Vol. 12/2 (2012) 151-156

JOURNAL OF NATURAL REMEDIES

A comparative study of antioxidant potential of successive chloroform and methanolic extracts from leaves of *Mitragyna parvifolia*.

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Abstract

The tree *Mitragyna parvifolia* (Rubiaceae), commonly known as Kadamb, belongs to a small genus comprising of 10 species. The plant has been used in folklore medicine since times immemorial. It possess diverse medicinal and therapeutic properties such as analgesic, antipyretic, anti-inflammatory, antiarthritic, antinociceptive, anticonvulsant etc which have been proved scientifically. The leaves were subjected to successive extraction using solvents in increasing order of polarity. Working dilutions of 20, 40, 60, 80 and 100µg/ml of successive chloroform and methanolic extracts were prepared. Similar working dilutions of the standard, L-ascorbic acid were also prepared. The dilutions of the test extracts and standard were subjected to different antioxidant assays (radical scavenging assay and reducing power assay) and for estimation of total phenolic content. The antioxidant activity for all dilutions of successive methanolic extract was found to be higher when compared to that of successive chloroform extract. The IC_{50} values for the successive chloroform and methanolic extract were found to be >100mcg/ml and <20mcg/ml respectively whereas the total phenolic content in the successive chloroform and methanolic extract was found to be 44 and 160 mg of GAE/g of dry extract. Thus, from the results obtained it may be concluded that high levels of antioxidant activity in the successive methanolic extract may be attributed to the high phenolic content found in the same extract.

Keywords: Radical scavenging assay; reducing power assay; total phenolic content; *Mitragyna parvifolia.*

1. Introduction

Molecular oxygen is essential for the survival of all, but the process of oxygen utilization in normal physiological and metabolic processes reduces oxygen to oxygen derived free radicals like superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals. All these radicals known as reactive oxygen species (ROS), exert oxidative stress towards the cells of human body [1]. Under normal conditions, the ROS generated are detoxified by the antioxidants present in the body as there is a balance between the ROS generated and the antioxidants present. But in case of ROS overproduction this equilibrium is hindered favoring the ROS gain and accumulation in the body. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids and nucleic acids [2]. This oxidative process thus leads to a number of physiological disorders. These have been implicated in the pathogenesis

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of diabetes, liver damage, nephrotoxicity, inflammation, cardiovascular disorders, neurological disorders and in the process of aging [3,4]. Several studies demonstrate that a great number of medicinal and aromatic herbs, as well as fruits and leaves of some plants biosynthesize phytochemicals possessing antioxidant activity and may be used as a natural source of free radical scavenging compounds [5]. Almost all plants and their parts possess antioxidant activity of which some examples may be cited as *Morinda citrifolia* [6], *Byrsonima crassifolia, Davilla kunthii, Davilla rugosa, Inga edulis* [7], *Cordia dichotoma, Luffa aegyptiaca, Stevia rebaudiana, Brassica oleracea* etc.

Mitragyna parvifolia (Roxb) Korth is a medium to large size tree belonging to the family Rubiaceae [8]. It is popularly known as Kaim and is found growing gregariously throughout the drier parts of India, Pakistan and Srilanka. *M. parvifolia* has got diverse medicinal and therapeutic properties such as analgesic, antimicrobial [9], antipyretic, antiarthritic [10], anti-inflammatory, antinociceptive [11], anticonvulsant [12], antioxidant [13] etc. The antioxidant activity has been reported in the whole ethanolic extract of leaves [13] but extracts prepared by means of successive extraction have not been evaluated for antioxidant potentials. Thus, evaluation of successive extracts is being carried out here using multiple assay procedures besides determining their total phenolic content as several studies are available which correlate antioxidant activity with total phenolic content [14, 15].

2. Materials and Methods

2.1. Plant material

The leaves of *M. parvifolia* were collected during the month of July from Bhakra Nangal, Punjab. The leaves were authenticated from Forest Research Institute (FRI), Dehradun bearing a voucher specimen no 172/2011-Bot-15-1 and was deposited in the institution for future reference. The collected leaves were washed, shade dried and ground to a coarse powder using a mechanical grinder.

2.2. Extract preparation

The leaf extracts were prepared by successive extraction using solvents in increasing order of polarity. Dried leaf powder (1.5 kg) was extracted using petroleum ether (60-80) followed by extraction with chloroform, ethyl acetate, acetone and methanol in a soxhlet extractor. The various extracts prepared were concentrated using a rotary vacuum film evaporator at about 40°C and stored in a refrigerator at about 4° C till further usage.

2.3. Preliminary Phytochemical Screening

The prepared extracts were subjected to different qualitative phytochemical screening tests to evaluate the chemical constituents present in the leaves [16].

2.4. Antioxidant Assays

2.4.1. Radical Scavenging Assay (RSA)

This assay procedure employs the use of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical (HIMEDIA, INDIA). The method employed was according to the procedure of Kaushik *et al* [13]. Solution of DPPH (0.1mM) in methanol was prepared and kept in darkness for 30 minutes. The stock solution (1 mg/ml) of the standard (Ascorbic acid) and the samples (successive chloroform and methanolic extract) were also prepared in methanol. Working dilutions (20, 40, 60, 80 and 100 µg/ml) were prepared from the stock solution. 1ml of various dilutions of standard/ samples were added to 5ml of prepared DPPH solution (0.1mM). Control solution was also prepared in similar manner adding 1ml of methanol instead of the

standard/sample. The contents of all flasks were shaken vigrously and allowed to stand in dark for 30 minutes following which the absorbances were measured at 517 nm against a blank (methanol) on a UV-Visible spectrophotometer (Shimadzu UV 1650 PC).

The radical scvanging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\% \text{ RSA} = 100 \text{ X } (1 - A_T / A_C)$$

A_T : Absorbance of DPPH solution containing test sample/ standard.

A_C : Absorbance of DPPH solution without sample or standard (control).

The assay procedure was carried out in triplicate.

2.4.2. Reducing Power Assay

The assay procedure employed was according to the method of Kaushik *et al* [13]. The stock solution (1 mg/ml) of the standard (Ascorbic acid) and the samples (successive chloroform and methanolic extract) were prepared in Phosphate buffer (pH= 7.4). The working dilutions (20, 40, 60, 80 and 100µg/ml) in buffer were prepared from the stock. 1ml of different dilutions (20, 40, 60, 80 and 100µg/ ml) of extract/standard was mixed with phosphate buffer (2.5 ml, pH 7.4) and potassium ferricyanide (2.5 ml, 1 %). The mixture was incubated at 50°C for 20 minutes. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture followed by centrifugation at 3000 rpm for 10min. Upper layer of the centrifuged solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared ferric chloride solution (0.5 ml, 0.1%). Absorbance was measured immediately at 700 nm against a blank solution prepared in a similar manner omitting standard/sample and ferric chloride solution. A similar procedure was repeated with distilled water which served as control. Ascorbic acid was used as standard. The assay was performed in triplicates.

2.4.3. Total Phenolic Content (TPC)

The stock solution (1 mg/ml) of the standard (Gallic acid) and the samples (successive chloroform and methanolic extract) were prepared in water : methanol (1:1) and water respectively. The working dilution (100 µg/ml) of samples was prepared from the stock. Total soluble phenolics in the successive chloroform and methanolic extract have been determined using the Folin- Ciocalteau reagent. The Folin-Ciocalteau's reagent for TPC assay was prepared by dilution with water in ratio 1:10. 1.0 ml of the extract solution was added to 1.0 ml of the Folin- Ciocalteau reagent and mixed throughly. Three minutes later, 3.0ml of a 2% sodium carbonate solution was added to the mixture which was allowed to stand for 3 hours with intermittent shaking. The absorbance of the prussian blue color that developed was measured at 760 nm. The concentration of the total phenolics present was expressed as mg of GAE /g of dry extract.

2.4.4. Statistical analysis

Results of the assays are expressed as mean value ± standard error mean (SEM).

3. Results

The IC_{50} values for the successive chloroform and methanolic extracts in radical scavenging assay was found to be >100 mcg/ml and <20 mcg/ml respectively. Results of the radical scavenging assay and reducing power assay for both the extracts have been summarized Table 1 and results of the total phenolic content assay have been presented in Table 2.

Table 1. Antioxidant activity of successive chloroform and successive methanolic leaves extracts of *Mitragyna parvifolia.*

Name of the Extract	Concentration(ig/ml)	DPPH Assay	Reducing Power Assay
Chloroform	20	15.9 ± 0.01	0.288 ± 0.02
	40	19.03 ± 0.02	0.292 ± 0.01
	60	20.64 ± 0.01	0.294 ± 0.01
	80	23.73 ± 0.03	0.325 ± 0.02
	100	25.71 ± 0.01	0.346 ± 0.04
Methanolic	20	51.78 ± 0.18	0.291 ± 0.06
	40	64.39 ± 0.18	0.300 ± 0.05
	60	74.90 ± 0.18	0.348 ± 0.03
	80	81.99 ± 0.36	0.469 ± 0.13
	100	95.96 ± 0.04	0.543 ± 0.01

Each value represents the mean±SEM, n=3.

Table 2. Results of the total phenolic content assay for successive chloroform and methanolic leaves extracts of *Mitragyna parvifolia.*

Extract	mg of GAE/g of dry extract
Successive chloroform extract	44.6
Successive methanolic extract	160.0

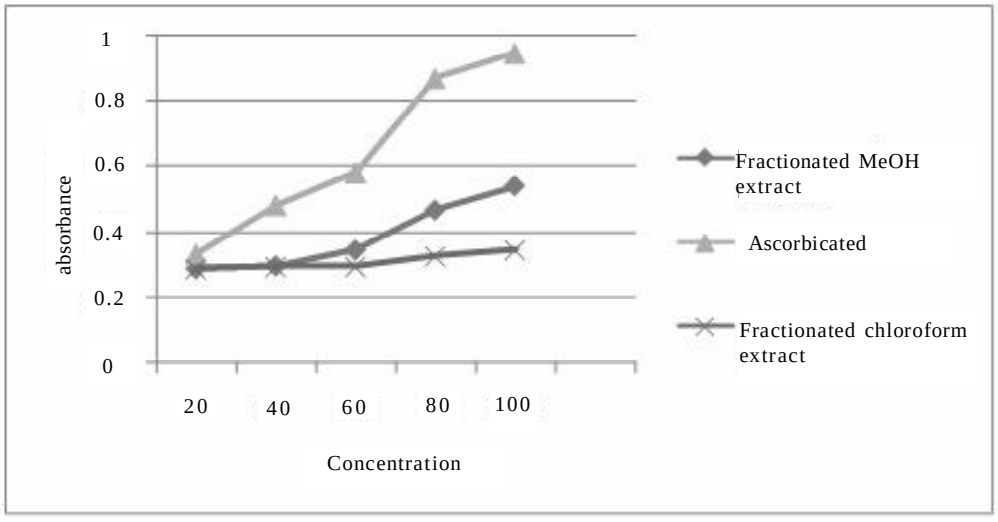
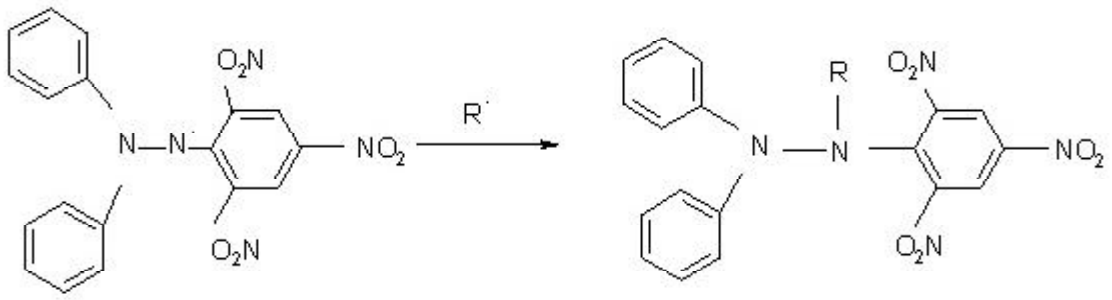


Figure-1 Reducing Power Assay



2,2-diphenyl-1-picrylhydrazyl (purple)

2,2-diphenyl-1-picrydrazine (yellow)

4. Discussion

The earlier literature reveals the presence of alkaloids, steroids, flavanoids, and glycosides in the leaves of the plant [10]. The phytochemical screening tests performed indicate the presence of polyphenolics and tannins in the leaves in addition to the previously reported phytoconstituents. The polar phytochemicals namely polyphenolics, flavanoids and tannins are believed to be associated with antioxidant activity. The DPPH assay is the most commonly employed model for assessing the antioxidant activity. DPPH is a nitrogen centered stable free radical which upon reaction with antioxidants undergoes conversion from 2,2-diphenyl-1-picrylhydrazyl to 2,2-diphenyl-1-picrylhydrazine accompained with a color change from purple to yellow.

The larger the decrease in absorbance of the reaction mixture, the higher the antioxidant activity in the test substance. The successive methanolic extract depicts a large change in color from purple to yellow with the increse in concentration and hence a potent antioxidant activity (Table 1).

Another model of assessing antioxidant activity is the determination of the reductive ability of the test sbstance by means of reducing power assay. In this assay the presence of antioxidants in the sample results in reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. Fe (III) reduction is often used as an indicator of electron-donating activity. The initial yellow colored solution changes to different shades of green and blue resulting from conversion of Fe^{3+}/ferricyanide complex to ferrous form accompained with increase in absorbance.

Antioxidants

Potassium ferricyanide, + Ferric chloride → Potassium ferricyanide + Ferrous chloride [17].

Increase in absorbance is therefore considered as an indicator of increase in reductive ability. In this case also the successive methanolic extract shows higher reductive ability as compared to successive chloroform extract (Table 1). The successive methanolic extract thus showed high antioxidant activity in both the assays as compared to the successive chloroform extract.

Plant polyphenols, are a diverse group of phenolic compounds which possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors.The polyphenol derived radical has the ability to stabilize and delocalize the unpaired electron (chain-breaking function), chelate metal ions (termination of the Fenton

reaction) [18], inactivate lipid free radical chains and prevent hydroperoxide conversion into reactive oxyradicals [3]. The amount of total phenolics present in the successive chloroform and methanolic extract was found to be 44.0 mg and 160.0 mg of GAE/g of dry extract respectively (Table 2). The successive methanolic extract showed high phenolic content in comparison to the successive chloroform extract. Thus, the successive chloroform extract which showed lesser antioxidant activity was also deprived of high phenolic content. On the other hand the high antioxidant activity in the successive methanolic extract was accompained with high polyphenolic content in the same extract. The above study thus proves the potentials of successive methanolic extract of leaves of *Mitragyna parvifolia* as an antioxidant substance. The confirmation as an antioxidant emphasizes the utility of the plant in treatment of various disorders (neuropsychatric and neurodegenerative ailments, inflammation, aging etc) for which free radicals is the underlying cause. Such findings thus suggest the importance of medicinal plants in future treatments of degenerative disorders. However studies regarding the isolation and purification of the compounds actually responsible for this activity need to be investigated.

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