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In-vitro Re-evaluation of Antioxidant activity by 2, 2-Diphenyl-1picrylhydrazyl Free Radical (DPPH) Assay in Medicinal Plants of Andhra Pradesh, India

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Abstract

Medicinal plants are a major source of raw materials on the globe from ancient to present century for the traditional system like Ayurveda, Siddha and Unani. Even the modern system of medicine has more than 25 percent of drugs in use which are either plant based or plant products. In day to day life, particularly in India people are suffering from menacing health diseases from common cold to amnesia and poisonous snake bites. These diseases can be treated by using some herbal extracts from plants which has antioxidant activity. Medicinal plants like *Azadirachta indica*, *Ocimum sanctum*, *Lawsonia intermis*, *Murraya koenigii*, *Curcuma longa* and *Cuminum cymium* belonging to different families play a vital role in day to day usage of different indigenous communities due to its sacred and medicinal value. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. In the course of finding potential antioxidant from plant source, six medicinal tree species belonging to different families has been selected. Leaves were dried and extracted with methanol solvent systems. Antioxidant activity using 1, 1-diphenyl-2-picryl hydrazyl radical scavenging assay, of six extracts from six genus of different families are reported and a comparison of the free radical scavenging ability of the extracts is emphasized. The highest percentage of 1,1-diphenyl-2-picryl hydrazyl free radical scavenging activity is found in Azadirachta *indica (*81.8% of inhibition) which shows antimicrobial, anti inflammatory and anticancerous properties where Ascorbic acid (68.5 µg/ml) is taken as standard for comparison.

1. Introduction

India has one of the richest plant medical cultures on the globe. The term antioxidant in the beginning was used to refer indicatively to a chemical that averted the consumption of oxygen (**Shahidi and Naczk, 2004; Tachakittirungrod et al., 2007**). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption (**Tomaino et al., 2005**). The recent growth in knowledge of free radicals and reactive oxygen species (ROS) in biology (**Bravo, 1998; Martinez-Valverde et al., 2000**) is producing a medical rebellion that promises a new age of health. Free radicals and related species have attracted a great deal of attention in recent years. They are mainly extracted from oxygen, ROS and nitrogen (reactive nitrogen species/RNS), and are generated in our body ,various endogenous systems, exposure to different physico chemical conditions or patho physiological states However ROS levels are elevated by exogenous factors such are pollution, smoke, radiation, pesticide, drug consumption. In the case of disturbed balance between formation of free radicals and antioxidant shielding (**Roginsky and Lissi, 2005**), there is an oxidative stress which can leads to development of various diseases (**Arganosa et al., 1998**), including cardiovascular diseases , diabetes, cancer, Alzheimer's diseases, retinal degeneration, ischemic dementia, neurodegenerative disorders aging, trauma, stroke, and infection. ROS are entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors. In addition to antioxidant enzymes, non-enzymatic molecules, including thio-redoxin, thiols, and disulfide-bonding plays an important role in antioxidant shielding systems. Antioxidant based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's diseases, and cancer have appeared during the last three decades.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Antioxidants shielding system (**Farombi et al., 2000; Jaffel et al., 2011**), act as radical scavenger, hydrogen donors, electron donor, peroxide decomposer, single oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. To provide maximum intracellular protection these scavengers are strategically compartmentalized thought the cell. According to some plant secondary products are of particular interest are Plant phenolics, polyphenolic, alkaloids, non-proteins amino acids,

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isothiocyanate, indoles phytosterols, carotenoids, chlorophyll derivatives.

1.1 Antioxidant Based on Defense Mechanism: These are of three types:

1. Prevention antioxidants- These suppress the free radical formation.

Ex. Enzymes such as peroxidase, catalase, lactoferrin, carotenoids, etc.

2. Radical scavenging antioxidants – These suppress the chain initiation reaction. Ex. Vitamin-C & Carotenoids.
3. Enzyme inhibitor antioxidants – These induce production and reaction of free radicals and the transport of appropriate antioxidants to appropriate active site.

1.2 Formation of Free radicals

Oxygen radicals **(Gilbert DL, 1981)** are generated from the triplet state oxygen by excitation or reduction. "Superoxide theory of oxygen toxicity" states that oxygen is toxic because it can form superoxide radical, peroxynitrite and hydroxyl radicals which can initiate autoxidation, polymerization and fragmentation.

Before we understand the working, it is necessary to have a brief idea about free radicals (**Roginsky and Lissi, 2005**). During a chemical reaction (oxidation), one reactant loses an electron and is called oxidant or free radical, while the other gains an electron. In living organisms oxygen in unstable from is the most common free radical. This is called Reactive Oxygen Species and is generated during various metabolic activities. Contaminants as well as normal metabolism of cell can change molecule into a free radical. The examples of ROS are OH, O_2, H_2O_2, O_3, HOCI, RO_2, and RO.

Any molecule can become a free radical by either losing or gaining an electron. Once initiated these free radicals get involved in chain reaction with stable types. The compounds thus formed have longer stability and in body and increase the potential for cellular damage. Free radicals damage the cell at the site of their operation causing serious disorders. Plaque may accumulate in arteries on oxidation. LDL Cholesterol functions as free radical and damages the free artery lining. It hampers the blood circulation which may lead to heart attack.

As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations. Free radical damage to protein can result in loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis. Redox signaling is a major area of free radical research that is attracting attention. ROS are an entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, inducing superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors.

2. Material and methods

2.1 Materials

2.1.1 Chemicals and Reagents

The chemicals and reagents obtained from the biochemistry laboratory for calculating the in-vitro antioxidant activity of six medicinal plants extracts are:

1, 1-Diphenyle-2-picryl hydrazyl (DPPH), Ascorbic acid, methanol, Sodium carbonate (Na2CO3), phosphate buffer (pH-6.6) and distilled water.

2.1.2 Apparatus

Absorbance spectrophotometery was carried out using a UV-VIS spectrophotometer. Wavelength scans and absorbance measurements were in 1ml quartz cells of 1cm path length. Beakers, test tubes, measuring cylinders, analytical balance, micro pipettes, motor and pestle, glass rods, centrifuge, pipettes, micro-tips, conical flasks, centrifuge tubes, incubator, hot air oven, test tube stand, magnetic stirrer, filter papers, foils, marker and shaker.

2.1.3 Plant materials

The plant leaves of *Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii,* the rhizome of *Curcuma longa* and the seeds of *Cuminum cyminum* are the medicinal plant materials collected from different regions for calculating the antioxidant activity by free radical scavenging assay.

Botanical Name : *Ocimum sanctum*
Family : *Lamiaceae*
Vernacular Name : Tulsi are Manjari, Krishna Tulsi, Trittavu, Tulshi and Thulsi
Part used : Leaves

Habitat: Tulsi is a heavy branched having hair all over. It attains the height of about 75 – 90 cm. It has round oval shaped leaves which are up to 5 cm long. The leaves are 2- 4 cm in length. Its seeds are flat. Its flowers are purple – creamish in colour. The Tulsi with the green leaves is called the Shri Tulsi and one with the reddish leaves is called the Krishna Tulsi. Its seeds are yellow to reddish in colour. Leaves of Tulsi contain very essential oil.

Medicinal properties: The fresh leaves of Tulsi are taken by the millions of people every day. The leaves gives relief in cold, fever, bronchitis and cough. "Modern scientific research offers impressive evidence that Tulsi reduces stress, enhances stamina, relieves inflammation, lowers cholesterol, eliminates toxins, protects against radiation, prevents gastric ulcers, lowers fevers, improves digestion and provides a rich supply of antioxidants and other nutrients. Tulsi is especially effective in supporting the heart, blood vessels, liver and lungs and also regulates blood pressure and blood sugar.

Botanical Name : *Azadirachta indica*
Family : *Maliaceae*
Vernacular Name : It is popularly known as the miracle tree. Nimba and Neem
Part used : Leaves

Habitat: Neem tree is found throughout India. It is a popular village tree. Neem tree can easily be grown in the dry, stony, shallow and clayey soils.

Medicinal properties: Neem leaves to cure skin diseases such as boils, ulcers, eczema, and ring worm, anti inflammatory, antipyretic and hypoglycemic **(Porter WL, 1986)** and also exhibits antimicrobial and anticancerous properties. Neem leaves are traditionally being used as curative against certain fungal and bacterial diseases. However, evaluation of its antiviral properties is limited to few viruses viz. Measles, Chicken pox, HSV and HIV.

Botanical Name : *Lawsonia intermis*
Family : *Lythraceae*
Vernacular name : *Henna*
Part used : *Leaves*

Habitat: Henna is a tall shrub or small tree, 2.6 m high.

Medicinal properties: Henna also acts as an anti-fungal and a preservative for leather and cloth.

Botanical Name *: Murraya koenigii*
Family : *Sprengel Rutaceae*
Vernacular Name : *Curry leaves*

Part used : *Leaves*
Habitat: Curry leaf is a traditional spice used in south India for all the curry preparations. The plant *Murraya koenigii* (L.) *Spreng,* belonging to the family *Rutaceae* is native to India and distributed in most of Southern Asia.
Medicinal properties: The leaves increase digestive secretions and relieve nausea, indigestion, and vomiting. This species is known to possess anti inflammatory, antidysenteric, antioxidant, antidiabetic and diverse pharmacological properties.
Botanical Name : *Curcuma longa*
Family : *Zingiberaceae*
Vernacular Name : *Turmeric, Yellow zinger, Curcuma, haldi, pasupu*
Part used : *Rhizome*
Habitat: Turmeric is the rhizome or underground stem of a ginger-like plant. It is usually available ground, as a bright yellow, fine powder. The main rhizome measures 2.5 - 7 cm (1" – 3 ") in length with a diameter of 2.5 cm (1"), with smaller tubers branching off.
Medicinal properties: Turmeric is currently being investigated for possible benefits in **Alzheimer's disease**, **cancer**, arthritis, and other clinical disorders.
Botanical Name : *Cuminum cyminum*
Vernacular Name : *Cumin, Jeera*
Family : *Apiaceae*
Part used : *Seeds*
Habitat: Cultivation of cumin requires a long, hot summer of 3–4 months, with daytime temperatures around 30 °C (86 °F); it is drought-tolerant, and is mostly grown in Mediterranean climates. It is grown from seed, sown in spring, and needs fertile, well-drained soil.
Medicinal properties: Jeera acts as an anti-obesity, anti-inflammatory, blood purifier, diuretic, glactogauge (that enhances milk engendering during lactation) and uterine stimulant medicine. Jeera is also a very good medicine for mucous diarrhoea and non-specific colitis and is used in combination with other medicines to cure the irritable bowel syndrome.

2.2 Methods

2.2.1 Preparation of Extracts
Fresh leaves of Plants were cut into small pieces, dried in the sun for seven days. And finally in an oven below 60 °C .The dried plant materials (1 kg) was ground into fine powder using motor and pestle and then exhaustively extracted with methanol. The extract was concentrated to a dark greenish residue. This crude extract was used for further investigation for potential antioxidant properties.

2.2.2 Antioxidant Assay
The **antioxidant activity** of Plant extracts were determined by **in- *vitro*** methods (**Zou et al., 2004**). Such as:

- The DPPH free radical scavenging assay

The assay (**Proestos et al., 2006**) was carried out in triplicates and average values were considered.

2.2.3 DPPH radical scavenging activity
The antioxidant activity of the methanolic extracts was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical (**Hanato et al., 1988**). DPPH is a stable free radical containing an odd electron in its structure and utilized for detection of the radical scavenging in chemical analysis. Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured as shown in Fig.1. One ml of each solution of different concentrations (10-100µg/ml) of the extracts was added 3ml of 0.004% methanolic DPPH free radical solution and reaction mixture was shaken vigorously. These solution mixtures were kept in dark for about 30 minutes and the absorbance of the preparations were measured at 517 nm using a UV spectrophotometer which was compared with the corresponding absorbance of the standard ascorbic acid of different concentrations (10-100 µg/ml). Then the % of inhibition was calculated by the following equation:
% DPPH radical scavenging activity = [(Absorbance of control – Absorbance of test sample) / (Absorbance of control)] × 100.

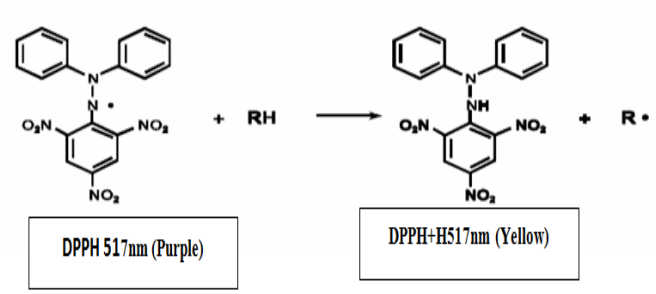

Figure 1. DPPH radical scavenging activity.

3. Results and discussion

Free radicals are inextrabically linked to various disorders, causing different diseases (**Cook and Samman, 1996**). Many plant products have exact antioxidant effect by quenching various free radicals and the singlet form of molecular oxygen**,** (**Liu et al., 2007; Salem et al., 2011**).Various methods have been proposed to evaluate antioxidant characteristics and to explain antioxidant function of plant products. Of those antioxidant activity, different types of free radical scavenging activity is most commonly used for the evaluation of the total antioxidant behavior of extracts.

3.1 DPPH Radical Scavenging Activity
DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (**Kumaran and Karunakaran, 2007**). Hence DPPH is usually used as substrate to evaluate the antioxidant activity (**Molyneux, 2004**). The strength of this scavenging activity of methanol extract and standard on DPPH radical revealed the scavenging activity in terms of percentage of inhibition. The following Table 1. shows the results of DPPH radical scavenging activity of selected six medicinal plants and ascorbic acid as standard. The Figure 2. shows the DPPH radical scavenging activity of Ascorbic acid, Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii, Curcuma longa, and Cuminum cyminum.

Table 1. Antioxidant activities of medicinal plants in Methanol.

Concentration of plant extracts (µg/ml)	% of inhibition						
	Ascorbic acid	Azadirachta indica	Ocimum sanctum	Lawsonia intermis	Murraya koenigii	Curcuma longa	Cuminum cyminum
10	58.3	40	50	54.5	28.5	37.5	40
20	61.5	50	50	57	55.5	58.3	57.1
40	64.2	50	57.1	57.1	58.3	64.2	64.2
60	66.6	66.6	62.5	58.3	55.5	61.5	66.6
80	68.5	66.6	62.5	58.3	55.5	75.0	68.7
100	68.5	81.8	66.5	66.6	63.6	75.0	75.0

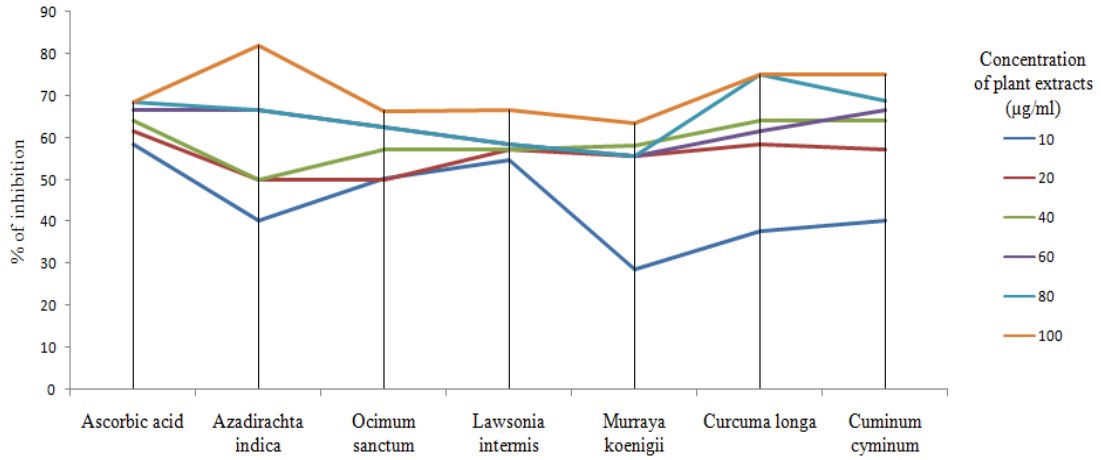

Figure 2. DPPH radical scavenging activity of Ascorbic acid, Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii, Curcuma longa, and Cuminum cyminum

The percentage of inhibition shown by Azadirachta indica, Curcuma longa and Cuminum cyminum is 81.8%, 75% and 75% respectively (**Wangensteen et al., 2004**). Which is greater than the percentage of inhibition of standard i.e. ascorbic acid - 68.5 And the methanolic extracts of Ocimum sanctum, Lawsonia intermis and Murraya koenigii has also shown significant activity i.e. 66.5%, 66.6% and 63.6% respectively, as shown in Table 2 as well as shown in Figure 3.

Table 2. Percentage of DPPH Radical Scavenging Activity

S.No.	Plant Species	Plant part used	%of inhibition
1	*Azadirachta indica*	Leaves	81.8
2	*Curcuma longa*	Rhizome	75
3	*Cuminum cyminum*	seeds	75
4	*Ocimum sanctum*	Leaves	66.5
5	*Lawsonia intermis*	Leaves	66.6
6	*Murraya koenigii*	Leaves	63.6

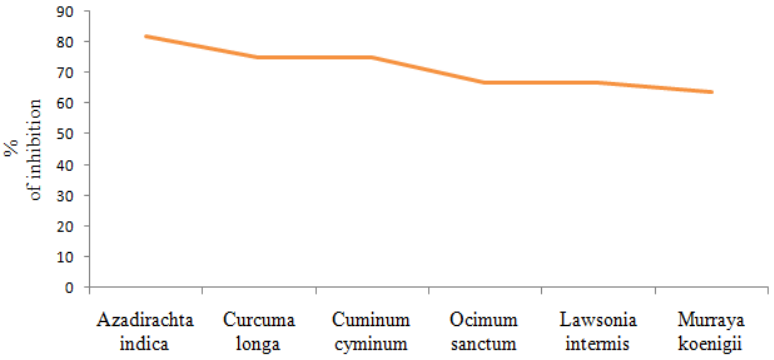

Figure 3. DPPH radical scavenging activity of selected six medicinal plants

Based on the data obtained from the study, methanolic extracts of these plants exhibited high scavenging activity and can minimize or reduce free radical damaging occurred in the human body (**Sriti et al., 2011**).

4. Conclusions

In conclusion, the results of the present study that the methanolic extracts of *Azadirachta indica, Curcuma longa , Cuminum cyminum, Ocimum sanctum, Lawsonia intermis and Murraya koenigii* exhibits significant antioxidant activity through the scavenging of different free radicals which participate in various pathophysiology of diseases. Standardized aqueous methanolic extracts from the selected

leaves and seeds having different target radicals, such as superoxide radical, nitric oxide, and peroxidative decomposition of phospholipids, were prepared and screened by *In-vitro* assays. Fresh leaves of Plants were cut into small pieces, dried in the sun for seven days. And finally in an oven below 60 °C .The dried plant materials (1 kg) was ground into fine powder using motor and pestle and then exhaustively extracted with methanol. The extract was concentrated to a dark greenish residue. This crude extract was used for further investigation for potential antioxidant properties. These extracts were tested for, DPPH free radical scavenging activity correlated with antioxidant capacity (**Cheung et al., 2003**). The highest percentage of DPPH free radical scavenging activity is found in *Azadirachta indica(*81.8 µg/ml % of inhibition) which shows antimicrobial, anti inflammatory and anticancerous properties where Ascorbic acid (68.5 µg/ml) is taken as standard for comparison. Overall, the plant extracts is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity of life. The antioxidants is important in industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines.

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