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Estimation of total phenolic content, cytotoxicity and *in-vitro* antioxidant activity of stem bark of *Moringa oleifera*

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ABSTRACT

Objective: To assess the phytochemical constituents, total phenolic content, cytotoxicity and *in-vitro* antioxidant activity of stem bark extracts of *Moringa oleifera* (*M. oleifera*) (Moringaceae). **Methods:** Brine shrimp lethality (BSL) bioassay was used to investigate the cytotoxic effects. DPPH and nitric oxide radical scavenging activity was used to demonstrate antioxidant activity. **Results:** Phytochemical analysis revealed the presence of tannins, flavonoids, steroids and alkaloids. The LC_{50} values were obtained for extracts as 850 μg/mL for petroleum ether extract, 800 μg/mL for chloroform extract and 900 μg/mL for methanol extract. The total phenolic content of the methanolic extract was 50.72% w/w, equivalent to gallic acid. Petroleum ether, chloroform and methanolic extracts of *M. oleifera* and standard ascorbic acid were found to be scavenger of DPPH radical with an IC_{50} of 124.75, 112.08, 54.34 and 13.86 μg/mL, respectively. Methanolic extract was found to be good scavenger of DPPH radical. Petroleum ether, chloroform, ethyl acetate soluble fraction of methanolic extracts of *M. oleifera* and ascorbic acid were found to be scavenger of nitric oxide radical with an IC_{50} of 93.32, 65.12, 54.83 and 12.59 μg/mL, respectively. Ethyl acetate soluble fraction was found to be good scavenger of nitric oxide radical. **Conclusions:** It can be concluded that the crude extracts of *M. oleifera* is a potential source of natural antioxidants, and this justifies its uses in folkloric medicines.

1. Introduction

The ongoing growing recognition of medicinal plants is due to several reasons, including increasing faith in herbal medicine. Allopathic medicine may cure a wide range of diseases; however, its high prices and side-effects are causing many people to return to herbal medicines which have fewer side effects[1]. Many oxidative stress related diseases are as an outcome of accumulation of free radicals in the body. A lot of researchers are working on finding natural antioxidants of plants genesis. The use of herbal extracts and nutritional supplements either as alternative or complimentary medicine to the conventional chemotherapy for treatment of inflammatory diseases is well documented in Ayurveda, which is an alternative medicinal system that has been practiced primarily in the Indian subcontinent for 5 000 years[2]. For most of the developing countries, the main issue of public health is still the acute need for basic health care, which is sadly lacking even at the most elementary level. This is true in both the rapidly growing cities and in the rural areas. The World Health Organization (WHO) indicates that more than half of the world's population do not have access to adequate health care services. This is due to the fact that poor people neither have access to nor can afford the present health care services. Therefore, innovative alternative approaches are needed to address this problem. Medicinal plants offer alternative remedies with tremendous opportunities. They not only provide access and affordable medicine to poor people; they can also generate income, employment and foreign exchange for developing countries. Many traditional healing herbs and plant parts have been shown to have medicinal value, especially in the rural areas and that these can be used to prevent, alleviate or cure several human diseases. The WHO estimates that more than 80% of the world's population rely

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either solely or largely on traditional remedies for health care. Interest in the exploitation of medicinal and aromatic plants as pharmaceuticals, herbal remedies, flavourings, perfumes and cosmetics, and other natural products has greatly increased in recent years[3].

Moringa oleifera (*M. oleifera*) is the most widely cultivated species of a monogeneric family, the Moringaceae, that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Figure 1).

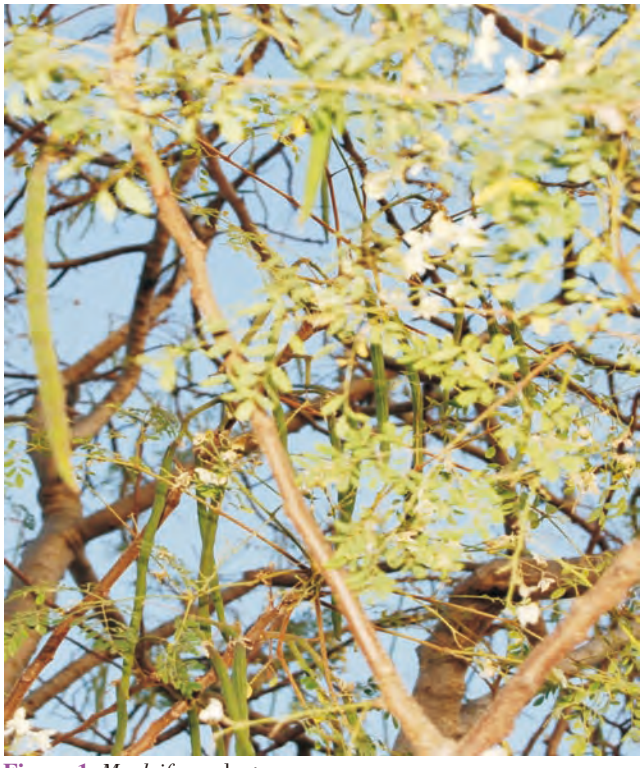

Figure 1. *M. oleifera* plant

This rapidly-growing tree (also known as the horseradish tree, drumstick tree, saijhan, sajna or Ben oil tree), was utilized by the ancient Romans, Greeks and Egyptians; it is now extensively cultivated and has become naturalized in numerous locations in the tropics[4]. The benefits for the treatment or prevention of disease or infection that may accrue from either dietary or topical administration of *Moringa* preparations (*e.g.* extracts, decoctions, poultices, creams, oils, emollients, salves, powders, porridges) are not quite so well known[5]. Its seeds have shown analgesic[6] and antipyretic activities[7]. Its leaves have shown wound healing[7], analgesic[8], hepatoprotective[9,10], antiulcer[11], hypotensive[12] and diuretic activities[13]. Roots have shown antifertility activity[14]. Various phytoconstituents have been isolated from seeds as alkaloid (moringines) by Agrawal *et al*[15], from flowers quarcetin, kaempferol by Selvakumar *et al*[9] and from leaves thiocarbamate by Murakami *et al*[16]. The *in-vivo* lethality in a simple zoological organism such as the brine shrimp lethality test (BST), developed by Meyer *et al*[17], might be used as a simple tool to guide for cytotoxic activity. Since ancient times, the medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities.

As antioxidants have been reported to prevent oxidative damage caused by free radical, it can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals and also by acting as oxygen scavengers[18]. The potentially reactive derivatives of oxygen, attributed as reactive oxygen species (ROS), are continuously generated inside the human body. The generated ROS are detoxified by the antioxidants present in the body. Recently there has been a rise of attention in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Besides well identified and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices, some natural antioxidant (*e.g.* rosemary and sage) are already exploited commercially also as antioxidant additives or a nutritional supplements[19]. Also many other plant species have been investigated in the search for novel antioxidants[20-23] but generally there is still a demand to find more information concerning the antioxidant potential of plant species. It has been mentioned the antioxidant activity of plants might be due to their phenolic compounds[24]. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action[25]. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity[26]. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1-diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases[27]. Derived polyphenols from plants are of great importance because of their potential antioxidant and antimicrobial proper ties. Phenolic compounds exhibit a considerable free radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen or electron-donating agents, the stability of the resulting antioxidant derived radical, their reactivity with other antioxidants and finally their metal chelating properties[27-29].

2. Materials and methods

2.1. Plant material

Stem bark of *M. oleifera* (Moringaceae) was collected from local region of Nashik, India in October 2008. The plant material was identified and authenticated by Dr. PG Diwakar Botanical Survey of India, Pune (Ref no. BSI/WC/Tech/2009/370).

2.2. Preparation of extract

The plant materials were cleaned, dried under shade and pulverized by using grinder. The powder of plant (500 g) was in succession extracted with petroleum ether, chloroform, and methanol in order of their rising polarity using Soxhlet apparatus. The yield of extracts obtained as petroleum ether as 0.89%, chloroform as 3.6%, methanol as 16.63%. Preliminary phytochemical study revealed the occurrence of sterols, glycosides, alkaloids, triterpenoids, flavonoids and tannins in the extracts.

2.3. Brine shrimp lethality

The *in-vivo* lethality in a simple zoological organism such as the brine shrimp lethality test (BST), developed by Meyer *et al*[17], might be used as a simple tool to guide for cytotoxic activity. Brine shrimp eggs were collected from Department of Fisheries, Government of Maharashtra, India.Brine shrimp eggs were placed in artificial sea water (3.8% w/v NaCl in distilled water) and incubated at 24-28 ℃. Eggs were hatched for 48 hours providing large number of larvae (nauplii). Ten nauplii were placed in 5 mL of sea water and different concentrations were prepared and placed in vials. Alive nauplii were counted after 24 hours and lethal concentration (LC_{50}) was calculated.

2.4. Antioxidant activity

2.4.1. DPPH free radical scavenging assay

2, 2-diphenyl-1-picryl-hydazyl (DPPH) is widely used to test the ability of compounds as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of phyto-constituents. DPPH is nitrogen centered free radical. It reacts similar as peroxyl radial. The reaction rates directly correlate with antioxidant activity. The odd electron in DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptivity of DPPH radical at 517 nm reduces when odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolouration is stochiometric with respect to number of electrons captured[30] (Figure 2).

Ascorbic acid was used as standard. Percentage inhibition was calculated using formula: % inhibition = $[(A_{blank} - A_{test})/A_{blank}] \times 100$ (A is absorbance).

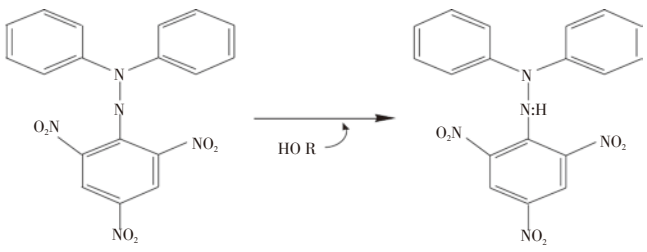

Figure 2. The derivation from DPPH to DPPH-H

2.4.2. Nitric Oxide Radical Scavenging Assay

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions. Scavengers of nitric oxide compete with oxygen and reduce the production of nitric oxide. Nitric oxide scavenging activity was performed by sodium Nitroprusside-Griess reagent. In this method sodium nitroprusside (1mM) in phosphate buffer saline solution was mixed with different concentration of extracts solution in methanol and incubated at 37 ℃ for 150 min. Blank solution was also prepared. After incubation 0.5 mL of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid add 0.1% N-(1-naphthyl)-ethylenediamine hydrochloride) was added. The absorbance was taken at 546 nm. Ascorbic acid was used as standard. Percentage inhibition was calculated as per above formula. IC_{50} was calculated for each extract[31,32].

2.5. Estimation of Total phenolic content[32,33]

Total phenolic content of methanol extracts of *M. oleifera* was evaluated with Folin-Ciocalteu method. The Folin-Ciocalteu reagent is sensitive to reducing compounds, polyphenols there by producing blue colored complex. The quantative phenolic estimation was performed at max 765 nm by change in intensity of Folin-phenolic compounds complex. To prepare a calibration curve 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mL of the gallic acid, stock solution was transferred to 100 mL flasks, and then diluted with water to produce gallic acid solutions, producing concentrations of 0, 25, 50, 7L of sodium carbonate solution was added in each flask and volume was adjusted with distilled water. Readings were taken after 1 hr at 765 nm by U.V. Spectrophotometer 1650 Shimadzu, Japan against reagent blank. The calibration curve of absorbance vs concentration was plotted. 1mL of stock solution of extracts was transferred in 25 mL flask; similar procedure was adopted as above described in preparation of calibration curve. With the help of calibration curve, the phenolic concentration of extracts was determined.

3. Results

3.1. Phytochemical screening

The crude petroleum ether, chloroform and methanolic extract of *M. oleifera* stem bark was qualitatively tested for the presence of sterols, glycosides, alkaloids, triterpenoids,

flavonoids, anthraquinones, carotenoids, tannins and the results were given in Table 1.

Table 1
Preliminary phytochemical study of stem bark extracts of *M. oleifera*.

Phytochemical components	Petroleum ether	Chloroform	Methanol
Test for Sterols	+	−	−
Test for Glycosides	−	−	+
Test for Alkaloids	−	+	−
Test for Triterpenoids	+	−	−
Test for Flavonoids	−	−	+
Test for Anthraquinones	−	−	−
Test for Carotenoids	−	−	−
Test for Tannins	−	−	+

+: present; −: absent

3.2. Cytotoxicity studies

In BSL bioassay, crude petroleum ether, chloroform and methanolic extract of *M. oleifera* stem bark showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations. The LC_{50} (μg/ml) values obtained for extracts as 850 μg/ml for petroleum ether extract, 800 μg/ml for chloroform extract and 900 μg/ml for methanol extract.

3.3. Antioxidant activity by DPPH free radical scavenging assay

DPPH radical scavenging ability is widely used as an index to evaluate the antioxidant potential of medicinal plants. *In vitro* antioxidant studies of the three extracts, the extent of DPPH radical scavenging at different concentrations (25−100 μg/ml) of *M. oleifera* extracts was measured, with ascorbic acid as the standard. The radical scavenging effect was found to increase with increasing concentrations. The control and the plant extracts showed their maximum activity: control (91.93 %), methanol (78.49%), chloroform (50.68 %), petroleum ether (34.14 %) with IC_{50} values of 13.86 μg/mL, 54.34 μg/mL, 112.08 μg/mL, 124.75 μg/mL respectively (Table 2).

3.4. Antioxidant activity by nitrous oxide free radical scavenging assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat[32]. *In vitro* antioxidant studies of the three extracts, the extent of NO radical scavenging at different concentrations (25−100 μg/mL) of *M. oleifera* extracts was measured, with ascorbic acid as the standard. The radical scavenging effect was found to increase with increasing concentrations. The control and the plant extracts showed their maximum activity: control (93.78 %), methanol (67.35%), chloroform (60.98%), petrolum ether (40.12%) with IC_{50} values of 12.59 μg/mL, 54.34 μg/mL, 65.12 μg/mL, 93.32 μg/mL respectively.

Table 2
DPPH and nitric oxide free radical scavenging activity of extracts of *M. oleifera*.

Test component	Concerntration (μg/mL)	Inhibition(%)	
		DPPH	Nitric oxide
Petroleum ether Extract	25	17.18	10.12
	50	25.89	20.45
	75	29.91	34.85
	100	34.14	40.12
Chloroform Extract	25	13.65	17.12
	50	25.58	32.47
	75	38.24	49.58
	100	50.68	60.98
Methanol Extract	25	20.63	15.14
	50	34.12	35.25
	75	45.73	50.68
	100	78.49	67.35
Ascorbic acid	5	15.64	25.17
	10	34.51	48.29
	15	51.45	63.18
	20	73.87	78.19
	25	91.93	93.78

3.5. Estimation of total phenolic content

Total phenolics content in methanolic extract of *M. oleifera* was found to 50.72% w/w, equivalent to gallic acid. Result is shown in Figure 3.

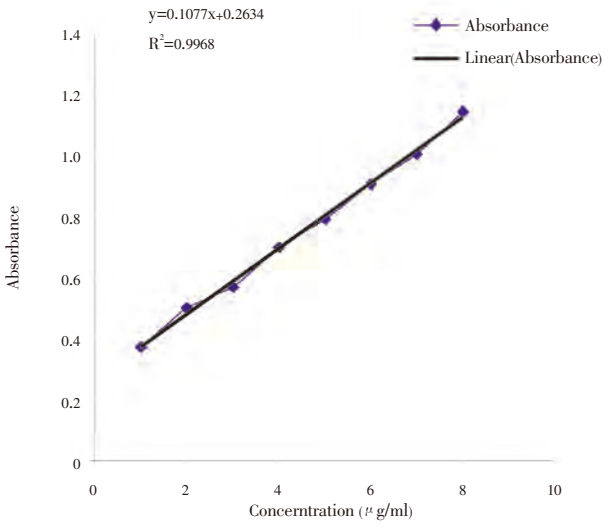

Figure 3. Calibration curve for gallic acid

4. Discussion

The crude petroleum ether, chloroform and methanolic extract of *M. oleifera* stem bark was qualitatively tested for the presence of sterols, glycosides, alkaloids, triterpenoids, flavonoids, anthraquinones, carotenoids, and Tannins. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity[37]. Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods existing which eradicate needless separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities[38]. In India the Ayurvedic system of medicines has been used for more than 3 000 years. Charaka and Susruta, developed samhitas based on herbal sources and is still valued even in this day as wealth of indigenous medicines. These indigenous medicines are favored above allopathic medicines since the latter cause lot of side effects due to its synthetic character. The selection of today's therapy is therefore investigation of plant drugs. Nevertheless, due to the over exploitation of the medicinal plants, many of them have become scarce. Plant biotechnology has played a vital task in the mass multiplication for conservation and also to procure numerous content of drugs.

The BSL assay represents a fast, economical and easy bioassay for testing plant extracts bioactivity which in the majority cases correlates reasonably well with cytotoxicity and anti-tumor properties[39]. In this paper, the BSL of extracts of stem bark of *M. oleifera* which is used in traditional medicine, was determined following the modified method of Solis *et al*[38]. Cytotoxic property by plant material is due to the presence of antitumor compounds[39]. Cancer is the main killer disease in most developed as well as developing countries, which is induced by oxidative stress[40,41]. Hence antioxidants which are very effective in combating cancer need thorough search especially safer compounds from plant sources. Increased oxidative stress encountered in body due to either environmental hazard, or impairment in the body metabolism due to varying disease conditions including drugs or having insufficient amount of dietary antioxidants, has to be curbed by exogenous supply of antioxidants as a choice of therapy or preventive measure. Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. Natural antioxidants are preferred in allopathic drugs to overcome the side effects. Most of the polar compounds such as phenolic and flavonoid substances are potent inhibitors of reactive oxygen species attack[42]. Phenolic and flavonoids also show cytotoxicity in Hoechst 33258 fluorescence assay by inhibiting cellular DNA in a concentration-dependent manner[43]. The biological properties, including cytotoxic and antioxidant properties, of flavonoids are considered in an evaluation of the medicinal and nutritional values of these compounds[44]. The antioxidant activity has correlation with total phenolic content. Methanolic, chloroform and petroleum ether extracts at various concentrations ranging from 25-100 μg/mL were tested for their antioxidant activity using DPPH radical scavenging assay method. DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. The results revealed that the methanolic extract of the species exhibited the highest radical scavenging activity with 78.49% followed by its chloroform extract with 50.68% and for petroleum, ether extract 34.14%. The DPPH radical scavenging activities of extracts increased gradually in a dose dependent manner. Smaller IC_{50} value corresponds to a higher antioxidant activity of the plant extract[45].

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through intermediates through NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. Free radicals are constantly produced in the living system, which can cause an extensive damage to bio-molecules and tissues thereby causing various diseases like extensive lysis and degenerative diseases[46]. The results obtained are shown in Table 4 and it indicates that the crude methanolic extract (67.35 %) , chloroform extract (60.98 %) and petrolum ether extract (40.12 %) of the plant possessed moderate no free radical scavenging activity. The no free radical scavenging activity was increased by increasing the concentration of the test samples.

Plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers. Therefore, it was reasonable to determine the total phenolic content in the plant extract. The result shows that the phenolic content of *M. oleifera* stem bark is higher and the radical scavenging activity is likely to be due to the phenolics, however, phenols may not be solely responsible for the antioxidant activity. In general, extracts with high antioxidant activity show a high phenolic content. Plant extracts with high phenolic contents also show high flavonoid content as reported for other plant species[47]. ROS have been considered to cause harm to living organisms and thus play a significant role in many human diseases such as arthritis, myocardial infarction , atherosclerosis, diabetes mellitus and cancer [48-51]. Phenols are a class of low molecular weight secondary metabolites found in most land plants. Phenol compounds have some

antioxidant activity[52, 53]. They are able to terminate free radicals and chelate metal ions that are capable of catalyzing formation of ROS that promote lipid peroxidation[54]. Phytochemicals have been of huge interest as a supply of natural antioxidants used for health promotion, food preservation, food flavoring and cosmetics as they are safer than synthetics[55]. The antioxidant activities of different extracts of *M. oleifera* are in accordance with their amount of phenolics contents. Currently there has been an increased interest worldwide to identify antioxidant compounds from plant sources which are pharmacologically potent and have small or no side effects for use in protective medicine and the food industry. Increasing acquaintance in antioxidant phytoconstituents and including them in daily uses and diet can give sufficient support to human body to fight those diseases. Phytochemical analysis reveals the presence of tannins, flavonoids, steroids and alkaloids. This study affirms the *in vitro* antioxidant potential of crude methanolic, chloroform and petrolum ether extracts of the stem bark of *M. oleifera* ,with results comparable to those of the standard compounds such as ascorbic acid. BSL assay is very useful and inexpensive way of assessing the bioactivity of plant extracts. These extracts can be regarded as a promising candidate for a plant derived antitumor agent.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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