulunmaktadır. Liken ekstresinin antibakteriyal aktivitesi agar kuyu difüzyon yöntemine göre belirlenmiştir. Maksimum antibakteriyal aktivite *Bacillus subtilis*'e, en düşük antibakteriyal aktivite ise *Escherichia coli*'ye karşı gözlenmiştir. Bu çalışma, *D. scruposus* likeninin doğal antioksidan ve antibakteriyal kaynağı olarak kullanılabileceğini göstermiştir.

Anahtar Kelimeler: Liken, Serbest radikal süpürme aktivitesi, Toplam fenolik içeriği, Antioksidan aktivite, Antibakteriyal aktivite.

Introduction

Free radicals play an important role in the pathogenesis of some serious diseases, such as neurodegenerative disorders (Singh et al. 2004), cancer (Valko et al. 2006), cardiovascular diseases (Kris-Etherton et al. 2004), carsinogenesis (Marnett 2000), atherosclerosis (Singh and Jialal 2006), diabetes (Robertson and Harmon 2006) and cataracts (Varna 1992).

The antioxidants are known to play an important protective role against disorders caused by oxidant damage. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reaction.

Lichens are symbiotic associations of fungi, green algae or cyanobacteria. Lichens have been used by various ethnic groups from the dawn of the civilisations.

Lichens produce secondary metabolites such as depsides, depsidones, dibenzofurans and phenolic compounds, most of which are not known from other groups of plants. These secondary metabolites have important biological activities such as antimicrobial (Marijiana et al. 2010), antipyretic (Ingólfsdóttir 2002), antiproliferative (Bucar et al. 2004), cytotoxic (Bezivin et al. 2003), antiinflamatuar (Süleyman et al. 2002), antitumour (Malhotra et al. 2008), analgesic (Bugni et al. 2009), and antioxidant (Luo et al. 2010).

In many European countries, lichens were used as a remedy for pulmonary tuberculosis and in the treatment of wounds and skin disorders. These medicinal uses have to some extent been confirmed by studies which showed that many lichen metabolites such as depsidones, depsides are active against mycobacteria and gram-positive bacteria (Vartia and Tervila 1952). Owing to pronounced antimicrobial activity of some of their secondary metabolites, lichens are attracting much attention among researchers as significant new sources of bioactive substances (Yılmaz et al. 2005; Halama and Haluwin 2004).

The aim of the present study was to explore antioxidant activity of ethanol extract of *D. scruposus* which was collected from Black Sea Region. It was also of interest to screen whether the extract possess antibacterial activity.

Materials and methods

Reagents

Butylated hydroxytoluen (BHT), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fluka Chemical Co. (Buchs, Switzerland). 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and rutin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Plant material

Lichen specimen was collected between 2275 to 2350 m on the Kalecik Plateau (Araklı-Trabzon) which is located in the eastern part of the Black Sea region of Turkey, in 2004, between 40°35' N and 40°06' E and authenticated by Dr. Kadir Kınalıoğlu, Giresun University, Faculty of Science and Arts, Department of Biology (Botany). The lichen sample was stored in the herbarium of Giresun University, Arts and Science Faculty, Giresun (Herbarium No. 1818).

Preparation of the lichen extract

Lichen sample was dried at room temperature for 48 h and powdered with a blender. Powdered lichen (20 g) were extracted with 200 mL of ethanol by using a Soxhlet apparatus for 72 h at a temperature not exceeding the boiling point of the solvent (Lin et al. 1999). The extract was filtered using Whatman filter paper (No.1) and then concentrated in vacuo at 40˚C using a rotary evaporator. Extract was stored at -80˚C for further assays.

Test microorganisms

Two gram negative and three gram positive bacteria strains were used to determine the

antibacterial activity of ethanol extract of *D. scruposu*s. Bacteria are used in the study as follows: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* 25922 and *Salmonella enterica serovar typhimurium* ATCC 14028.

Antibacterial activity determination

The dried lichen extract was dissolved to obtain 30 mg/mL final concentration in ethanol. Then, ethanol extract was sterilized by filtration through 0.45 μm Millipore filters (Murray et al. 1995). Antibacterial tests were carried out by the agar well diffusion method. Inocula, corresponding to a value of 0.5 on the Mc Farland optical density scale, was prepared in Mhüller Hinton Broth and cultivated (100 μL) onto Müeller Hinton agar plates in three directions by sterile swabs. Wells, 5 mm diameter, were punched in each agar plate. Wells were filled with 30 μL of ethanol extract of lichen, ethanol (for negative control) separately (Boyanova et al. 2005). All the plates were incubated at 37˚C for 24 h. After incubation the antibacterial activity was evaluated by measuring the inhibition zone diameter observed. Each test was performed twice. The solvent (ethanol) was not affected by the growth of any of the bacteria.

The minimal inhibition concentration (MIC) were also studied for the microorganisms which were determined as sensitive to *D. scruposus* extract. The inocula of microorganisms were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

The 96 well plates were prepared by dispensing into each well 95 μL of Mhüller Hinton Broth and 5 μL of the inoculum. 100 μL *D. scruposus* extract initially prepared at the concentration of 1 mg/mL was added into the first wells. Then, 100 μL from their serial dilutions were transferred into seven consecutive wells. The last well containing 195 μL of nutrient broth without compound and inoculum on each strip was used as negative control. 96 well plates were incubated at 37°C overnight. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms (Güllüce et al. 2004).

Antioxidant activity

Determination of total phenolic compounds

Total phenolic compounds were determined with Folin–Ciocalteu reagent, according to the method of Slinkard and Singleton (Slinkard and Singleton 1977) with some modifications. Lichen extract was dissolved to obtain final concentration 1 mg/mL in ethanol. Aliquots (0.1 mL) of the extracts (1 mg/mL) were transferred into test tubes and their volumes were made up to 4.6 mL with distilled water. After addition of 0.1 mL Folin–Ciocalteu reagent (previously diluted 3-fold with distilled water) and 0.3 mL 2% Na_2CO_3 solution, tubes were vortexed and the absorbance of the mixture was recorded after 2 h at 760 nm, using a Shimadzu 1240 UV–Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan), against a blank containing 0.1 mL of extraction solvent. The amount of total phenolic compounds was calculated as mg of gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solution (covering the concentration range between 0.02 mg/mL and 0.1 mg/mL) and expressed as mg gallic acid per mg of extract of the plant material. The data were presented as the average of triplicate analyses.

Reducing power

The reducing powers of the lichen extract from *D. scruposus*, BHT, Trolox and rutin were determined according to the method described by Oyaizu (Oyaizu 1986). Different amounts of extracts (25-100 μg/mL) in 1 mL of ethanol were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%), and then incubated at 50°C for 30 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, and the mixture was centrifuged at 3000g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.1% $FeCl_3$ (0.5

mL), then the absorbance was measured at 700 nm.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the lichen extract was measured according to the procedure described by Brand-Williams et al. (Brand-Williams et al. 1995). Appropriate dilution series (25-100 µg/mL) were prepared for each ethanol extract in ethanol 0.1 mL of each dilution was added to 3.9 mL of a $6x10^{-5}$ M methanolic solution of DPPH followed by vortexing. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was measured spectrophotometrically at 517 nm against methanol. The DPPH radical scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control

A_1 is the absorbance of the sample

Extract concentration providing 50 % inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagent BHT, Trolox and rutin were used as positive control and all tests were carried out in triplicate.

$ABTS^{\cdot+}$ radical scavenging activity

The $ABTS^{\cdot+}$ scavenging activity of the lichen extract was measured according to the procedure described by Arnao et al. (Arnao et al. 2001). The stock solutions included 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\cdot+}$ solution with 60 mL methanol to obtain an absorbance of 0.701±0.02 units at 734 nm using the spectrophotometer. Fresh $ABTS^{\cdot+}$ solution was prepared for each assay. 150 µL of different concentrations of lichen extract (25-100µg/mL) were allowed to react with 2850 µL of the $ABTS^{\cdot+}$ solution for 2 h in the dark. Then the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{\cdot+}$ scavenging activity was calculated using the following equation:

ABTS radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control

A_1 is the absorbance of the sample

Results and discussion

Antibacterial activity

As seen in Fig. 1, ethanol extract of *D. scruposus* showed varying antibacterial activities depending on the microorganisms tested.

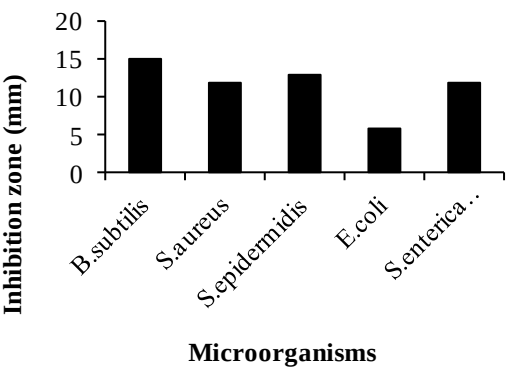

Figure 1. Inhibition zones which were created by lichen extract against test microorganisms.

Saenz et al. (2006) investigated acetone extracts of *D. scruposus* against *S. aureus* and *E. coli* and found that acetone extract exhibited 6 mm inhibition zone against *S. aureus*. In this study, ethanol extract of the lichen exhibited 12 mm inhibition zone against *S. aureus*. According these findings, it can be suggested that, ethanol extract of lichen is more efficient than acetone extract against *S. aureus*. Also, inhibition zones created by ethanol lichen extract against *S. aureus* and *S. epidermidis* are the same. Saenz et al. (2006) found that acetone extract of *D. scruposus* did not exhibit any

inhibition zone against *E. coli*. In our study, ethenol extract of the lichen exhibited 6 mm inhibition zone against *E. coli* it can be suggested that ethanol extract of lichen is more efficient than acetone extract against *E. coli*

Briefly, the maximum antibacterial activity was observed against *B. subtilis* and the minimum antibacterial activity was observed against *E. coli*. In addition, *B. subtilis* is more susceptible than other test microorganisms to ethanol extract of lichen and *E. coli* is the most resistant microorganisms to ethanol lichen extract among the other test microorganisms.

Our results differ from the literature results. These differences are based on different solvent used in extraction, different amounts of lichen extract and collected lichen from different regions.

The basic quantitative measurement of in vitro activity of antibacterial agents with antibacterial potential is the MIC. Demonstration of low MIC values by the ethanol extracts is an indication that the phytoconstituents of the plant have the therapeutic properties (Abubakar 2009). MIC values of *D. scruposus* lichen extract are shown in Fig. 2. Ethanol extract of lichen showed minimum inhibitory concentration (MIC) at 62.50 µg/mL for *S. enterica serovar typhimurium*, at *125* µg/mL for *B. subtilis*, *S. aureus* and *S. epidermidis*. Ethanol lichen extract also showed minimum inhibitory concentration (MIC) at 250 µg/mL for *E. coli*.

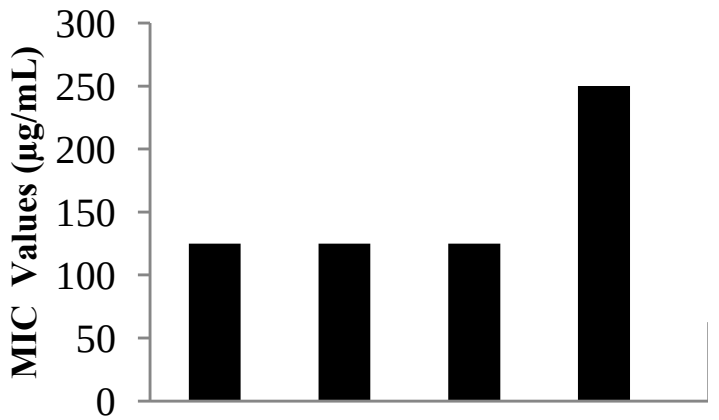

Figure 2. MIC values of lichen extracts against test microorganisms.

Antioxidant activity

Total phenolic activities

Phenolic compounds have antioxidant properties because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen (Hall and Cuppett 1997). In our investigation, a linear calibration curve of gallic acid, in the range of 20-100 µg/mL with R^2 value of 0.9953, was constructed and it was determined that there was 161.4±0.01 µg/mL gallic acid equivalent of phenolic compound in the 1 mg of ethanol extract of *D. scruposus*.

Lichens produce secondary metabolites which are mainly phenolic compounds (Nash 1996). It is suggested that some polyphenolics have inhibitory effects on mutagenesis and carsinogenesis in humans, when up to 1.0 g from a diet rich in fruits and vegetables are ingested daily (Yen et al. 2005).

Reducing power

The reducing power has been used as one of the antioxidant capability indicators of plants (Bhandari and Kawabata 2004). Fig. 3 shows the reducing power of ethanol extract of lichen. The reducing power of lichen extract, BHT,

Trolox and rutin increased steadily with increasing concentration of samples. The reducing power of lichen extract and standard compounds were as follows: BHT>Rutin=Trolox>lichen extract at 1000 µg/mL. The ethanol extract showed lower reducing power than the standards. This indicates that the lichen extract was electron donor and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction.

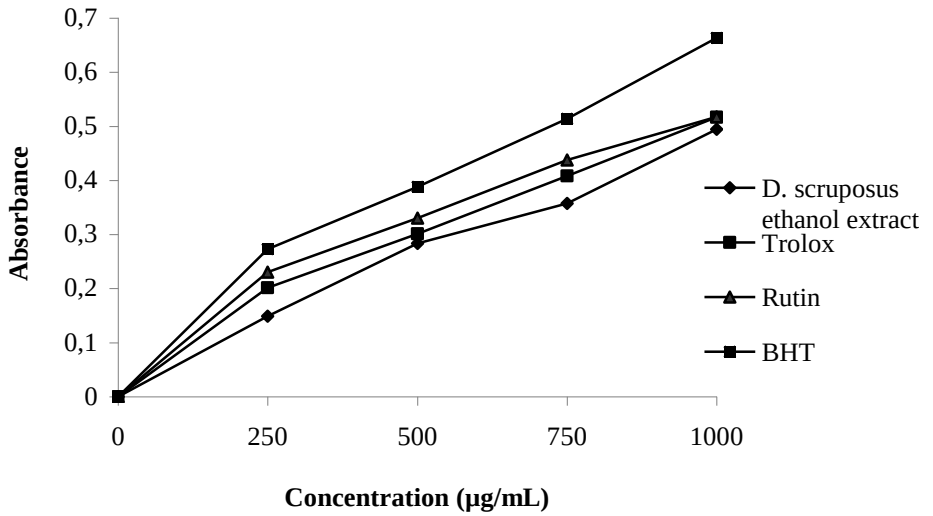

Figure 3. Reducing power of ethanol extract of *D. scruposus*.

DPPH radical scavenging activity

In DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine (Oyaizu, 1986). Fig. 4 shows the dose response curves of DPPH radical scavenging activity of the extract from lichen. The extract was capable of scavenging DPPH radicals in a concentration-dependent manner. BHT, Trolox and rutin were used as references for radical scavenger activity. The scavenging activity of lichen extract, BHT, Trolox and rutin on DPPH radicals increased between 25-100 µg/mL and were 85.0±1.46%, 90.73±1.96%, 92.83±1.96% and 85.33±1.15% at a concentration of 100 µg/mL, respectively. Lichen extract and rutin showed similar DPPH radical scavenging activity, while BHT and Trolox were more effective DPPH radical scavenger. DPPH scavenging activity is best presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. A higher DPPH radical scavenging activity was associated with a lower IC_{50} value. IC_{50} values for lichen extract, BHT, Trolox and rutin on DPPH radical scavenging activity were found as 54.37±0.71, 48.11±0.51, 47.69±0.36 and 50.93±0.47 µg/mL. A lower IC_{50} value indicates a higher DPPH free radical scavenging activity.

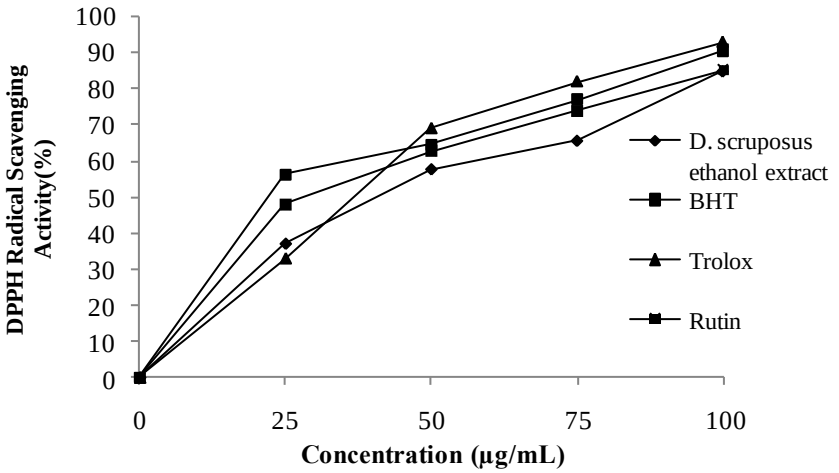

Figure 4. DPPH radical scavenging activity of ethanol extract of *D. scruposus*.

$ABTS^{\cdot+}$ radical scavenging activity

The $ABTS^{\cdot+}$ assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long-wavelength absorption spectrum showing absorption at 734 nm. Bleaching of a preformed solution of the blue-green radical cation $ABTS^{\cdot+}$ has been extensively used to evaluate the antioxidant capacity (Sanchez-Moreno 2002). The $ABTS^{\cdot+}$ radical scavenging activity of ethanol extract of lichen compared to rutin and Trolox are shown in Fig. 5. $ABTS^{\cdot+}$ radical scavenging activity increased with increasing concentration, reaching 96.77±1.37% at 100 µg/mL and this value was considerably higher than that of the positive controls, rutin 88.67±0.42% and Trolox 72.00±1.67% at a same concentration. IC_{50} values for lichen extract, rutin and Trolox on $ABTS^{\cdot+}$ radical scavenging activity were found as 45.45±0.80, 51.07±0.49 and 66.03±0.82 µg/mL, respectively.

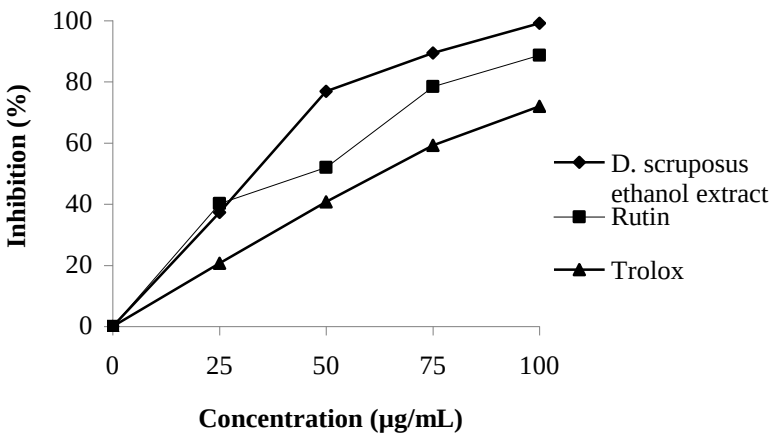

Figure 5. $ABTS^{\cdot+}$ scavenging activity of lichen extract.

In conclusion, ethanol extract of *D. scruposus* showed antibacterial and strong antioxidant activity. Therefore, *D. scruposus* lichen as a natural antioxidant source appears to be an alternative to synthetic antioxidants. The results suggest that the lichen extract tested possess compounds with antibacterial properties as well as antioxidant activity, which require further studies to determine antibacterial agents for therapy of infectious diseases in human and plant diseases.

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