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Radical scavenging activity and preliminary phytochemical screening on aerial part extracts of *Cineraria abyssinica* sch. bip. EXA

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Abstract
Though the aqueous decoction of the aerial part of *Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) has been used for the treatment of various diseases associated with oxidative stress in Ethiopia, there appear to have been no report on the phytochemistry and antioxidant activity of the aerial part of this plant. The aims of this study were, therefore, to carry out radical scavenging activity and preliminary phytochemical screening on the aerial part of the plant. The aqueous crude extract, ethyl acetate, butanol and dichloromethane fractions of the aerial part of *C. abyssinica* were studied for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The crude extract and fractions showed promising DPPH activity with IC_{50} ranging from 3.29 µg/ml to 9.18 µg/ml. The ethyl acetate fraction showed the most potent DPPH scavenging activity (IC_{50} = 3.29 µg/ml) better than the standard, ascorbic acid (IC_{50} = 5.21 µg/ml). Preliminary phytochemical screening of the aqueous extract of the aerial part of the plant revealed the presence of alkaloids, polyphenols, flavonoids, coumarins, saponins, tannins and phytosterols. Hence, the present study revealed the radical scavenging activity of phytochemicals present in the aerial part of *C. abyssinica* that scientifically support the traditional claim and its potential as invaluable source of natural antioxidant.

Keywords: *Cineraria abyssinica*, radical scavenging, DPPH, preliminary phytochemical screening, aerial part, Ethiopia

1. Introduction
Free radicals are atoms or molecules with lone pair of electrons in their outer most shell. They can be endogenous or exogenous in origin. Though they have some important physiological roles, excessive free radicals damage proteins, lipids and nucleic acid and they are implicated in the pathogenesis of myriads of diseases [1, 2]. Although there are a number of synthetic antioxidants, their safety is questionable [3] and hence, it is prudent to look for potent, safe and cheaper antioxidants from natural sources to replace these synthetic chemicals. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported the importance of plants against oxidative stress in biological systems [4].
Cineraria abyssinica Sch. Bip. exA (Asteraceae) locally known as 'Esemefirh' in Amharic [5] and 'Baluketel or Fatu kitel' in Harari and Oromifa [6], is an erect or scrambling, annual or perennial herb that can grow up to 20-100 cm high. It has repeatedly branched stem, with alternate, simple to lyrate pinnatifid petiolate leaves and radiate capitula with yellow florets. It extends from Ethiopia into Yemen and Saudi Arabia [5]. In Ethiopian traditional medicine, the aqueous decoctions of the leaves and aerial parts of *C. abyssinica* are used as remedy for various ailments, such as cancer, liver and kidney diseases, hypertension, diabetes, malaria and gastrointestinal disorders including diarrhea. Recently we have established the antibacterial, hepatoprotective and antioxidant activities of its leaf extracts and rutin isolated from it. Phytochemical investigation on its leaf part also showed the presence of polyphenols, flavonoids, saponins, tannins, coumarins and phytosterols [6-9]. Though traditionally the aerial part is used for myriads of diseases associated with oxidative stress, to the best of our knowledge, there is no previous research on the antioxidant activity of the aerial part of the plant. Hence the main objectives of this study were to investigate the DPPH radical scavenging activity and to carry out preliminary phytochemical screening on the aerial part extracts of the plant.

2. Material and methods

2.1. Plant material

The aerial part of *C. abyssinica* at its flowering stage were collected from Harar in the Harari People Region, 525 km East of Addis Ababa, Ethiopia in October 2013 and authenticated by Mr. Melaku Wondafrash at the National Herbarium, Addis Ababa University, Biology Department and a voucher specimen was deposited at the laboratory of Pharmacognosy Course and Research Unit, College of Health Sciences, Mekelle University for future reference.

2.2. Chemicals and instruments

DPPH (SIGMA), methanol (Natason, India), n-butanol (Unichem, India), ethyl acetate (CarloErba) and dichloromethane (CarloErba) were used. All other chemicals and solvents were analytical and laboratory grade. JENWAY 6405 UV/Vis, UK was used for DPPH analysis.

2.3. Preparation of crude extract and fractions

The dried and powdered aerial part of *C. abyssinica* (500 g) was extracted by decoction using 3 L of distilled water for 1 h. The extraction was carried out three times. The filtrates were pulled together and dried in an oven at 40 °C to get the aqueous crude extract [9]. For preparation of the fractionations, 10 g of the aqueous crude extract was suspended in distilled water and treated successively with 200 ml of different solvents of increasing polarities starting from dichloromethane, ethyl acetate and n-butanol, each three times. Each of the individual solvent fractions were then concentrated in rotary evaporator at 40 °C and dried in oven at 45 °C.

2.4. DPPH scavenging activity

The method described by Braca *et al.* (2001) [10] was used with slight modification. 3 ml of 0.004% DPPH (SIGMA) in methanol was mixed with 1 ml of various concentrations (100, 50, 25, 12.5, 6.75, µg/ml) of the crude aqueous extract and fractions of the aerial part of *C. abyssinica* and ascorbic acid (a reference compound) separately. Then after 30 min incubation at room temperature in the dark, the absorbance of the mixture in the samples was measured using a spectrophotometer (JENWAY 6405) at 517 nm against methanol as blank. The percentage radical scavenging activities of the samples were evaluated by comparing with a control (3 ml DPPH solution and 1ml methanol). Each sample was measured in triplicate and averaged.

The percentage radical scavenging activity (RSA) was calculated using the following formula:

$$\% \text{ RSA} = [(A0 - A1)/A0] \times 100$$

Where $A0$ is the absorbance of the control, and $A1$ is the absorbance of samples after 30 min.

The free radical scavenging activity of the crude extract, fractions and ascorbic acid is expressed as IC_{50}. The IC_{50} value is defined as concentration (in µg/ml) of sample that inhibits 50% of the DPPH radical.

2.5. Preliminary phytochemical screening

The aqueous decoction of the aerial part of the plant was tested for the presence of secondary metabolites such as phytosterols, polyphenols, flavonoids, coumarins, tannins, saponins and anthraquinones following the standard procedures with some modifications [11].

2.5.1. Test for alkaloids

500 milligrams of the extract was treated in a test tube with 10 ml of 1% HCl for 30 minutes in a water bath and then filtered through cotton in to a test tube. Small portion of the extract was transferred into two test tubes and to one of the test tubes, five drops of Mayer's reagent and to the second five drops of Wagner's reagent were added and the formation of whitish opalescence (Mayer's reagent) or reddish brown precipitate (Wagner's reagent) was inspected.

2.5.2. Test for anthraquinones

2.5.2.1. Free anthraquinones

100 mg of the extract was shaken vigorously with 10 ml of benzene and the extract was filtered. The filtrate was treated with 5 ml 10% ammonia solution and shaken. The formation of pink, violet or red colour in the ammonia phase was considered positive for free anthraquinones.

2.5.2.2. Anthraquinone-O-glycoside

To about 1 g of the extract, 10 ml of 2 N HCl was added and boiled for 1 hr. It was then cooled, filtered, and the filtrate was extracted with 10 ml of benzene. To 5 ml of benzene extract, equal volume of 10% ammonia solution was added and shaken. The formation of pink, red or violate colour in the aqueous (ammonia) phase was taken as positive for anthraquinones-o-glycoside.

2.5.3. Test for polyphenols

To 100 mg of the extract dissolved in methanol or water, three drops of a mixture (prepared immediately before the reaction of one ml 1% $FeCl_3$ and one ml 1% $KFe(CN)_6$) were added and the formation of green blue colour was inspected.

2.5.4. Test for flavonoids

2.5.4.1. Shinoda reduction test

100 mg of the extract dissolved in five ml of 50% methanol was divided into two test tubes and to one of the test tube metallic magnesium and to the other zinc was added then five drops of concentrated HCl were added to each test tubes and the formation of an orange or red colour was taken as positive for the presence of flavonoids.

2.5.4.2. Lead acetate test

To 2 ml of the extract in the methanol five drops of 2% lead acetate solution were added and the development of yellow or orange precipitate was inspected.

2.5.5. Ammonia test for coumarins

To 100 mg of the extract dissolved in 5 ml of ethanol, 2 ml of 10% ammonia was added and the occurrence of an intensive fluorescence under UV light was inspected. Comparison was made by taking another 5 ml of the extract in ethanol without 10% ammonia as a reference.

2.5.6. Test for saponins

2.5.6.1. Formation of honeycomb froth

500 mg of the extract in 10 ml of distilled water was shaken in a test tube and the formation of honeycomb froth that persists for half an hour was considered as positive for saponins.

2.5.6.2. Chemical test

To 2 ml of the aqueous solution of the extract, 1 ml of 10% solution of sodium nitrate and 3 drops of concentrated H_2SO_4 were added and the formation of a bloody red colour was inspected.

2.5.7. Test for tannins

1 gm of the extract was heated in a test tube with 10 ml of distilled water for five minutes. After cooling, the solution was

filtered through filter paper and 5 ml of 2% NaCl was added to the clear filtrate. Then 5 ml of 1% gelatin was added and the formation of precipitate was inspected.

2.5.8. Test for phytosterols
One gram of the extract was macerated with petroleum ether, filtered and the filtrate was concentrated and treated with the following reagents.

2.5.8.1. Salkwoski reaction
The residue was dissolved in chloroform and to it 5 drops of concentrated H_2SO_4 were added carefully and the production of a red or violet colour was regarded as positive for the presence of steroidal compounds.

2.5.8.2. Liebermann and Burchard's reaction
The concentrated residue was dissolved in chloroform and it was treated with a solution of a cold mixture of concentrated H_2SO_4 (five drops) and acetic anhydride (1 ml) and the formation of rose or reddish brown colour which turns to green or blue was taken as positive for steroidal skeleton.

3. Results and discussion
Table 1 shows that the aqueous crude extract and the different solvent fractions prepared from the aerial part of *C. abyssinica* possess strong DPPH radical scavenging activity with 50% inhibition concentration (IC_{50}) value of 3.29 µg/ml to 9.18 µg/ml.

Table 1: DPPH scavenging activity of the crude extract and solvent fractions prepared from aerial part of *Cineraria abyssinica* in comparison with ascorbic acid.

Test groups	IC_{50} (µg/ml)
Aqueous crude extract	7.18
Butanol	6.75
Dichloromethane	9.18
Ethyl acetate	3.29
Ascorbic acid	5.21

Antioxidants protect us from free radical damage and they are critically needed for good health and for the augmentation of our body defense mechanism. Free radical scavenging is one of the mechanisms by which antioxidants inhibit cellular damage. In Ethiopian traditional medicine the aqueous decoctions of the leaf and aerial part of *C. abyssinica* has widely been used for the management of a number of diseases that are associated with oxidative stress [6-9] and the purpose of this study was to justify the traditional use by examining the DPPH radical scavenging activity of the aerial part extracts of the plant. DPPH free radical scavenging method is colorimetric assay and it is one of the most commonly used methods to assess the radical scavenging activity of compounds or extract in a short time [12].
DPPH is a purple-coloured, stable nitrogenous radical with a maximum absorbance at 517 nm. In the presence of a free radical scavenger, electron or hydrogen donors, the odd electron becomes paired off to form a stable diamagnetic molecule, 2,2,-diphenyl-1-picrylhydrazine, that will result in reduction in the absorbance and discoloration of DPPH solution from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract [13, 15]. IC_{50} (the concentration of substrate that brings about 50% loss of the DPPH or the concentration of the sample that is necessary to decrease the absorbance of DPPH by 50 %) is typically employed to express the antioxidant activity and to compare the antioxidant capacity of various samples [16]. The lower the IC_{50} value, the higher is the scavenging potential. The IC_{50} values of the aqueous crude extract and fractions were calculated from the graph of percentage radical scavenging activity versus concentrations and the results are given in Table1. In the present study the aqueous crude extract showed DPPH scavenging activity with an IC_{50} value of 7.18 µg/ml. This is slightly higher than the one reported on the aqueous leaf extract of *C. abyssinica* [9] which implies that the antioxidant compounds are mainly concentrated in the leaf part of the plant. As the aqueous crude extract showed good DPPH radical scavenging activity and in order to characterize the nature of the active compounds, it was further fractionated in different solvents of various polarities. The different solvent fractions showed various degree of DPPH scavenging activity. While the ethyl acetate fraction exhibited the lowest IC_{50} (3.29 µg/ml) hence the most potent radical scavenging activity, even better than the standard, Ascorbic acid (IC_{50} = 5.21 µg/ml), the dichloromethane fraction showed the largest IC_{50} (9.18 µg/ml), lowest radical scavenging activity compared to the others (Table 1). This variation in the antioxidant activities of the different extracts of the plant could be attributed to difference in the nature and/or amount of phytochemicals extracted by the different solvents. Future antioxidant activity-guided isolation study on the aerial part of *C. abyssinica* should, therefore, preferably consider the ethyl acetate fraction that displayed the most potent DPPH scavenging activity.
Preliminary phytochemical screening of the aqueous decoction of the aerial part of *C. abyssinica* (Table 2) revealed the presence of polyphenols, flavonoids, coumarins, tannins and phytosterols while anthraquinones both free and O-glycosides were absent. Similar result was reported on the 80% methanolic leaf extract of *C. abyssinica* except that alkaloid was absent in the leaf part of the plant [9]. This variation in the alkaloid content between the leaf and aerial part of the plant may indicate the ability of the plant to store the alkaloids at specific organs in this case in its stem or flowers other than its leaves or the alkaloid may exist in very minute amount in the leaf part that they were unable to be detected by the alkaloidal precipitating reagents.

Table 2: Preliminary phytochemical screening on the aqueous extract of the aerial part of *Cineraria abyssinica*

Secondary metabolite	Test reagents	Result
Alkaloids	Wagner's reagent	Positive
	Mayer's reagent	Positive
Anthraquinones	Borntrager's reagent	Negative
	Borntrager's reagent	Negative
Coumarins	Ammonia fluorescence test	Positive
Flavonoids	Shinoda reagent	Positive
	Lead acetate	Positive
Phytosterols	Libermann-Burchard's	Positive
	Salkowski test	Positive
Polyphenols	1 % $FeCl_3$ and 1 % $KFe(CN)_6$	Positive
Saponins	Froth test	Positive
	10% sodium nitrate and conc. H_2SO_4	Positive
Tannins	Gelatin test	Positive

Polyphenols like flavonoids, tannins and coumarins are well known DPPH scavengers. It is reported that the antioxidant and free radical scavenging activities of phenolic compounds

like flavonoids depend on the arrangement of functional groups around the nuclear structure. Hence the radical scavenging activity of this plant could be ascribed to the presence of phenolic compounds viz. flavonoids, coumarins and tannin which may act singularly or in combination [17, 18]. Flavonoids vary in their polarity and the flavonoids composition of an extract does vary with the solvents, whether water, methanol, ethanol, acetone, or ethyl acetate used for extraction. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures [19]. In the present study, the ethyl acetate fraction showed the most potent DPPH radical scavenging activity compared to the other fractions as well as the standard, ascorbic acid. This potent DPPH scavenging activity can be attributed to the ability of the ethyl acetate to dissolve less polar flavonoids, aglycones of phenolic compounds [19]. Studies showed that flavonoidal aglycones are more potent antioxidants than their corresponding glycosides [20]. Other phytoconstituents such as coumarins, alkaloids and saponins may also contribute for the radical scavenging activity of the plant [21-25]. Hence further study to identify the compound(s) responsible for the potent radical scavenging activity of the plant particularly the ethyl acetate fraction is required.

4. Conclusion

In conclusion, this study suggests that the aerial part of *C. abyssinica* possess different phytochemicals which in isolation or in combination could be responsible for the radical scavenging activity of the plant. Hence it justifies its traditional use and its potential in preventing or slowing the progress of varies oxidative stress-induced disease, oxidation of foods and pharmaceuticals. Further studies that involve bioactivity guided isolation, *in vivo* antioxidant activity and toxicity study are very important to exploit the potential benefit of this plant.

5. Conflict of interest

None of the authors have conflict of interest.

6. Acknowledgements

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