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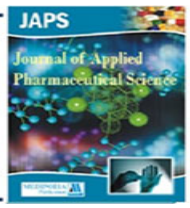

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Radical scavenging activities of the leaf extracts and a flavonoid glycoside isolated from *Cineraria abyssinica* Sch. Bip. Exa. Rich

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ABSTRACT

The aqueous decoction of the leaves of *Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) is used for treatments of myriads of diseases associated with oxidative stress. However, there appear to have been lack reports on its phytochemistry and pharmacological activities. The main purpose of this study was, therefore, to determine the radical scavenging activities of the leaf extracts of *C. abyssinica.* The aqueous and 80% methanolic crude extracts, chloroform, acetone and methanol fractions of the leaf extracts of C. abyssinica showed potent radical scavenging activities (IC_{50} = 5.27-12.41 µg/ml). Activity-guided fractionation of the most active, methanol fraction resulted in the isolation of a flavonoidal glycoside, rutin. The structure was assigned on the basis of spectroscopic methods including ultraviolet spectroscopy (UV), infra red (FT-IR), mass spectrometry (MS) and one and two dimensional nuclear magnetic resonance spectrometry (1D- and 2D-NMR). Rutin showed the most potent DPPH scavenging activity (IC_{50} = 3.53 µg/ml) slightly higher than ascorbic acid (IC_{50} = 3.57 µg/ml). Acute toxicity studies on the crude extracts showed the nontoxic nature of the plant up to 3 g/kg. Therefore, the present study revealed for the first time the presence of antioxidant phytochemical in the leaves of *C. abyssinica* that scientifically validated the traditional use and its great potential to be used as a source of antioxidant phytochemicals.

Keywords: free radical scavenging, DPPH, Oxidative stress, C. abyssinica, Asteraceae, Rutin.

INTRODUCTION

Free radicals are atoms or groups of atoms that have at least one unpaired electron, which make them highly unstable and reactive. Living organisms accumulate free radicals through both normal metabolic processes and exogenous sources. Although radicals have beneficial effect during energy production and as antibacterial, excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death (Asres *et al*., 2006; Pham-Huy *et al*., 2008). Several lines of experimental and clinical evidences strongly suggest that free radicals are implicated in the pathogenesis of myriads of diseases, such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases, liver diseases, malaria, human immunodeficiency virus (HIV), etc (Baynes, 1991; Grieve and Shah, 2003; Beal, 2005; Singh and Jialal, 2006; Sas *et al.*, 2007). Thus, antioxidant agents that can slow or prevent the oxidation process by removing free radical intermediates are desired.

Several strong synthetic antioxidants have been reported (Shimizu *et al.,* 2001); however, they have proven to be highly toxic (Wichi, 1988). For this reason, in recent years, there is a tremendous interest in finding antioxidant phytochemicals for use as supplements to human health (Terao and Piskula, 1997). A wide range of natural compounds, including phenolic compounds, nitrogen compounds, and carotenoids have antioxidant properties and may serve as potential candidates for antioxidant resources (Velioglu *et al.,* 1998).

Cineraria abyssinica (Asteraceae) locally known as 'Esemefirh' in Amharic (Tadese, 2004) and 'Baluketel or Fatu kitel' in Harari and Oromifa, 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 lyrately pinnatified petiolate leaves and radiate capitula with yellow florets. It extends from Ethiopia into Yemen and Saudi Arabia (Tadese, 2004).

In Ethiopian traditional medicine, the aqueous decoction of the leaves and aerial parts of *C. abyssinica* is used as a remedy for various ailments, such as cancer, liver and kidney diseases, hypertension, diabetes and gastrointestinal disorders that are related with oxidative stress. Despite its wider use, there appears to lack scientific reports on its phytochemistry as well as on its pharmacological activities. It was, therefore, deemed prudent to assess the radical scavenging ability of the plant using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) spectrophotometric assay.

MATERIALS AND METHODS

Plant material

The leaves of *C. abyssinica* at its flowering stage were collected from and around the town of Harar in the Harari People Region, 525 km East of Addis Ababa, Ethiopia in September 2008. The plant was authenticated by Ato Melaku Wondafrash of the National Herbarium, Addis Ababa University, Biology Department and a voucher specimen has been deposited at the National Herbarium, Addis Ababa University (Collection number, B 01).

Animals

Wistar albino female mice (25-30 g) obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) animal house were used for the experiments. The animals were housed under standard laboratory conditions and were fed commercial rodent feed and tap water ad libitum. The animals were fasted overnight with free access to water and acclimatized for one week in the new environment before experiments were carried out. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996) and approved by the Institutional Review Board of the School of Pharmacy, Addis Ababa University.

Chemicals and instruments

All the chemicals and reagents used for the experiments are analytical grade. Ultraviolet (UV) spectra were run on a Shimadzu UV-1800 spectrophotometer. Infra red (IR) spectra were taken on a Shimadzu IR Prestige-21 spectrophotometer in KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 using a Bruker A-400 spectrometer with TMS as an internal standard. Electro spray mass spectra were obtained with LCQ Deca XP, ESI, negative mode spectrometer.

Preparation of crude extracts

Two different extractions were carried out. The leaves of *C. abyssinica* were shade dried and powdered. The powder (300 g) was extracted exhaustively at room temperature by maceration with 5 L of 80% methanol for 72 h with occasional shaking (3x) and the other by aqueous decoction for 30 min. The combined methanolic filtrates were then dried in a Rotary vacuum evaporator (< 40 °C) and the aqueous extract was lyophilized. The yields of the extracts were 20% and 17% for the 80% methanolic extract and for the aqueous extract respectively.

Preparation of solvent fractions and isolated compound

The air-dried powdered leaves of *C. abyssinica* (300 g) were successively extracted in Soxhlet apparatus using solvents of increasing polarity (5 L), starting from chloroform then acetone and methanol. The solvents were removed by placing the extracts in a Rotary vacuum evaporator (< 40 °C). The chloroform fractions yielded 9.5% black-coloured paste while the acetone and the methanol fractions gave 7.5% and 21% reddish brown powders respectively. Further fractionation of the most active methanol fraction by preparative thin layer chromatography (PTLC), silica gel F_{254} (Sigma) using butanol: acetic acid: water (4:1:5, upper phase) as mobile phase afforded one major band with retention factor (RF) of 0.45. The band was scraped and after desorption in methanol and concentration gave a yellowish powder. The powder was further purified by LH-20 column chromatography using methanol as solvent and the purity of the eluate was checked by analytical TLC.

Identification of the isolated compound

The identity of the isolated compound was done chemically by Mg-HCl (Shinoda test) and Molish test and by UV and shift reagents (Mabry *et al.,* 1970; Markham, 1982). The formation of red colour by Shinoda test and purple colour in Molish test indicated that the compound is a flavonoid glycoside.

Structural elucidation of the compound

Structure elucidation of the isolated compound from the methanol fraction was carried out by spectral techniques; UV, IR, MS and 1D- and 2DNMR. The UV spectra of the isolated compound were recorded in methanol (MeOH) and shift reagents were also used as shown in Table 1. In consistent with the result for the chemical tests, the isolated compound showed typical characteristic absorption spectra for flavonol. Addition of sodium methoxide (NaOMe) to the methanol solution of the isolated compound resulted in a bathochromic shift of 51 nm with an increase in intensity of absorption in band I, indicating the presence of free 4'-hydroxyl group on the isolated compound. A

bathochromic shift of 16 nm in band II upon addition of sodium acetate (NaOAc) indicates the presence of free 7-hydroxyl group. Addition of NaOAc/H_3BO_3 to the methanol solution of the isolated compound resulted in a bathochromic shift of 20 nm indicating the presence of ortho-dihydroxyl group in the B-ring. The bathochromic shift of 55 nm in band I in the presence of $AlCl_3$/HCl indicates the presence of free 5- hydroxyl group. The bathochromic shift of 70 nm in band I region upon the addition of $AlCl_3$, as shown in Table 1, indicted the substitution in position 3 (Markham, 1982). Therefore based on these UV spectra, the isolated compound appeared to be a 3-substituted-3',4',5,7-flavonol. In consistent with the data obtained from the UV spectra, the IR spectrum of the isolated compound (KBr pellet) revealed the presence of broad hydroxyl absorption at 3450 cm^{-1} and conjugated carbonyl absorption of at 1650 cm^{-1}. The negative-ion electrospray ionization (ESI) mass spectrum of the isolated compound showed a quasi-molecular ion $[M-H]^-$ at *m/z* 609, indicating a relative molecular weight (*M*) of 610. The negative-ion ESI-MS also gave a prominent fragment ion at *m/z* 301 $[[M-H]^- - 308]^-$, suggesting a loss of diglycoside moiety from the quasi-molecular ion. The MS data along with UV, ^1H, ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra made it possible to come up with a molecular formula $C_{27}H_{30}O_{16}$ for the isolated compound.

Table. 1: UV absorption maxima (λ max) of the compound isolated from the methanol fraction of the leaves of *Cineraria abyssinica* in methanol before and after addition of shift reagents.

MeOH	NaOMe	NaOAc	NaOAc+H_3BO_4	$AlCl_3$	$AlCl_3$+HCl
205 nm	272 nm	273 nm	262 nm	209.6 nm	209.2 nm
257 nm	330.2 nm	323.2 nm	297.6 nm	274.4 nm	269 nm
359 nm	410.4 nm	326.8 nm 403 nm	379.8 nm	339.6 nm 429.2 nm	401.4 nm

The ^1H NMR spectrum of the isolated compound exhibited a characteristic proton signal at δH 12.56 corresponding to a chelated hydroxyl group at C-5. In addition to this, the presence of five aromatic protons were seen in the ^1H NMR spectrum; two ortho-coupling protons assignable to H-6' (δ7.55, 1H, d, *J* = 8.4 Hz) and H-5' (δ6.85, 1H, d, *J* = 8.4 Hz); two-meta coupling protons at H-6 (δ6.20, 1H, d, *J* =1.6 Hz) and H-8 (δ6.39, 1H, d, *J* = 1.6 Hz); a singlet aromatic proton at H-2' (δ7.52, 1H, s). The occurrence of five aromatic protons signals in the ^1H NMR further supported the tentatively assigned chemical structure of the isolated compound by UV. The ^1H NMR spectrum also supported the presence of rhamnose and glucose moieties with the rhamnose anomeric proton signal at δH 4.38 and glucose H-1 signal at δH 5.33. A doublet of methyl group of rhamnose was observed at high field δH 1.11 (3H, d, J = 6 Hz). The rest of protons in the sugar moiety resonated between 3.32 and 3.81 ppm.

The ^{13}C NMR spectrum (150 MHz, DMSO-*d*6) showed 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. The DEPT spectrum of the isolated compound, revealed the presence of one methyl carbon (δC = 18.2 ppm) of rhamnose, one methylene carbon (δC = 67.4 ppm), 15 methine carbons and 10 quaternary carbons. In the aliphatic region of ^{13}C NMR, 12 carbon resonances are assigned for a rutinoside moiety among which the most downfield signals at 101.2 and 101.6 are assigned for the two anomeric carbons C1'" and C1" of rhamnose and glucose, respectively (Agrawal, 1992). The chemical shift of each carbons of the isolated compound in the ^{13}C NMR spectrum (Table 2) was assigned by comparing with the literature data (Agrawal, 1992; El-Sawi and Sleem, 2010).

Confirmation of the position of attachment of the sugar moiety was obtained from the HMBC spectrum of the isolated compound in which key ^3J correlations was seen between the anomeric proton signal of glucose (δH 5.33) and C-3 resonance of the aglycone (δC 133.72) indicating that C-3 is the site of glucosilation. In addition, a cross-peak between the δH 4.4 (H-1'", rhamnose) and the δC 67.43 (C6" of the glucose) confirmed that the glycosylation of the glucose unit by the rhamnose took place on the 6″-hydroxyl.

Proton and ^{13}C NMR data recorded in DMSO-d6 were in a good agreement with published values **(**Agrawal 1992; El-Sawi and Sleem, 2010**).** Furthermore, comparison of ESI-mass spectra of the isolated compound with those of standard rutin revealed that they are superimposable on each other. Hence the compound was identified unequivocally as rutin (Figure 1).

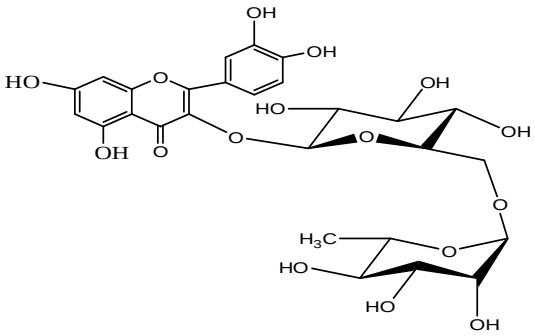

Fig. 1: Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside)

DPPH scavenging activity

The method of Sokmen *et al.*, (2004) was used in this experiment. Firstly, 5 ml of 0.004% DPPH (SIGMA) (in methanol) was mixed with 50 µl of various concentrations (1000, 500, 250, 125, 50, µg/ml) of the crude extracts, fractions, the isolated compound or 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 (Unico^{TM}2100) at 517 nm against methanol as blank. The percentage radical scavenging activity of the samples was evaluated by comparing with a control (5 ml DPPH solution and 50 µl 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 *A*0 is the absorbance of the control, and *A*1 is the absorbance

of samples after 30 min. The free radical scavenging activities of the crude extracts, fractions, rutin and ascorbic acid were expressed as IC_{50}. The IC_{50} value was defined as concentration (in µg/ml) of sample that inhibits 50% of the formation of DPPH radical.

Table 2: ^{13}C NMR spectral signals of the compound isolated from the methanol fraction of *Cineraria abyssinica* in comparison with those of rutin and rutinose.

	^{13}C NMR (ppm) (isolate)	^{13}C NMR of rutin (ppm) (literature)*	^{13}C NMR rutinose (ppm) (literature)**
C2	157.1	156.9	
C3	133.7	133.8	
C4	177.8	177.9	
C5	161.6	161.6	
C6	99.1	99.1	
C7	164.5	164.8	
C8	94.1	94.3	
C9	156.9	156.6	
C10	104.4	104.5	
C1'	121.6	121.1	
C2'	115.7	115.8	
C3'	145.2	146.1	
C4'	148.8	148.8	
C5'	116.7	116.9	
C6'	122.1	121.3	
C1"	101.6	101.3	101.4
C2"	74.5	73.7	74.3
C3"	76.9	77.4	76.6
C4"	71.0	71.1	70.3
C5"	76.3	75.9	76.1
C6"	67.4	67.1	67.3
C1'"	101.2	101.5	100.9
C2'"	70.8	70.9	70.6
C3'"	70.4	70.7	70.6
C4'"	72.3	72.3	72.1
C5'"	68.7	68.7	68.5
C6'"	18.2	18.2	18.0

* El-Sawi and Sleem (2010).
** Agrawal (1992).

Table. 3: DPPH scavenging activity IC_{50} values of the crude extracts, solvent fractions, and rutin from the leaves of *Cineraria abyssinica* in comparison with ascorbic acid.

Test groups	IC_{50} (µg/ml)
Aqueous extract	6.27
80% methanol extract	5.78
Chloroform fraction	12.41
Acetone fraction	8.53
Methanol fraction	6.82
Rutin	3.53
Ascorbic acid	3.57

Acute toxicity tests

Acute toxicity studies were carried out on the aqueous and 80% methanolic leaf extracts of *C. abyssinica* according to Daisya *et al.*, (2009). Normal healthy male mice fasted for 12 h were randomly divided into drug-treated 'test' groups and vehicle-treated 'control' group, totally making up 7 groups of 6 mice per cage. 0.5, 2.0 and 3.0 g of each of the extracts suspended in 1% carboxyl methyl cellulose (CMC)) was separately administered orally to the mice in each of the test groups. The mice in the control group were treated with vehicle alone (1% CMC). 2 h after treatment, the mice in both the test and control groups were allowed access to food and water, and behavioral changes were observed over a period of 24 h. The mortality caused by the extract within this period of time was also observed.

RESULTS AND DISCUSSION

As shown in Table 3, the aqueous and 80% methanolic crude extracts, fractions, rutin and the standard, ascorbic acid showed significant DPPH radical scavenging activities. However, by far the most potent radical scavenging activity was displayed by rutin IC_{50} = 3.53 µg/ml.

DPPH is a purple-coloured stable radical of organic nitrogen with a maximum absorbance at 517 nm and it is widely used to study radical scavenging activities of extracts and pure compounds. When the odd electron becomes paired off in the presence of a free radical scavenger to form hydrazine, the absorption reduces and the DPPH solution is decolourised from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract (Molyneux, 2004; Dehpour *et al.*, 2009). IC_{50} (concentration required to obtain a 50% antioxidant capacity or is the concentration of substrate that brings about 50% loss of the DPPH) is typically employed to express the antioxidant activity and to compare the antioxidant capacity of various samples (Locatelli *et al.*, 2009). In the present study, the crude extracts, fractions and rutin were shown to serve as potent antioxidant agent or hydrogen donor that can scavenge radicals. The 80% methanolic crude extract showed stronger DPPH scavenging activity than the aqueous as well as fractions. This could be due to the ability of methanol to extract non polar, medium polar and polar phytochemicals that can act synergistically. Among the fractions, the methanol fraction showed stronger radical scavenging activity hence it was further fractionated by PTLC and LH-20 column chromatography and resulted in the isolation of rutin. Rutin showed the most potent DPPH scavenging activity IC_{50} = 3.53 µg/ml even slightly stronger than ascorbic acid (3.57 µg/ml). The antioxidant and free radical scavenging activities of phenolic compounds like flavonoids are dependent upon the arrangement of functional groups around the nuclear structure. Thus, both the number and configuration of H-donating hydroxyl groups are the main structural features that influence the antioxidant capacity of flavonoids (Cao *et al.*, 1997; Pannala *et al.*, 2001).

The presence of ortho-dihydroxyl group in the B-ring of rutin as well as the sugars at the 3 position makes it strong free radical scavenging activity. Rutin is a flavonoid glycoside widely distributed in nature. To the best of our knowledge, this is the first report on the isolation of rutin from *C. abyssinica*. Rutin has been extensively studied and it is known to exhibit multiple pharmacological activities including antiviral (Panasiak *et al.*, 1989; Yarmolinsky *et al.*, 2012), antitumor (Deschner *et al.*, 1991; Choiprasert *et al.*, 2010; Araújo *et al.*, 2011), antiallergic (Chen *et al.*, 2000), anti-inflammatory (Guardia *et al.*, 2001; Kazłowska *et al.*, 2010), antihypertensive (Matsubra *et al.*, 1985; Lapa Fda *et al.*, 2011), antidiabetic (Prince and Kamalakkannan, 2006), gastroprotective (La-Casa *et al.*, 2000; Abdel-Raheem, 2010), anticonvulsant (Nassiri-Asl *et al.*, 2008), Cardioprotective (Ali *et al.*, 2009; Challa *et al.*, 2011), hepatoprotective (Janbaz, 2002; Wu *et al.*, 2011), neuroprotective (Tongjaroenbuangam *et al.*, 2011;

Moshahid Khan *et al.,* 2011), nephroprotective (Chen *et al.,* 2011) and antioxidant. The antioxidant activity of rutin is due to its radical scavenging activity as well as metal-chelating properties (Kim *et al.,* 2002; Nagai *et al.,* 2005; Asres *et al.,* 2006). The acute toxicity study revealed the non-toxic nature of the aqueous and 80% methanolic leaf extracts of *C. abyssinica* up to 3 g/kg. No mortality was observed in the extract-treated mice and the extracts also did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions and coma.

The plant is locally used for the management of cancer, liver and kidney diseases, diabetes, and cardiovascular problems for which reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a crucial role in the induction and progression of the diseases. For instance, in all forms of liver disease there is a consistent evidence of enhanced production of free radicals and/or significant decrease of antioxidant defense. As a consequence, a large number of studies have focused on the pathogenetic significance of oxidative and nitrosative stress in liver injury as well as on therapeutic intervention with antioxidant and metabolic scavengers (Yamamoto *et al.,* 1998; Kapiowitz, 2000; Loguercio and Fedrico, 2003).

Therefore, the potent radical scavenging activities of the leaf extracts and its rutin as well as the previously reported pharmacological activities of rutin may partly justify the traditional claims of the plant. The plant also merits further researches such as activity-guided isolation of the other active fractions, sub-acute and chronic toxicity studies.

CONCLUSION

In conclusion, our result demonstrated that the leaves extracts of *C abyssinica* have potent radical scavenging activities and this effect could be attributed at least in part to the presence of a potent flavonoid glycoside, rutin. The acute toxicity study showed the nontoxic nature of the plant and hence the present study justifies the traditional use of the plant for the management of diseases associated with oxidative stress.

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