Research Article

Phytochemical screening and *in vitro* antioxidants activities of ethanolic extract of *Acokanthera schimperi* leaves

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ABSTRACT

Objective: *Acokanthera schimperi* belonging to the family Apocynaceae is well known in Ethiopia to treat skin diseases, tonsillitis, and snake bites for its antimicrobial, antiseptic, and anti-inflammatory properties, and it has a long history of being used as an herbal remedy. The present study was aimed to analyze the phytochemical screening and *in vitro* antioxidant activities of the organic leaf extracts, to find out novel antioxidants in a pharmaceutical formulation to treat oxidative stress-related diseases without side effects. **Methods:** Leaves of *A. schimperi* were subjected to successive solvent extraction using petroleum ether, chloroform, ethyl acetate, and ethanol, to obtain the respective extracts, these extracts were used for phytochemical screening using standard qualitative phytochemical methods, then ethanolic extracts were tested for antioxidant activity using various *in vitro* antioxidant methods such as 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), hydroxyl (OH), superoxide radical (SOR) scavenging, and reducing power activity. **Results:** Qualitative phytochemical analysis of leaves of *A. schimperi* organic extracts revealed that the maximum presence of flavonoids, terpenoids, phenolic compounds, tannins, saponins, glycosides, and phytosterol. Further *in vitro,* antioxidant activity of ethanolic extract from leaves of *A. schimperi*, among various antioxidant assays performed the maximum inhibition observed for DPPH for 80% with IC_{50} of 53±12µg/ml, followed by NO for 73% with IC_{50} 53.48 µg/ml, superoxide radical for 60% with IC_{50} of 91.08µg/ml. **Conclusion:** The results of the present study demonstrated that ethanolic leaf extract of *A. schimperi* possess significant bioactive secondary metabolites with potential antioxidant activity, so these leaves of the plant used as a viable source of natural antioxidants for industrial and pharmaceutical preparations.

KEY WORDS: Free radical, Oxidative stress, Antioxidant, *Acokanthera schimperi*

INTRODUCTION

Medicinal plants are traditionally used for thousands of years. Herbs are now very popular in developing countries due to improved knowledge about the safety, efficacy, and quality assurance of ethnomedicine. In recent years secondary plant metabolites (phytochemicals) have been extensively investigated as a source of medicinal agents.[1] Oxidative stress is an imbalance of the radical species, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) and antioxidant system, upregulation of ROS contribute the pathogenesis of the oxidative stress-related disease.[2] In Ethiopia, *Acokanthera schimperi* belongs to family Apocynaceae, (locally called "Yemerz ETS") is used alone or in combination with other species of plants for the treatment of headache (root and bark), epilepsy, amnesia, eye disease, scabies, leprosy (leaf), syphilis (leaf), Tinea capitis (leaf), wound (leaf), eczema (unspecified part), swelling (root), warts (unspecified part), common cold (leaf), rheumatic pain (stem) elephantiasis (root), and *schimperi* traditionally used for curing of skin infection, against snake bite as anti-venom, and tonsillitis.[3]

Dried tender single stem's leaf or roots of *A. schimperi* crushed with water and squeezed a drop through the nasal region. Based on the previous pharmacological studies from Ethiopian researchers, 80% methanol extract *A. schimperi* leaves showed anti-microbial

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activity against the strain of *Staphylococcus aureus*.[4] Tadeg *et al*.[5] reported that the leaves and bark are applied to the skin to treat skin disorders, inflammatory-related disease and an infusion of the leaves is gargled to treat tonsillitis. Mohammed *et.al*.[6] reported that *A.schimperi* is having antimalarial activity against Plasmodium berghei in Swiss albino mice. Traditional users lack of awareness about *A. schimperi,* no proper documentation and active bioactive compounds of the plant are mostly unknown. The local people are unable to exploit the valuable medicinal importance of this traditional medicinal plant in their locality.

The present study was aimed to evaluate the phytochemical constituents, *in vitro* antioxidant activity of ethanolic extract of *A. schimperi* leaves for exploitation of its medicinal importance for curing oxidative stress-related diseases.

MATERIALS AND METHODS

Plant Material Collection

The leaves of *A. schimperi* were collected from a Central Zone of Tigray region, Northern Ethiopia in February 2017. The plant material was authenticated, and specimen herbarium was deposited at Addis Ababa University, Biology Department, National Herbarium of Ethiopia and it was given the voucher specimen number of BA 001. The leaves of *A. schimperi* were dried at 25° C for 10 days in the absence of sunlight and powdered well-using mortar and pestle and stored in an airtight container.

Preparation of the Extracts

The powdered medicinal plant material was taken and subject to serial exhaustive solvent extraction methods, during these solvents of increasing polarity from a low polar (Petroleum ether) to a high polar solvent (ethanol) to ensure that wide polarity range of compounds could be extracted, during extraction solvents were diffuse into the plant material and solubilize the phytocompounds with similar polarity. For qualitative determination, the extracts were placed in pre-weighed flasks before drying. The remaining plant parts residues were extracted with other solvents sequentially.

Percentage Yield and Phytochemical Screening

The qualitative preliminary phytochemical analysis was performed for Petroleum ether, chloroform, ethyl acetate, and ethanol extracts by the following methods as per the standard methods.[7,8]

In vitro Antioxidant Scavenging Assays

The antioxidant activity of ethanolic leaf extract of *A. schimperi* was analyzed for *in vitro* antioxidant scavenging assays based on HAT (Hydrogen Atom Transfer) and ET-(Electron Transfer) based reactions. The antioxidants activities were determined by 1-diphenyl-2-picrylhydrazyl (DPPH) radical, reducing power, hydroxyl radical scavenging, nitric oxide radical scavenging, and superoxide radical scavenging assay. All the analysis was done in triplicates, and average values were taken.

DPPH Radical Scavenging Assay

The DPPH free radical scavenging capacity of ethanolic extract of *A. schimperi* was measured using a standard protocol with slight modifications developed by Yen and Chen.[9] Briefly, 4 ml of varying concentrations of ethanolic leaf extract of *A. schimperi* and ascorbic acid (20, 40, 60, 80, and 100 µg/ml) were mixed with 1.0 ml of an ethanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.1 mM. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm against control. L-Ascorbic acid was used as positive control, and IC_{50} values were also calculated. The percentage of DPPH scavenging of the sample was calculated according to the equation.

(%) DPPH radical scavenging = ([Abcontrol$_{517nm}$-Abtest$_{517nm}$]/Ab control$_{517nm}$)×100

Reducing Power Activity

Reducing power activity was measured by the method of Oyaizu.[10] Briefly, 2.5 ml of varying concentrations of ethanolic leaf extract *A. schimperi* and ascorbic acid (20, 40,60, 80, and 100 µg/ml), 2.5 ml of 1% potassium ferric cyanide and 2.5 ml of 0.2 M sodium phosphate buffer were mixed and incubated at 50°C for 20 min and reaction was terminated by the addition of 2.5 ml of 10% (w/v) of trichloroacetic acid followed by centrifugation at 3000 rpm for 10 min. 5.0 ml of the supernatant upper layer was mixed with 5.0 ml of deionized water and 1.0 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blank that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing the power of the plant extract. Ascorbic acid was used as positive control, and IC_{50} values were also calculated.

% of radical reducing power=([Ab control$_{700nm}$-Ab test$_{700nm}$]/Ab control$_{700nm}$) ×100

Where, Abs control = Absorbance of potassium ferricyanide solution (potassium ferricyanide + sodium phosphate buffer) Abs test = Absorbance of potassium ferricyanide solution + plant extracts or ascorbic acid solutions. All the tests were performed in triplicates, and the results were averaged.

OH^- Radical Scavenging Assay

OH^-radical scavenging activity was described by Smirnoff and Cumbes.[11] The OH^-radicals were generated from $FeSO_4$, and hydrogen peroxide is

detected by their ability to hydroxylate the salicylate, and the hydroxylated salicylate complex was measured at 562 nm. The reaction mixture (3 ml) contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of hydrogen peroxide (6 mM), 0.3 ml of sodium salicylate (20 mM), and varying concentrations of 20, 40, 60, 80, and 100 µg/ml of ethanolic leaf extract *A. schimperi,* and ascorbic acid were allowed for incubation for 1 h at 37°C, after incubation the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as follows:

% of OH radical scavenging = ([Ab control$_{562nm}$-Ab test$_{562nm}$]/Ab control$_{562nm}$) ×100

Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity was described by Green *et al.*[12] Briefly, varying concentrations 20, 40, 60, 80, and 100 µg/ml of ethanolic leaf extract *A. schimperi* and ascorbic acid were mixed with 2.5 ml of sodium Nitroprusside and made up to 3.0 ml with phosphate-buffered saline. Then, the mixture was incubated for 15 min at 25°C. After incubation, 0.5 ml of the reaction mixture was removed, and 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO4, and 0.1% naphthyl ethylenediamine dihydrochloride) was added. Then, the absorbance was measured at 546 nm.

The percentage inhibition was calculated by comparing the results of the test with those of controls not treated with the extract, as per the following formula:

% of NO radical scavenging = ([Ab control$_{546nm}$-Ab test$_{546nm}$]/Ab control$_{546nm}$) ×100

Superoxide Radical Activity

Superoxide radical activity was described by Liu *et al.,*[13] briefly, the reaction mixture consists of 20 µl of varying concentration of 20, 40, 60, 80, and 100 µg/ml ethanolic leaf extract *A. schimperi* and ascorbic acid, to which 0.2 ml of EDTA, 0.1 ml of nitro blue tetrazolium, 0.05 ml of riboflavin were added, and the reaction was started by adding 2.25 ml of phosphate buffer to reaction mixture and control tubes were set up without the extract. Similarly, the activity of the standard ascorbic acid was also carried out, allowed for incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

The percentage inhibition was calculated by comparing with the optical density of the control tubes.

% of O_2^-radical scavenging=([Ab control$_{560nm}$-Ab test$_{560nm}$]/Ab control$_{560nm}$) ×100

Statistical Analysis and IC_{50} Value

All experiments were performed in triplicate (n = 3), and results were expressed as mean ± SD. The simplest estimate of IC_{50} is to plot x-y and fit the data with a straight line (linear regression) that were done in Microsoft Excel 2015.

The simplest estimate of IC_{50} was to plot x-y and fit the data with a straight line (linear regression).

IC_{50} value is then estimated using the following formula:

$$Y=a*x+b$$

$$IC_{50}= \frac{(0.5-b)}{a}$$

Where IC_{50} = Half-maximal inhibitory concentration.

RESULTS AND DISCUSSION

Plants are a source of a large number of drugs comprising to different groups of secondary metabolites exhibit anticancer, antimicrobial, anti-inflammatory, and anti-diabetic, etc. A large number of the plants are claimed to possess the biological properties in the traditional system and are also used extensively by the people worldwide for curing diseases.[14]

The Percentages Yield of Different Organic Solvent Extracts of *A. schimperi* Leaves

As shown in Table 1, the highest percentage of yield was obtained using ethanol (2.671%) as a solvent by the process of serial exhaustive solvent extraction followed by petroleum ether (0.876 g), chloroform (0.872 g), and ethyl acetate (0.782 g). The highest extraction of yield was obtained using ethanol from *A. schimperi* leaves because ethanol as polar solvent dissolved polar compounds present in the *A. schimperi* leaves. Mohanasundari and Suja[15] reported that ethanolic extract of *Alpinia speciosa* and *Alpinia calcatara* is promising sources of potential antioxidant agents in some diseases, due to the presence of different primary and secondary metabolites.

Qualitative Phytochemical Analysis of *A. schimperi*

The preliminary qualitative phytochemical screening of *leaf* extracts *A. schimperi* was done to assess the presence of the major classes of secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, tannins, steroids, and terpenoid other bioactive compounds responsible for antioxidant activities.

The results of preliminary phytochemical screening showed that the organic leaf extracts of *A. schimperi* have various classes of secondary metabolites as showed in Table 2, carbohydrates, tannins, cardiac glycoside, steroids, amino acids, and proteins present

in all organic extracts. In addition, the ethanolic extract alone the presence of all secondary metabolites except alkaloids. Based on the highest of yield secondary metabolites present in the ethanolic extract of *A. schimperi* further, it was selected for further *in vitro* antioxidant assay and HPTLC analysis.

In vitro Antioxidant Assays

Phenolic compounds are plant secondary metabolites with diverse beneficial biological activities such as anti-inflammatory, anti-allergic, antibacterial, anti-atherosclerotic, anticarcinogenic, antimutagenic, antitumor, and antiviral activities.[16,17] The antioxidant activity of flavonoids is due to their ability to reduce the free radical formation and to scavenge free radicals.[18]

DPPH Radical Scavenging Activity

The antioxidant activity with reference to DPPH assay of ethanolic leaf extract of *A. schimperi* was tested with concentration ranging from 20 to 100 µg/ml, as shown in Figure 1, the ethanolic leaf extract of *A. schimperi* exhibited a significant concentration-dependent free radical scavenging activity from 39.21% to 80.17%, respectively, compared with that standard ascorbic acid. The IC_{50} value of ethanolic leaf extract *A. schimperi* leaf extract was 53.12 µg/ml while the IC_{50} value of standard antioxidant ascorbic acid was 47.5µg/ml. The observed *in vitro* antioxidant activity of ethanolic extracts of leaves is due to the neutralization of free radicals (DPPH), by transfer of an electron or hydrogen from secondary metabolites present in them. It is well known that the antioxidant activity of plant extracts is due to the capacity to be donors of hydrogen atoms or electrons and to capture the free radicals, our results are correlated with Rajamanikandan *et al*.[19] they reported that DPPH radical scavenging activity of ethanolic extract of *Mollugonudicaulis* was compared with BHT, at a concentration of 2.5 mg/ml, the scavenging activity of ethanolic extract of *Mollugonudicaulis* reached 74.96%, while at the same concentration; the standard was 87%.

Hydroxyl Radical Scavenging Activity

The concentrations ranging from 20 to 100 µg/ml ethanolic leaf extract of *A. schimperi* were assessed for their hydroxyl radical scavenging activity. The ethanolic extract *A. schimperi* significantly scavenged the hydroxyl radical generated by the $FeSO_4$ and hydrogen peroxide system; hydroxyl radical scavenging activity was increased with increasing concentration of extracts. As shown in Figure 2, the percentage of hydroxyl radical scavenging activity of ethanolic leaf extract of *A. schimperi* leaves and ascorbic acid 100µg/ml was found to be 52.78 and 79%, while the IC_{50} values were 90.13 µg/ml and 48 µg/ ml, respectively.

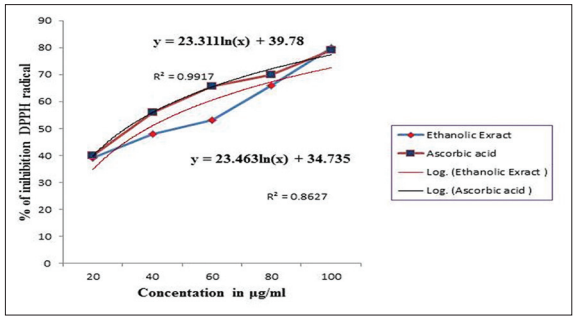

Figure 1: DPPH radical Scavenging activity of the ethanolic extract of *A.schimperi* compared to that of standard ascorbic acid.Each value is expressed as Mean ± Standard deviation (*n*=3)

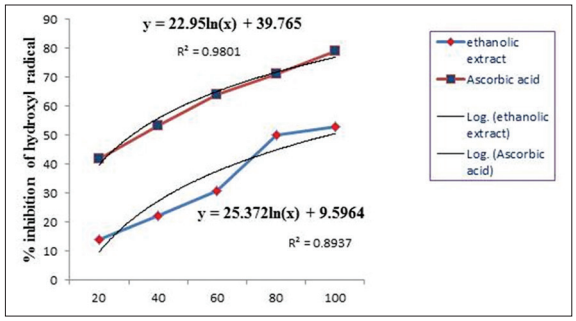

Figure 2: Hydroxyl radical scavenging activityof theethanolic extract of *Acokanthera schimperi* compared to that of standard ascorbic acid. Each value is expressed as Mean ± Standard deviation (*n*=3)

Table 1: Percentage yield and residue of leaves extracts of *A. schimperi* in different solvents

Solvent extract	% yield g/100 g	% residues g/100 g
Petroleum ether	0.876	99.124
Chloroform	0.872	99.128
Ethyl acetate	0.782	99.218
Ethanol	2.671	97.327

A. schimperi: Acokanthera schimperi

Table 2: Qualitative phytochemical analysis of different organic solvent crude extracts of *A. schimperi*

Phytoconstituents	Solvent			
	Petroleum ether	chloroform	ethyl acetate	ethanol
Alkaloids	--	--	--	--
Flavonoids	--	--	--	+
Tannins	+	+	+	+
Cardiac glycosides	+	+	+	+
Terpenoids	--	+	--	+
Saponins	--	--	+	--
Steroids	+	+	+	+
Carbohydrates	+	+	+	+
Fixed oils and fats	+	+	--	--

+ indicates the presence of secondary metabolites. - indicates the presence of secondary metabolites

OH^- radical is one of the potential inducers of DNA damage; the OH^- radical can attack purine and

pyrimidine bases to form an OH radical adduct, these consequences of DNA damage are the modification of genetic material resulting into cell death, mutagenesis, carcinogenesis, and aging. Therefore, it is important to discover some of the plant-derived bioactive secondary metabolites with good scavenging capacity on these ROS from plant sources. In this study, ethanolic leaf extracts of *A. schimperi*, was found to scavenge OH^- radical significantly in a dose-dependent manner and exhibit significant scavenging of 73% with IC_{50} of 53 µg/ml. The scavenging of the hydroxyl radicals was due to the presence of phenolic compounds in the ethanolic leaf extracts; these compounds act against oxidative damages leading to various degenerative diseases such as cardiovascular diseases, inflammation, and cancer by protecting the DNA, protein, and lipid from damage.[20] Sasikumar and Kalaisezhiyen[21] reported that all the extracts of *Kedrostis foetidissima* when added to the reaction mixture, scavenge hydroxyl radicals in a concentration-dependent manner. Therefore, it is important to discover plant-derived antioxidant with good scavenging capacity on these reactive oxygen and nitrogen species from ethanolic leaf extract of *A. schimperi.*

Nitric Oxide Radical Scavenging Assay

Nitric oxide is a potent pleiotropic mediator of various physiological processes, it is a diffusible free radical, which plays many roles as an effectors molecule in diverse biological systems.[22] The ethanolic extract effectively reduced the generation of nitric oxide from sodium nitroprusside in concentration depended manner. As showed in Figure 3, the percentage of nitric oxide radical scavenging activity of ethanolic extract of *A. schimperi* leaves and ascorbic acid 100 µg/ml was found to be 73.77% and 85.39%, while the IC_{50} values were 53.48 µg/ml and 41.23 µg/ ml, respectively.

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes, but excess production of NO is associated with several pathological diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use.[23] In the present study, ethanolic leaf extracts of *A. Schimperi* exhibited potent nitric oxide radical scavenging activity 73% with IC_{50} value of 53.48 µg/ml. Secondary metabolites in ethanolic extracts compete with oxygen to react with nitric oxide and thus inhibits the generation of nitrite. According to Ahirwal *et al*.,[24] the methanolic extract of *Gymnema Sylvestre* showed a significant antioxidant activity of 11.4, 13.3, 15.2, and 17.7% inhibition at 50, 100, 250, and 500 µg/ml, respectively, when compared with butylated hydroxyacetone.

Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity of ethanolic extract of *A. schimperi* as shown in Figure 4, exhibited the concentration depended scavenging activity of 60.33% at 100 µg/ml compared with standard ascorbic acid 73.33% at 100 µg/ml. The IC_{50} value of the standard was 37.77 µg/mL while that of the ethanolic extract was 91.07 µg/ml. As shown in Figure 5, the abilities of the ethanolic extract and ascorbic acid to quench superoxide radicals from the reaction mixture are reflected in the decrease of the absorbance.

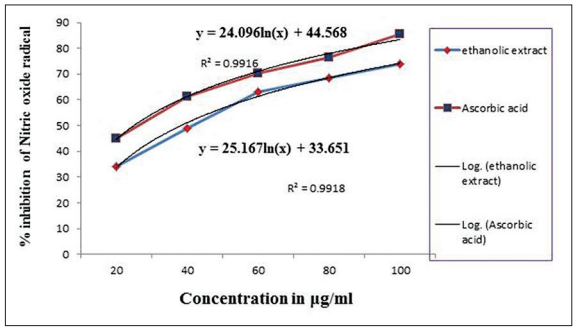

Figure 3: Nitric oxide scavenging activityof theethanolic extract of of *Acokanthera schimperi* compared to that of standard ascorbic acid. Each value is expressed as Mean ± Standard deviation (*n*=3)

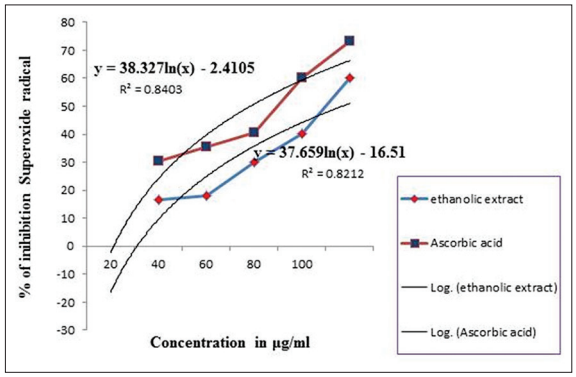

Figure 4: Superoxide radical scavenging activityof the ethanolic extract of *Acokanthera schimperi* compared to that of standard ascorbic acid. Each value is expressed as Mean ± Standard deviation (*n*=3)

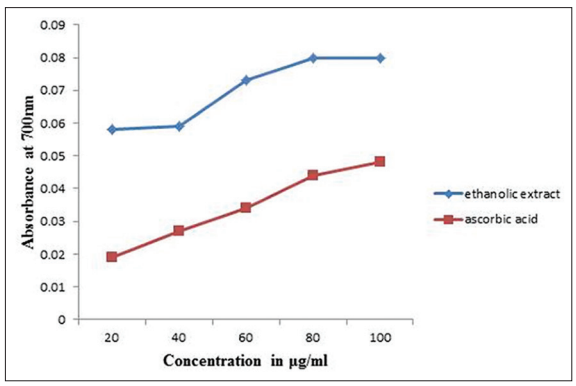

Figure 5: Reducing power activity of the ethanolic extract of *Acokanthera schimperi* compared to that of standard ascorbic acid. Each value is expressed as Mean ± Standard deviation (*n*=3)

Superoxide anion radical is one of the strongest ROS among the free radicals that are generated after oxygen is taken into living cells. Superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radical, damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids then induce oxidative damage.[25] The ethanolic extract of *A. schimperi* was found to be efficient scavengers of superoxide radical generated in PMS–NADH–NBT system *in vitro* and their activities are comparable to that of ascorbic acid. In the present study, the antioxidant activity was found to be positive for ethanolic extract which can be attributed to the presence of phenols and flavonoids as shown in the phytochemical screening test.

Reducing Power Activity

In this assay, the yellow color of the test solution changes to green or blue depending on the reducing power of the ethanolic extract, greater absorbance at 700 nm indicated greater reducing power. As shown in Figure 5, the ethanolic leaf extract of *A. schimperi* demonstrated that reducing power increased linearly with concentration. At 20, 40, 60, 80, and 100 µg/ml, reducing power of leaf extract was found to be 0.058, 0.059, 0.073, 0.080, and 0.082, respectively. The reducing power of the ethanolic leaf extract is due to their electron donating ability of secondary metabolites; these secondary metabolites react with cation compounds to stabilize and terminate chain reactions.

Anti-oxidative activity has been proposed to be related to reducing power; therefore, the antioxidant potential of ethanolic leaf extract of *A. schimperi* was estimated for their ability to reduce Fe (III) complex to Fe (II) along with standard ascorbic acid. The reducing power of the ethanolic leaf extract was observed that the maximum inhibition exhibited at a concentration of 100 µg/ml, compared with reducing the power of the standard ascorbic acid these results were correlated with increasing the concentration of water, ethanol, and aqueous ethanol extract of *Aerva lanata* having reducing power activity reported by Ragavendran *et al*.[26] The reducing power of the ethanolic leaf extracts of *A. schimperi* is due to the presence of electron donating secondary metabolites such as phenolics, flavonoids, and terpenoids, react with the free radicals to stabilize and terminate from the formation of free radical reaction, from the studies of Halvorsen *et al*.[27]reported that most of the secondary metabolites are redox-active compounds that can be picked up by the reducing power assay.

CONCLUSION

The ROS and RNS are double-edged swords and display dual functions in the living organism, increase in these reactive species causes damages to the metabolites such as proteins, lipids, and nucleic acids, in under pathophysiological conditions, endogenous antioxidants may not counteract excess free radicals. Hence, there is a continuous demand for exogenous antioxidant supplementation. Most of the ethnobotanical information of this medicinal plant is remaining undocumented. The preliminary phytochemical investigations revealed, that the presence of considerable amount of bioactive compounds such as flavonoids, steroids, terpenoids, and phenols but alkaloids were not detected in the all organic solvents, these secondary metabolites which are used in treatment of different chronic disease and therefore *A.* schimperi *is* a good source of natural antioxidant for medical application in antioxidant therapy.

Synthetic antioxidant therapy has been implicated to be effective in preventing diseases resulted from oxidative stress, but long-term use of synthetic antioxidant such as BHA and BHT contribute to carcinogenicity or tumorigenicity due to their oxidative characteristics, and high doses of this ingredient to cause significant damage to the lungs, liver, and kidneys. Oral consumption of BHT ingredient has also been shown to have toxic effects on the body's, due to their considering the advantages of lower side effects of natural antioxidants, plant, or their extracts have been extensively selected for development of natural bioactive antioxidant drugs.

The results of the present study revealed that the presence of secondary metabolites in the ethanolic extracts of *A. schimperi*. It also possesses potent antioxidant and free radical scavenging activity which was evidenced by the presence of glycosides, steroids, tannins, and phenolics. In future, these compounds may lead to the development of novel drug agents for the treatment of various diseases.

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