Asian Journal of Immunology

Volume 6, Issue 1, Page 252-259, 2023; Article no.AJI.107027

Activity Guided Isolation of Phytoconstituents of *Moringa oleifera.* Lam Leaves to Study their *In-vitro* Free Radical Scavenging Activity

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Authors' contributions

This work was carried out in collaboration between both authors. Author DVC designed the study, performed the experiments, analyzed and interpreted the data. Authors DVC and BG wrote and reviewed the manuscript and approved the final version of the manuscript. Both authors read and approved the final manuscript.

Article Information

Open Peer Review History:
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Original Research Article

Received: 28/07/2023
Accepted: 03/10/2023
Published: 06/10/2023

ABSTRACT

Aim: To isolate the antioxidative components of *Moringa oleifera.* L leaves by screening their free radical scavenging potential.
Methodology: The leaves of *Moringa oleifera.* L were dried in the shade, followed by grinding and cold macerating with 95% alcohol. The crude alcoholic extract was then fractionated with organic solvents like ether, chloroform, and ethyl acetate. The *in-vitro* free radical scavenging potential was assessed by α the α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging and Nitric oxide scavenging methods.
Results: Among the different leaf fractions of *Moringa oleifera* assessed, the ethylacetate fraction exhibited potential free radical scavenging potentiality, hence these fractions were further

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progressed for fractionation and refining using advanced chromatographic techniques. The sequential fractionation and purification resulted in the isolation of three components which were structurally characterized and identified employing advanced analytical techniques like UV, IR, Mass spectroscopy, and NMR spectroscopic methods. In comparison with the available literature data, the isolated compounds were named quercetin (1), kaempferol (2), and quercetin-3-O-rutinoside (3). The compounds isolated from the leaves of *Moringa oleifera* exhibited potential free radical scavenging activity on testing for their antioxidant property.

Conclusion: The compounds isolated from the *Moringa oleifera* leaves exhibited a strong free radical scavenging potential proving the antioxidant property of the plant.

Keywords: Moringa oleifera Lam; antioxidant activity; DPPH; nitric oxide.

1. INTRODUCTION

It's well known that plants have been the base for several traditional systems of medicine over the globe for several years and continued to serve manhood with novel remedies. On average three-quarters of the global population count on herbal extracts and herbs for their health care needs [1]. It's well recognized that free radicals including the reactive oxygen species (ROS) play a vital role in the development and progression of several diseases of mankind including but not limited to cancer, metabolic disorders, respiratory disorders, liver disorders, inflammatory disorders, and various forms of dementia [2]. The reactive oxygen species combine with the free radicals to form active radicals like superoxide anion radicals (O_2^-), hydroxyl radicals (OH), non-free radical species (H_2O_2), and singlet oxygen ($^1O_2$) [3].

The reactive oxygen species (ROS) are known to cause major damage to the cell membranes and DNA, causing lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to various degenerative diseases. Antioxidants are known to play an important role in health protection, there are scientific reports that evidence that antioxidant foods lower the risk of chronic ailments [4]. Antioxidants like Vitamin A, Vitamin C, vitamin E, phenolic acids, and flavonoids from herbal sources are known to have the potential to protect from the disease menace. Hence, the antioxidant components from the plant source have gained importance in research, particularly the polyphenols, phenolic acids, and flavonoids which are present enormously in fruits, seeds, vegetables, and leaves [5-10]. These compounds have also been reported for various pharmacological activities [11-12].

Moringa oleifera Lam belonging to the family Moringaceae generally known by its common name 'Drumstick' or 'Horseradish' tree is grown in different parts of the world, especially for its edible fruits and leaves. Apart from being used as food, different parts of the *Moringa oleifera* have scientific reports claiming different pharmacological properties like hypotensive, antispasmodic, diuretic, antioxidant, analgesic, hypolipidemic, anthelmintic, hepatoprotective, antimicrobial, antidepressant, anti-inflammatory, antiulcer, antifertility, and anticancer properties [13]. Taking into the consideration of the above-mentioned pharmacological properties and the different phytoconstituents reported with *Moringa oleifera* leaves we aimed in evaluating the free radical scavenging activity of various extracts and isolated the compounds from leaves of the plant using DPPH and nitric oxide scavenging methods.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Identification

Moringa oleifera leaves were procured from the market area of Bangalore and was the botanically authenticated by Prof. Balakrishna Gowda, GKVK, Bangalore.

2.2 Chemicals

1-diphenyl, 2-picrylhydrazyl radical (DPPH), and ascorbic acid were purchased from Sigma, USA. While the extraction solvents, and the chemicals of high standards employed for our phytochemical estimations were purchased from the local vendors.

2.3 Extraction Process

The leaves of *Moringa oleifera* were dried in shade and grinded using a mechanical grinder to collect the coarse powder. The powdered leaves were cold macerated for seven days employing

95% alcohol, followed by filtering, and concentrating the crude extract in rotary evaporator. The resulting crude alcoholic extract was further partitioned with different solvents like petroleum ether, chloroform, and ethyl acetate to yield their respective leaf fractions.

2.4 Isolation and Characterization of Phytoconstituents

Additionally, the ethyl acetate leaf fraction of *Moringa oleifera* that exhibited the lowest IC50 value in the DPPH radical scavenging activity was jeopardized to column chromatography employing different mobile phases like ethylacetate, toluene at different compositions. Collected different fractions i.e., 10 fractions of ethylacetate, 20 fractions of ethyl acetate: toluene (70:30), and 20 fractions of ethylacetate: toluene (85:25) that were set at a flow rate of 6 drops/min. The collected fractions were subjected to thin layer chromatography (TLC) employing readily available silica plates as stationary phase and the mobile phase was ethylacetate: toluene (70:30). The phytochemical constituents separated from the fractions on the TLC plates were viewed in the UV chamber employing ammonia vapors. For each group of fractions collected i.e., F1 (40-50), F2 (55-75) and F3 (80-100) yielded one phytoconstituent respectively. The main phytoconstituents separated from each of the fractions were purified employing Waters high performance liquid chromatography system, using Bonda Pak 20 µm C_{18} (300 x 7.8 mm, 125 A) column, and the mobile phase of methanol: water (40:60) at a flow rate of 2.00 mL/min for a total of 40-minute run time to obtain three pure compounds. Further the characterization of the isolated compounds was done by different analytical techniques like UV-Visible, NMR, IR, and Mass spectroscopic means.

2.4.1 UV-Visible analysis

The isolated compounds (1–3) were solubilized in 100% methanol solution at a strength of 0.1 mg/mL and subjected to UV-Visible spectrophotometric analysis by scanning them at a wavelength ranging between 200–600 nm.

2.4.2 Fourier transform infra-red spectral analysis

The FT-IR spectra was recorded employing Shimadzu's FTIR spectrometer using IR grade potassium bromide (KBR). The isolated compounds were mixed with KBR and enforced with hydraulic pressure to form pellets which were then scanned in the FTIR instrument with different wavelengths ranging from 4000 – 400cm^{-1}.

2.4.3 Mass spectroscopy

The molecular weight detections was performed using an MDS-Sciex triple quadrupole mass spectrometer (Sciex, Foster city, USA) equipped with an Turbo ion source interface that operates at 500^0C. The isolated pure compounds were solubilized in methanol at concentration of 10 µg/mL and infused into the mass spectrometer operated at a scan range between 100 to 1000 m/z in positive ionization mode at flow of 10µL/min using a syringe pump to record the mass spectra.

2.4.4 Nuclear magnetic resonance (NMR) spectroscopy

The proton (^{1}H) NMR spectra was recorded using Bruker's advanced FT-NMR spectrometer (Bruker ARX 400, Bruker, Germany), using tetramethyl silane (TMS) as our internal standard. The isolated compounds solubilized in deuterated solvents were syringe filtered and then subjected to NMR scanning.

2.5 Antioxidant Activity

2.5.1 DPPH free radical scavenging activity

The different leaf extracts and the isolated phytoconstituents of *Moringa oleifera* were tested for the free radical scavenging activity employing 2, 2-diphenyl-1- picrylhydrazyl (DPPH) a stable radical according to the method described by Molyneux [14]. The incubation mixtures containing 900 µL of DPPH (100 µM) solution in methanol, and 100 µL of different leaf extracts or isolated compound solutions with varied concentrations ranging from 5 to 500 µg/mL were incubated for 30-minutes at room temperature followed by measuring the absorbance at 517 nm using UV-Visible spectrophotometer. Ascorbic acid solution was used as a reference standard for the assay with concentrations ranging from 5-50 µg/mL. The following equation was used to calculate the percentage free radical scavenging activity:

$$\% \text{ activity} = \frac{Ac - At}{Ac} \times 100$$

Where, Ac- Absorbance of the control; At- Absorbance of the test sample

2.5.2 Nitric oxide (NO) scavenging activity

The nitric oxide scavenging activity was determined according to Griess Illosvoy reaction [15], where the spontaneous nitric oxide generated by the aqueous sodium nitroprusside reacts with the oxygen to generate nitrite ions which shows an absorbance at 546 nm. The incubation mixture containing different leaf extracts / isolated compound solutions (with varied concentrations), and 10mM sodium nitroprusside solutions were incubated at room temperature for 3-hours, followed by addition of sulfanilic acid reagent, and 0.1% of naphthyl ethylenediamine dihydrochloride. The above mixture was further incubated for 30-min at room temperature, followed by measuring the absorbance at 546 nm. The nitric oxide scavenging activity was computed according to the following equation:

$$\% \text{ activity} = \frac{Ac-At}{Ac} \times 100$$

Where, **Ac** = absorbance of the control (blank, without extract), **At**= absorbance in the presence of the sample/standard.

2.6 Data Analysis

The values of the all the experimental data executed in triplicates were represented as mean ± SD. The IC_{50} values were calculated by linear regression analysis.

3. RESULTS

Among the different leaf extracts tested at different concentrations ranging from 5 to 500 µg/mL, the ethanolic (EMO) and the ethylacetate (EAMO) leaf extract of *Moringa oleifera* recorded the significant free radical scavenging activity (Table 1).

The column chromatography of EAMO yielded three fractions which were subjected to preparative HPLC, to yield three compounds. The different leaf extracts and the isolated compounds were tested for the free radical scavenging activity by the DPPH and nitric oxide scavenging methods. The structural characterizations of the isolated compounds were done by spectroscopic means. The λ-max, IR spectra, molecular weight (m/z) and proton-NMR data of isolated compounds (1-3) were assigned as follows:

3.1 Structural Characterization of Compound (1)

The physical appearance of the isolated compound-1 was yellow amorphous powder; the UV spectra of the isolated compound-1 was 265 nm (λ-max); the IR spectra for the compound-1 revealed aromatic ring stretching bands at 1614, 1550, and 1523 cm^{-1} for C=C, along with the wavenumbers 1408, 1168, 1014, 795, 603, 491 cm^{-1},for the compound-1; the positive ionization mass spectra for the compound-1 revealed a m/z 303; the ^{1}H NMR spectra for the compound-1 exhibited a strong resonance at 12.05 ppm attributing to the C-5 OH protons of flavonoids, like wise unresolved broad resonances at 7.0 to 7.5 ppm attributed to C-3 and C-4 resonances. The signal at 6.19 and 6.41 ppm relates to the protons in the meta position (H-6, & H-8). Comparing the physical and spectral characteristics of the compound (1) with that of the literature confirmed the presence of functional groups present in the flavonoids and was characterized as quercetin [16-17].

3.2 Structural Characterization of Compound (2)

The compound-2 exhibited as yellow crystalline powder with maximum UV absorbance at 267 nm; IR spectra showed a characteristic band for the hydroxyl groups at 3425 cm^{-1} and 3322 cm^{-1}, likewise for the C=O group a band was observed at 1660 cm^{-1} and band at 1500 cm^{-1} confirms the aromatic ring. The intense band at 2995 cm^{-1} shows the aromatic C-H bending; The methanolic solution of the compound-2 infused into triple quad mass spectrometer exhibited a m/z 286 in positive ionization mode; $^{1}HNMR$ spectrum for compound-2 showed a prominent signal between 6.19 and 6.44 ppm for the two aromatic protons in the meta position (H-6, & H-8), the signal at 6.94 and 8.05 ppm corresponded to the 4 aromatic protons confirming the presence of tetra substituted flavones. The comparison of the spectral data of the compound-2 to that reported in the literature along with the physical characteristics revealed that the compound-2 was kaempferol [18-19].

3.3 Structural Characterization of Compound (3)

The physical appearance of the isolated compound-3 was yellow amorphous powder; the UV spectra of the isolated compound-1 was 258 nm (λ-max); the IR spectra exhibited

characteristic band at 3408 cm^{-1}; 3321 cm^{-1} for the OH-stretching, sharp bands at 2924 cm^{-1}; 2843 cm^{-1} represents CH2 stretching. Intense absorption bands at 1462 cm^{-1}; 1383 cm^{-1}; 1018 cm^{-1} relates to C=O, C-OH, and C-C vibrations and stretching respectively. The positive ionization mass spectra for the compound-3 revealed a m/z 611; ^{1}HNMR spectrum for the compound-3 isolated from ethylacetate leaf fractions of *Moringa oleifera* exhibited a characteristic signal at 7.56, 7.58, and 6.86 ppm, representing aromatic protons on the B-ring (H-2, H-6, & H-5). The signals at 6.38, and 6.19 ppm corresponds to protons (H-8, & H-6) on the A-ring. The signal at 5.3 ppm represents the anomeric proton for β-glucose, likewise the signals at 4.3 ppm, and 0.9 ppm symbolizes the protons for rhamnose moiety. Compound (3) was characterized as Quercetin-3-O-rutinoside by comparison of the physical and spectral data with those reported in literature [20].

The compounds (1-3) isolated from the ethylacetate leaf fractions assessed for their antioxidant activity showed a potent free radical scavenging activity in the tested models (Table 1).

4. DISCUSSION

Antioxidants are recognized to exert beneficial effects on human health hence they gain a substantial importance, and it has been proved that polyphenols like flavonoids act as antioxidants due to their hydrogen-bestowing ability [21]. The different leaf extracts and the compounds isolated from *Moringa oleifera* were assessed for their free radical scavenging activity using DPPH, and nitric oxide radial scavenging methods. Due to the complexity, and the oxidative processes of the phytochemicals the single method of free radical scavenging activity would not be sufficive to evaluate the total antioxidant activity. Hence, we had adopted two different free radical scavenging methods for our study. Ascorbic acid was used as the reference standard in both methods.

DPPH, considered as the stable free radical with central nitrogen accepting the electrons/hydrogen to form a stable diamagnetic molecule. Scavenging DPPH radical is a simple, rapid, and precise method for establishing the antioxidant proficiency of the substances. The assay involves measurement of change in DPPH concentration resulting from the reaction with antioxidants by spectrophotometer at 517 nm. The IC_{50} value was used to evaluate the antioxidant potential of the samples, the lower the IC_{50} higher the scavenging potentiality. The IC_{50} values ranged from 24.8 ± 0.17 µg/mL for ethylacetate leaf fraction to 185.2 ± 0.14 µg/mL for petroleum ether leaf fraction (Table 1). The strongest free radical scavenging potential (i.e., lower IC_{50}) was seen for the ethylacetate fraction which is ~7 folds stronger than petroleum ether, and chloroform fractions, and ~2 fold stronger than the ethanolic leaf extract. The isolated compounds from the ethylacetate leaf fractions had a much lower IC_{50} ranging between 4.5 ± 0.10 to 15.2 ± 0.13 µg/mL (Table 1) which is lower or equivalent to the reference standard ascorbic acid having IC_{50} value of 11.6 ± 0.19 µg/mL proving the strong DPPH free radical scavenging potential of the isolated compounds.

The nitric oxide (NO) is a potent chemical mediator released by neurons, macrophages, and endothelial cells, that takes part in controlling several physiological activities like inhibition of

Table 1. *Free radical scavenging* activity of various leaf extracts and the isolated compounds of *Moringa oleifera*

Extract	IC_{50} Values (µg/mL)	
	DPPH radical scavenging activity	**Nitric oxide radical scavenging activity**
EMO	40.5±0.20	95.5±0.31
PEMO	185.2±0.14	1828.9±0.22
CMO	180.1±0.28	1737.5±0.08
EAMO	24.8±0.17	72.7±0.27
Compound-1	4.5±0.10	26.7±0.43
Compound-2	15.2±0.13	28.9±0.39
Compound-3	10.1±0.37	37.5±0.18
AA	11.6±0.19	26.8±0.37

MO: Moringa oleifera. L, EMO: Ethanolic extract of MO, PEMO: Petroleum ether extract of MO, CMO: Chloroform extract of MO, EAMO: Ethyl acetate extract of MO, AA: Ascorbic acid

platelet aggregation, neuronal signaling, vasodilation, and regulation of cell mediated toxicity [22]. NO scavenging capacity is ascertained by the reduction in the absorbance at 550nm, induced by antioxidants. It is reported that the number of hydroxyl groups is important for radical scavenging activity of the flavanol glycosides. Though NO radicals are implicated with host defense, increased production of these radicals promotes pathogenesis of some inflammatory diseases [23]. However, in pathological conditions, NO reacts with superoxide anions and form potentially cytotoxic molecules. Nitric oxide inhibitors have been shown to have beneficial effects on some aspects of inflammation and tissue damage seen in inflammatory diseases. Like DPPH free radical scavenging activity, the ethylacetate leaf fractions of *Moringa oleifera* showed strongest NO free radical scavenging potential as compared to that of the petroleum ether, and chloroform leaf fractions. The IC_{50} values ranged from 72.7 ± 0.27µg/mL for ethylacetate leaf fraction (~25 fold lower) to 1828.9 ± 0.22µg/mL for petroleum ether leaf fraction (Table 1). The compounds isolated from the ethylacetate leaf fractions of *Moringa oleifera* exhibited lower IC_{50} ranging between 26.7 ± 0.43 to 37.5 ± 0.18µg/mL (Table 1) which is equivalent to the reference standard ascorbic acid having IC_{50} value of 26.8 ± 0.37µg/mL proving the strong NO free radical scavenging potential of the isolated compounds. These results showed the ability of the isolated components to reduce free radicals which may stop the free radical initiation or retard free radical chain reaction in the propagation of the oxidation mechanism. Our results are consistent with the former reports, where in which it has been documented that the antioxidant action of flavonoids is reliant on the position and total number of hydroxyl moieties in the molecule [24]. The lower IC_{50} of the isolated compounds indicates that the 3-4-dihydroxy structural elements in the compounds are important for radical scavenging activity. The scientific evidence reported establishes the link between the damaging actions of reactive free radicals and human aliments including but not limited to cancer, metabolic disorders, neurodegenerative diseases, and cardiovascular diseases [24]. Ascorbic acid is a potent free radical scavenger, so when compared to such pure component, IC_{50} of isolated compounds and extracts shows that *Moringa oleifera* is potent free radical scavenger. In very recent years, potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases. Moreover, flavonoids are known to be the natural immunomodulators from the dietary source and could protect the human body from infectious diseases by improving the intestinal immune system [25]. Hence, the leaves of *Moringa oleifera* proved to have the flavonoids quercetin, kaempferol, and quercetin-3-rutinoside with the free radical scavenging potential is an natural immunomodulator that could protect the human body from infectious diseases.

5. CONCLUSION

The results of the current study indicates that the significant free radical scavenging potential of *Moringa oleifera* is due to the presence of the flavonoids (quercetin, kaempferol, & quercetin-3-rutinoside) which act by donating the electrons and reacting with free radicals to form more stable product, and there by terminate the free radical chain reaction. Additionally, these results could also lay a platform for the selection of *Moringa oleifera* for further pharmacological investigations. The current study also confirms the folkloric usage of this plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

This research project was supported by the All-India Council for Technical Education **(**AICTE) scholarship granted to Devaraj VC, and the management of Bio-needs Pre-Clinical Services Pvt. Ltd., Bangalore, India. The authors are also thankful to Prof. Balakrishna Gowda, GKVK, Bangalore for identifying the plant and for his valuable suggestions during the work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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