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Full Length Research Paper

Comparative study of moringa leaves from different regions on antioxidant and hemolytic activity

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Moringa oleifera **is one of the 14 species of family Moringaceae and is commonly known as 'Drumstick tree' or the 'horse radish tree'. It is a medicinal Indian herb which is rich in secondary metabolites such as terpenoids, flavonoids, phenolics etc. It has the following properties such as- antitumor, antipyretic, anti-inflammatory, antihypertensive, cholesterol lowering, anti-atherosclerotic, anti-diabetic, hepatoprotective, antibacterial and antifungal activities. Despite of above mentioned application, this study showcases the antioxidant and hemolytic activities. Leaves from three different region exhibits antioxidant activity - DPPH and MDA assays. The results obtained for antioxidant property DPPH was found to be higher in the samples collected from Bengaluru and for the MDA Assay we obtained higher values in the Rajasthan sample. Furthermore, leaf extracts are non-toxic to RBC in lower concentration.**

Keywords: *Moringa oleifera*, antioxidant, direct hemolytic activity, recalcification time

INTRODUCTION

Moringa oleifera is a type of local medicinal Indian herb which has turn out to be familiar in the tropical and subtropical countries (Franklin and Snow, 1989). The leaves are the most nutritious part of the plant, being a significant source of B vitamins, vitamin C, provitamin A as beta-carotene, vitamin K, manganese and protein. Traditionally, it is used to treat many diseases throughout the world and many of them are scientifically proved, which mainly include; antitumor, antipyretic, antiepileptic, antiinflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, anti-atherosclerotic, anti-diabetic, hepatoprotective, antibacterial and antifungal activities and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia[3].

Moringa oleifera Lam (Marum) is one of the most widely cultivated plants in the Moringaceae family which can be used as vegetable and medicinal plants. Moreover, *M oleifera* leave could serve as the nutritional source of some vitamins and minerals such as calcium (Babu, 2000).

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Moringa is one such genus whose various species have not been explored fully despite the enormous reports concerning the various parts of a few species' potentials such as: cardiac and circulatory stimulants; antitumor; antipyretic; antiepileptic; anti-inflammatory; antiulcer; antispasmodic; diuretic antihypertensive; cholesterol lowering; antioxidant; anti-diabetic; hepatoprotective; antibacterial and antifungal activities. These are also being used for treatment of different ailments in the indigenous system of medicine. The Moringa species are currently of wide attention because of their outstanding economic potential. Amongst these species, *M oleifera* is the most ubiquitous for its nutritious and numerous medicinal uses that have been appreciated for centuries in many parts of its native and introduced ranges. Almost all the parts of this plants have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepato-renal disorders (Bandana et al., 2000; Mahajan et al., 2007) . Leaves of *M oleifera* have been reported to regulate thyroid status and possess radioprotective (Sulaiman et al., 2008) and antitumor activities (Rathi et al., 2006). Fruits are found to have hypocholesterolaemic activity in Wistar rats and rabbits, respectively (Jain et al., 2010 Chumark et al., 2008).

Pod has been reported to shown hypotensive effect (Karadi et al., 2006). Seeds have been reported for coagulative, antimicrobial and antitumor activity (Das et al., 2011). Roots possess antimicrobial and antiinflammatory activity (Caceres et al., 1991). *M oleifera* Lam. has been reported to contain various phytochemicals which includes carotenoids, vitamins, minerals, amino acids, sterols, glycosides, alkaloids, flavonoids and phenolics (Karadi et al., 2006; Dahiru et al., 2006).

Moringa leaves and pods are helpful in increasing breast milk in the breastfeeding months. A measure of one tablespoon of leaf powder provides 14% of the protein, 40% of the calcium, 23% of the iron and most of the vitamin A needs of children aged 1-3 years. A measure of six tablespoons of leaf powder will provide nearly all of the woman's daily iron and calcium needs during pregnancy and breastfeeding. It has been optimised and reported that 25 g daily of the Moringa leaf powder will give a child the following recommended daily allowances: protein 42%, calcium 125%, magnesium 61%, potassium 41%, iron 71%, vitamin A 272% and vitamin C 22%. Scientific research confirms that the Moringa leaves are a powerhouse of nutritional value. Vitamin content in the Moringa leaves is comparable with most of the fruits that we regularly have in our diet (Arun and Subha, 2012).

MATERIALS AND METHODS

Extraction of the Sample: Fresh leaves of moringa oleifera were shade dried and powdered with the help of a mixer.10% aqueous, methanol and ethanol-water extracts were prepared. The solutions were centrifuged at 8000 rpm for 15 minutes at room temperature and were filtered using Whatmann No.1 filter paper. These were further subjected to analysis. The leaf samples were collected from Malleshwaram, KR market and Rajasthan. The chemicals and solvents required were provided by the laboratory, Department of Biochemistry, Mount Carmel College, Bangalore, India.

Phytochemical Screening: Phytochemical evaluation of leaf extracts of *Moringa oleifera* was conducted with methanol, ethanol-water in a ratio 8:2 and water to check the solubility and activity. 10% extract of methanol, ethanol-water and water was prepared and centrifuged at 8000 rpm, 15 mins, and 24°C. These solutions were filtered using Whatmann No 1 filter paper. The obtained clear supernatants was subjected to various phytochemical tests like carbohydrates, proteins, alkaloids, glycosides, tannins/phenolics, flavonoids and terpenoids (See table 1).

Estimation of proteins by Lowry's method: Aliquots of standard BSA solution was taken (0-1 mL) which was made upto 1 mL using distilled water. 5 mL of alkaline copper reagent was added to the tubes and it was incubated at room temperature for 10 minutes. 0.6 mL of 1:1 Folin Ciocalteu Reagent was added to the tubes and the tubes were incubated at room temperature for 30 minutes. The absorbance was recorded at 660 nm. The plant extract with varying aliquots (25-100 µL) were taken and the above procedure was followed.

MDA- Measure of free radical activity: Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, the most abundant of which is malondialdehyde (MDA). MDA is a highly reactive three carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. MDA readily combines with several functional groups on molecules such as proteins, lipoproteins, and DNA. MDA- modified proteins may show altered physico-chemical behavior and antigenicity (Requena M *et al.* 2000). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation (Esterbauer H and Cheeseman K H. 1990). The oxidative destruction of polyunsaturated fatty acids is known as lipid peroxidation. It proceeds as a self- perpetuating chain reaction. The damage inflicted by these ROS is referred to as oxidative stress which increases the production of free radicals. Both the oxidative stress and the production of ROS have been implicated in the pathogenesis of a variety of disease states, like cancer and cardiovascular disease (Brown M S and Goldstein J L 1983). The purpose of our study was to assess and compare the level of MDA in patients with coronary heart disease (CHD) and in other stress related disorders and to check the effect of antioxidants on them (Kavitha et al., 2014).

Reagents: 1,1,3,3-Tetramethoxypropane (MDA) working standard solution, 0.5% TBA Solution- 500 mg of thiobarbituric acid was dissolved in 100 mL of distilled water, 5 M HCl solution -59.6 mL of distilled water was dissolved in 40.38 mL of concentrated hydrochloric acid, Glacial acetic acid.

Methodology: 10µL- 200µL aliquots of MDA working standard solution were pipetted out into different test tubes and the volume in each case was made up to 5 mL with distilled water. To each of test tube 5 mL of glacial acetic acid and 0.5 mL of 0.5% TBA was added. The test tubes were kept for incubation in boiling water bath for 45 minutes. The test tubes were then cooled, and to this 0.05 mL of 5 M HCl was added. A suitable

Table 1. Phytochemical screening

Sl no	Region	Malleshwaram			KR market			Rajasthan		
	Test	Aqueous Extract	Ethanol-water Extract	Methanol Water	Aqueous Extract	Ethanol-water Extract	Methanol Water	Aqueous Extract	Ethanol-water Extract	Methanol Extract
1	Alkaloids	+	+	+	+	+	+	+	+	+
2	Flavonoids	+	+	+	+	+	-	+	-	-
3	Protein	+	+	-	+	+	-	+	+	-
4	Carbohydrates	+	+	+	+	+	+	+	+	+
5	Terpenoids	+	+	+	+	-	-	+	+	+
6	Steroids	-	+	+	-	+	+	+	+	+
7	Coumarin Glycosides	+	+	+	+	-	+	+	+	+
8	Cardiac Glycosides	+	+	+	-	+	+	+	+	-
9	Tannins	+	+	+	+	+	+	+	+	+
10	Phenols	+	+	+	+	+	+	+	+	+

blank was prepared containing 5 mL of distilled water, 0.5 mL of glacial acetic acid, 0.5 mL of 0.5% TBA solution and 0.05 mL of 5M HCl. The absorbance was read at 535 nm using a colorimeter.

DPPH method: DPPH (2, 2-diphenyl-2-picrylhydrazyl) is a stable red colored free radical that absorbs at 517 nm. If free radicals are scavenged, DPPH change its color to yellow due to decolourisation (reduction). This assay uses this character to show free radical scavenging activity. Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. As this electron gets paired in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolourisation is stoichiometric with respect to the number of electrons captured (Sharma O P and Bhat T K. 2009). The stock solution was prepared using 0.1g ascorbic acid in 100 mL of distilled water (1000 µg/ mL). The stock was diluted suitably in order to obtain different concentrations of ascorbic acid ranging from 10 µg-100 µg. The plant sample was taken of varying aliquots (5-200 µL). To all the above tubes 3 mL of DPPH (20 µg/ml) was added. The mixture was incubated at room temperature for 10 minutes and the absorbance was recorded at 517nm (Kavitha et al., 2014)..

Recalcification time: To 1 mL of trisodium citrate, add 9 mL of blood (freshly drawn). Centrifuge the tubes at 900-1000 rpm for 12 minutes. The supernatant obtained (plasma) is used. 100 µL of plasma is added to 10 µL of tris buffer to different test tubes and kept in the incubator for 37℃. A control is set up using 10 µL of 0.25 M Calcium Chloride to the plasma and the time was noted for the clot to be formed. The sample was prepared using volumes from (2 µL-25 µL) and incubated for 1 minute. To this 10 µL of calcium chloride was added and the time was recorded for the formation of the clot (Lakshmi et al., 2014).

Direct Hemolytic Activity: 1 mL of 3.2% trisodium citrate was added to 9 mL of blood. This was centrifuged at 1000 rpm for 12 minutes. The pellet obtained was resuspended in equal volumes of phosphate buffer saline (pH 7.4). This was centrifuged at 3000 rpm for 10 minutes. The pellet was washed 2-3 times using phosphate buffer saline. The hematocrit was obtained in the form of pellet which was resuspended in phosphate buffer saline (1:9) dilution. 1 mL of this mixture was used for carrying out the assay. The blank was prepared using the RBC and water which was also used as the positive control. Phosphate buffer saline was used as the negative control. To 1 mL of hematocrit aliquots of sample (50-200µL) was added and incubated at 37℃ for 30 minutes.5 mL of buffer was added after incubation and the solution was read at 540 nm (Lakshmi et al., 2014).

RESULTS

DPPH Assay: From Figure 1, highest DPPH scavenging activity was found in Malleshwaram sample

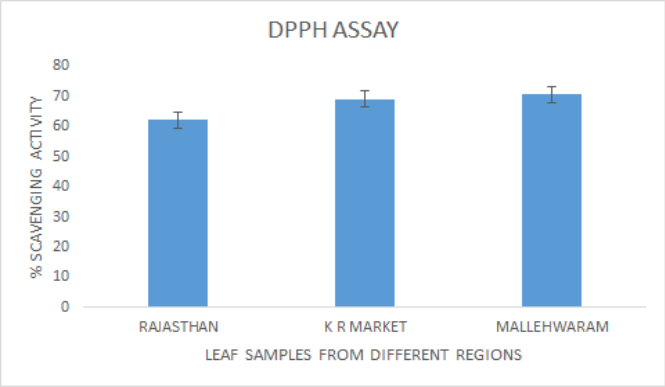

Figure 1. Estimation of scavenging activity by DPPH Assay

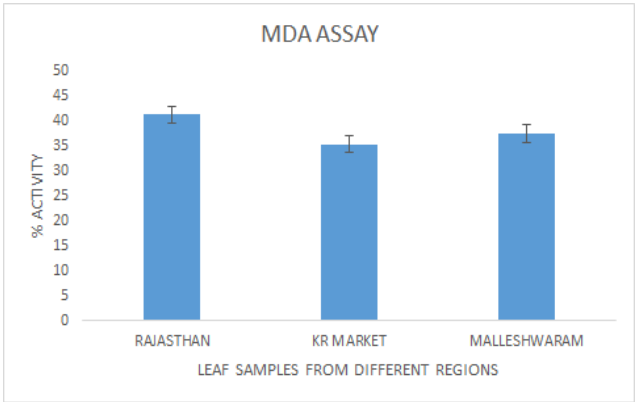

Figure 2. Lipid peroxidation activity by MDA Assay

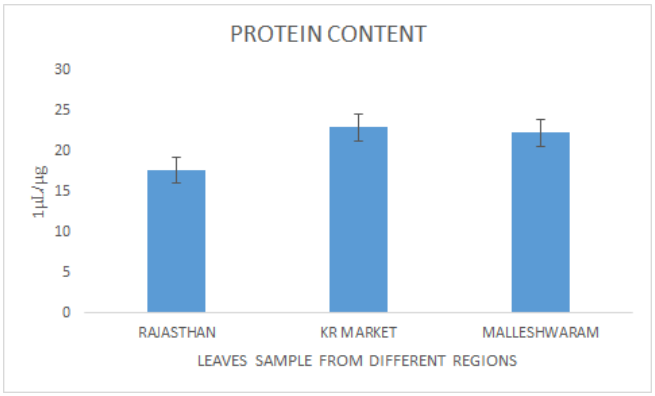

Figure 3: Estimation of protein content by Lowry's method

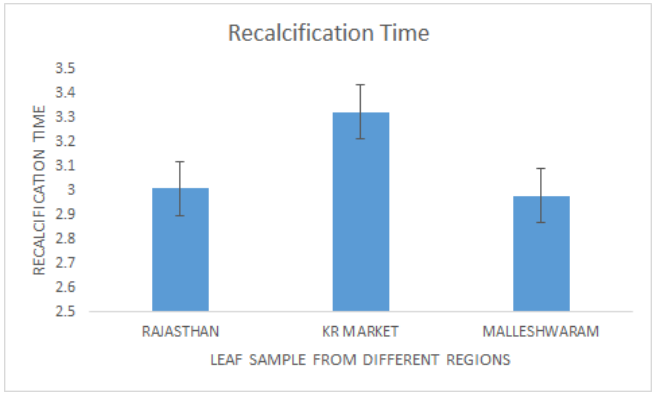

Figure 4: Recalcification time

followed by KR market and the least activity was found in Rajasthan.

MDA Assay: *From the above mentioned data (Figure 2), the Lipid Peroxidation activity was found higher in Rajasthan sample followed by Malleshwaram, and the least activity was found in KR Market.*

Protein estimation by Lowry's method: *24.5mg/µL of protein content was found in KR Market sample followed by 22.8 mg/µL of protein content was found in Malleshwaram and 17.7 mg/µL of protein content was found in Rajasthan (Figure 3).*

Recalcification time: The time taken for recalcification of the samples with lower volume was found to be a procoagulant. With increasing volume of the extract it was found to act as an anticoagulant (Figure 4). The sample collected from Malleshwaram was found to give better coagulating activity followed by Rajasthan and comparatively higher activity was found in KR Market sample.

Direct hemolytic assay: Control was taken as RBC and Water, the negative control was taken as saline. The sample collected from KR Market was found to have the least hemolytic activity hence, its toxicity was comparatively less (Figure 5).

DISCUSSION

Reactive oxygen species (ROS) are the most common natural byproducts of metabolic pathways that takes place in the body. ROS are chemically reactive species that causes cell damage and cell death (apoptosis). To prevent the oxidation chain reaction, antioxidants must be active to quench the free radicals and render them inactive (molecular form). The antioxidant assay was carried out by the DPPH method. In this method DPPH is reduced to diphenyl picrylhydrazyl, the rate of which determines the radical scavenging potential of the sample. The results revealed that ethanol water extract of the moringa leaves from Malleshwaram gave a higher antioxidant activity when compared to other sample extracts.

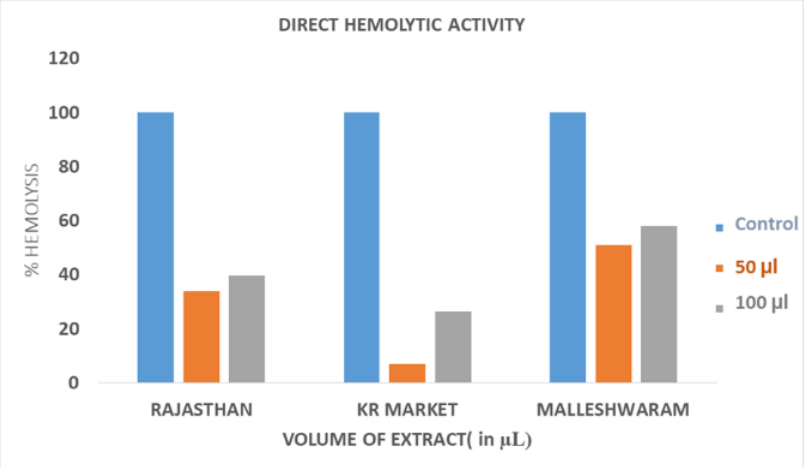

Figure 5: Direct hemolytic assay

Another method carried out to check for antioxidant properties was by lipid peroxidation assay. In this assay thiobarbituric acid is used for the detection of malondialdehyde (MDA). MDA is a natural byproduct of lipid peroxidation which causes oxidative stress and apoptosis. Results obtained showed that the ethanol water extract of Rajasthan had the maximum antioxidant activity when compared to other sample extracts.

Hemolysis is characterized by lysis of RBCs due to which there is a reduction in transport of oxygen. The samples were subjected to an assay that helps in knowing whether the samples have an adverse effect on the blood cells. The RBCs after centrifugation were subjected to different sample extracts. The spectrophotometric analysis was carried out at 540 nm. From the results obtained it was revealed that the ethanol-water extract of KR market to be of least value.

The study demonstrated a higher antioxidant activity in Malleshwaram sample (DPPH Assay) and Rajasthan sample (MDA Assay). KR Market sample exhibited a lower recalcification time when compared to other samples. The hemolytic assay can be further carried at the molecular level.

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