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Determination of antioxidant activities of different extracts of *in vitro* plantlets of *Murraya koenigii* L. Spreng

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Murraya koenigii (L) Spreng, locally known as "curry patta" or "mithaneem" (curry leaf tree) belonging to family Rutaceae, is commonly used as raw material for traditional medicinal formulation in India. Its aromatic leaves contain essential oil and is used as condiments. The fresh leaves of *Murraya koenigii* have been reported to possess anti-microbial, mosquitocidal, topo-isomerase inhibition and antioxidant properties. The present paper aims to undertake a comparative study of antioxidant potential of extracts of *in vitro* plant parts. Four different plant materials were tested for the determination of antioxidant activity. These plant materials include ADVS, ADVR, AXLS and AXLR. The stable DPPH was used for *in vitro* determination of free radical scavenging activity of the extracts. The strongest DPPH radical scavenging activity was exhibited by extract of AXLS and lowest activity was found in the ADVR. Present investigation reports first on comparative *in vitro* analysis of antioxidant potential of extracts ADVS, ADVR, AXLS and AXLR of *M. koenigii*.

Key words: Antioxidant; Flavonoids; *Murraya koenigii;* Phenols, *in vitro,* DPPH, GAE, QE AAE ADVS, ADVR, AXLS.

1. Introduction

Murraya koenigii found throughout tropical and subtropical East Asia, from India and China to New Caledonia and Northeastern Australia 1**.** Curry leaf is a small tree with dark grey bark and compound leaves 2. In India, this plant is commonly found in the outer Himalayas, from the Ravi eastwards, ascending to 5,000 feet, in Assam, Chittagong, Upper and Lower Burma. It is also found in evergreen and deciduous forests of peninsular India. The shrub is of common occurrence in Himachal Pradesh in areas lying between 800 and 1,450 meters above the sea level. Almost every part of this plant has a strong characteristic odour. A small spreading shrub, about 2.5 meters high, the main stem is dark green to brownish with numerous dots on it. Leaves, exstipulate, bipinnately compound, 30 cm long, each bearing 24 leaflets having reticulate venation. Leaflet is lanceolate, 4.9 cm long, 1.8 cm broad, having 0.5 cm long petiole. Flowers, bisexual, white, funnel-shaped, sweetly scented, stalked, complete, ebracteate, regular, actinomorphic, pentamerous and hypogynous. Inflorescence, terminal cyme, each bears 60 to 90 flowers, calyx, 5 lobed. Androecium is polyandrous, inferior, with 10 stamens, dorsifixed, arranged into circles of five each; smaller stamens, 4 mm. long whereas the longer ones, 5 to 6 mm; gynoecium, consists of 5 to 6 mm long stigma, bright, sticky; style, ovary, superior. Fruits, round to oblong, 1.4 to 1.6 cm long, 1 to 1.2 cm in diameter, fully ripe fruits are black with a very shining surface with blue pulp, the number of fruits per cluster varying from 32 to 80. Seed, one and/or more in each fruit, 11 mm long, 8 mm in diameter with spinach green in colour. Flowering starts from the middle of April and ends in the middle of May. The fruiting season was observed to continue from the middle of July to the end of August. The leaves, bark and roots of *Murraya koenigii* can be used as a tonic and a stomachache. They are also used externally to cure eruptions and the bites of poisonous animals. Strong odiferous oil occurs in the leaves and the seeds of this plant. An alkaloid, mukonine, is also found in this plant 3. The seed occupies the major part of the fruit and the edible portion is only 49.4 per cent of the whole fruit. The fruits are very sweet and are eaten fresh. They have a characteristic odour, which makes them slightly unpleasant. As revealed by the chemical composition of the fruits, they are very nutritious. The leaves are used as a spice in different curries and impart a very good flavour to the preparations. These fruits have also many medicinal properties. The branches of *Murraya koenigii* are very popular for cleaning the teeth as datun and are said to strengthen the gums and the teeth. This plant is quite ornamental due to its compound leaves. They have a distinct, spicy curry like flavor and odor. The tree prefers warm temperatures with full sun to partial shade.

1.1 Phenolic compounds

Phenolic derivatives represent the largest group known as 'secondary plant products' synthesized by higher plants. Many of these phenolic compounds are essential to plant life, e.g., by providing defense against microbial attacks and by making food unpalatable to herbivorous predators. Although a precise chemical definition may be given for plant phenolics, it would inevitably include other structurally similar compounds such as the terpenoid sex hormones. Therefore, an operational definition of metabolic origin is preferable, and thus the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoids metabolism, following the phosphoenolpyruvate → phenylalanine → cinnamate → 4-coumarate course, leading to chalcone, flavanone, dihydroflavonol and anthocyanin. Significant antioxidant, antitumoral, antiviral and antibiotic activities are frequently reported for plant phenols. They have often been identified as active principles of numerous medicines. In recent years, the regular intake of fruits and vegetables has been highly recommended, because the plant phenols and polyphenols, they play important roles in long term health and reduction in the risk of chronic and degenerative diseases. Recognition of the benefits brought by these natural products to human health has encouraged the inclusion in everyday diets of some typical plant derived food and beverages, among the most preferred examples being olive and vegetable oils, citrus and other fruit juices, chocolate, tea, coffee and wine. Over eight thousand naturally occurring phenolic compounds are known; these substances contain at least one aromatic ring with one or more attached –OH groups, in addition to other substituents and can be divided into 15 major structural classes. Major classes of plant phenolics with 'the type of carbon skeleton, class name (example)' format include: C6, simple phenols (resorcinol); C6-C1, phenolic acids (*p*-hydroxybenzoic acid); C6-C2, acetophenones and phenyl acetic acids; C6-C3, hydroxy cinnamic acids (caffeic acid); C6-C4, hydroxyanthraquinones.

1.2 Flavonoid

Personalvonoids may have existed in nature for over one billion years and thus have interacted with evolving organisms over the eons. Clearly, the flavonoids possess some important purposes in nature, having survived in vascular plants throughout evolution 4. The very long association of plant flavonoids with various animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities of these chemicals in mammalian and other biological systems. Unique examples are the inhibition of gamete membrane fusion in sea urchins caused by quercetin during egg fertilization and modulation of mammalian sperm motility by quercetin 5,6. Also, prenatal exposure to genistein does indeed influence sexual differentiation in rats and thus raises the question of analogous effects in humans 7.Over 4000 structurally unique flavonoids have been identified in plant sources 8,9,10. Primarily recognized as the pigments responsible for the autumnal burst of hues and the many shades of yellow, orange and red in flowers and food 11, 12. The flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, as well as tea and red wine. They are prominent components of citrus fruits and other food sources and are consumed regularly with the human diet13. These low molecular weight substances, found in all vascular plants, with an assortment of structures based on a common three ring nucleus. They are usually subdivided according to their substituents into flavanols, anthocyanidins and flavones, flavanones and chalcones. This basic structure is comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (c) in the middle. This subdivision is primarily based on the presence (or absence) of a double bond on position 4 of the C (middle) ring, the presence (or absence) of a double bond between carbon atoms 2 and 3 of the C ring, and the presence of hydroxyl groups in the B ring. In the flavonoid structure, a phenyl group is usually substituted at the 2-position of the pyrone ring. In isoflavonoids, the substitution is at the 3-position. Flavonoids and tocopherols (vitamin E) share a common structure, i.e., the chromane ring. There have been several efforts to quantitate the amounts of different flavonoids in assorted food plants. The flavonoid consumed most is quercetin and the richest sources of flavonoids consumed in general are tea (48% of total), onions and apples 14. It should be stressed that recent evidence indicates that flavonoid glycosides are much more readily absorbed (than the aglycones) by humans. Moreover, both the amount and the source could vary appreciably in different countries. For instance, the amount consumed could be considerably higher in the Mediterranean diet, which is rich in olive oil, citrus fruits and grains. These quantities could provide pharmacologically significant concentrations in body fluids and tissues. Nevertheless, flavonoid dietary intake far exceeds that of vitamin E, a monophenol antioxidant and that of β-carotene on a mg/per day basis. Flavonoids have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances and pigments and light screens. The commercial importance of secondary metabolites in recent years has created great interest in altering the production of bioactive plant metabolites by means of tissue culture technology. Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for both studying and producing plant secondary metabolites. Different strategies, using an *in vitro* system, have been extensively studied to improve the production of plant chemicals. Advanced biotechnological methods, especially *in vitro* propagation techniques cryopreservation and culturing plant cells and tissues should provide new means of conserving and rapidly propagating valuable, rare and endangered medicinal plants which may also help in conservation of biodiversity of locally used medicinal plants 15. Biotechnological approaches are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites 16. Plant cell suspension cultures involve growth of single or cell aggregates in liquid medium. Cultured cells are made to synthesize useful metabolites. Altering media composition and feeding of precursors and elicitors induce synthesis and accumulation processes. Induction of changes in the shikimic acid pathway plays central role in the synthesis of useful pharmaceuticals. Enhanced production by cell immobilization and biotransformation of new compounds are potential areas of this research. Alkaloids and terpenoids have extensively been produced *in vitro* conditions. Most of alkaloid compounds are pharmaceutical leads. Localization of specialized cells responsible for synthesis and accumulation of metabolites in

plant tissues help extensively to choose the cell type for culture. Altering the genetic make up of the cells to go for enhanced production and quality drugs are the theme focus of this field. Hairy root cultures and bioreactor production of secondary metabolites have resulted in remarkable yields. Many prospective drugs including taxol, ajmalicine, atropine, codeine, dopamine, digitoxin and morphine is being obtained enormously from *in vitro* cell cultures and thereby conserving natural resources.

1.3 Antioxidants

Antioxidants are gaining a lot of importance as to cure a number of diseases like aging, cancer, diabetes, cardiovascular and other degenerative diseases etc. owing to our sedentary way of life and stressful existence. Added to these are the deleterious effects of pollution and exposure to harmful chemicals. All the above can cause accumulation of harmful free radicals. Free radicals are the types of Reactive oxygen species (ROS), which include all highly reactive, oxygen containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical and various lipid peroxides. All these are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage. In living organisms, various ROSs can be formed in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes, macrophages and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells, whereas exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. Free radicals may be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally achieve stable configuration. They are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress among others. To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. Thus, antioxidants are capable of stabilizing or deactivating free radicals before they attack cells. Naturally, there is a dynamic balance between the amount of free radicals produced in the body and antioxidants to scavenge or quench them to protect the body against deleterious effects. The amount of antioxidant principles present under normal physiological conditions may be insufficient to neutralize free radicals generated. Therefore, it is obvious to enrich our diet with antioxidants to protect against harmful diseases. Hence there has been an increased interest in the food industry and in preventive medicine in the development of "Natural antioxidants" from plant materials. The plants with antioxidant properties are becoming more and more popular all over the world. Considering the importance of this area, we have listed some of the important medicinal plants having potent antioxidant property, which are traditionally used in the Indian subcontinent for various disorders where free radicals are thought to be involved. These medicinal plants include: *Terminalia chebula* (Harada),*Terminaliabelerica*(Bihara),*Emblicaofficinalis*(Amla), *Camellia sinensis* (Green tea),*Theobroma cacao* (Cocoa), *Vitex vinifera* (Grapes), *Panax ginseng* (Ginseng),*Withaniasomnifera*(Ashwagandha), *Ocimum sanctum* (Tulsi), *Asparagus racemosus*(Satavari),*Centellaasiatica*(Brahmi), *Terminalia arjuna* (Arjuna),*Glycyrrhizaglabra*(Liquorice), *Curcuma longa* (Turmeric)and *Piper longum*(Pipal) etc. Incorporation of these plant antioxidants into one's diet results in providing a measure of protection against oxidative damage from free radicals. When added to a regimen of exercise, balanced diet and a clean environment, could go a long way in providing protection against aging, cancer, liver diseases and other degenerative diseases. All the above-mentioned plants are being used in various formulations as antioxidant supplements. There is much more to be learnt regarding antioxidants and their applications in improving the health and well-being of humankind and needless to say there is a tremendous need to spread awareness about them. Conservation and sustainable utilization of Medicinal and Aromatic plants must involve a long term, integrated, scientifically oriented action program.

2. Materials and Methods

2.1 Plant Material Collection

The plant material of *Murraya koenigii* was collected from *in vitro* raised plantlets from the Plant Tissue Culture Lab Department of Botany, Govt. PG College Ajmer. The extracts prepared from *in vitro* plant materials (ADVS, ADVR, AXLS and AXLR) were used for analyzing total phenols, flavonoids and antioxidant activity *in vitro*. Four plant materials tested for determination of antioxidant activity, were ADVS, ADVR, AXLS and AXLR. One-gram plant material was extracted in 10 ml of 80% methanol by maceration. The solvent was then centrifuged at 14,000 rpm for 30 minutes at room temperature. The extract obtained was used for analysis. All solvents used were of analytical grades, DPPH and quercetin were procured from sigma chemical co. (st., Louise, US), Gallic acid, Ascorbic acid were procured from Merck co. (Germany), Folin Ciocalteu, Aluminum chloride, Methanol, Sodium carbonate and potassium acetate were purchased from Qualigens fine chemical co. (India). The absorbance measurements were recorded on Spectroscan-50, UV-VIS spectrophotometer (Biotech. engineering management Co. UK.)

2.2 Total phenols determination

Total phenols were determined by FollinCiocalteu reagent method 17. An aliquot of each plant extract (0.5ml 1:10 $mg.l^{-1}$) or gallic acid (standard phenolic compound) was added with follinciocalteu reagent (5ml 1:10 diluted with distilled water) and 4ml of 1M solution of Na_2CO_3. The mixture was allowed to stand for 30 minutes at room temperature and absorbance was measured at 710nm.Total phenolic contents of extracts were expressed as mg GAE/gm fresh weight. All samples were analyzed in triplicates.

2.3 Total flavonoids determination

Total flavonoids contents were analyzed by the aluminum chloride method 18. Each plant extract (0.5 ml of 1:10 gm.l^{-1}) mixed with 1.5 ml methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate and 2.8 ml distilled water. The mixture was allowed to stand for 30 minutes at room temperature and absorbance was measured at 415nm. Total flavonoids contents was expressed as mg QE g $^{-1}$ fresh mass. Samples were analyzed in triplicates.

2.4 DPPH- free radical scavenging activity determination

The stable DPPH was used for *in vitro* determination of free radical scavenging activity of the extracts 19. Different concentrations of each extract were mixed with methanolic solution of DPPH (0.004%). The mixture was allowed to stand for 15 minutes. The scavenging of free radicals by extract was evaluated spectrophotometrically at 517nm against the absorbance of DPPH radicals. The percentage discoloration was calculated by following formula. DPPH radical scavenging activity (%) = [AC_{517} – AE_{517} / AC_{517}] x 100. Where AC_{517} is absorbance of a DPPH solution without extract, $AE_{517\ is}$ the absorbance of the tested plant extract with DPPH. The degree of discoloration indicates the free radical scavenging efficiency of the substances. Ascorbic acid was used as a free radical scavenger reference compound.

3. Results

Total phenol and flavonoid content of methanolic extracts from different parts of *in vitroMurraya koenigii* and their free radical scavenging effect were determined spectrophotometrically (as shown in Table).

3.1 Total phenols & flavonoids determinations

Total phenol was measured as GAE and flavonoid as QE/gmfresh weight of *in vitro* plant part of *Murraya koenigii*. The observation of Spectrophotometric analysis of four *in vitro* extracts (ADVS, ADVR, AXLS and AXLR) of *Murray koenigii* for totalphenols and flavonoids contents are presented in Table. The results showed that the maximum phenols were observed **ADVS 1.57 ± 0.101mg)** followed by **AXLR 1.47 ± 0.10mg, ADVR 1.38 ± 0.120mg** and **AXLS 1.30 ± 0.070mg**. Flavonoid content was found to be maximum in ADVS **(0.750 ± 0.03mg)** followed by AXLS (0.710 ± 0.02mg) and AXLR **(0.70 ± 0.01mg)**whereas minimum flavonoid contents were exhibited by ADVR **(0.365 ± 0.01mg)**.

3.2 Total Antioxidant capacity determination

The antioxidant capacity of four samples of *in vitro*plant parts of *Murraya koenigii* was measured AAE/gmfresh weight AAE using DPPH free radicals. The highest antioxidant capacity was exhibited by AXLS which was **3.0 ± 0.11mg/ gm^{-1}** fresh weight. Followed by ADVS **(2.5 ± 0.06mg/ gm^{-1})** and AXLR **(2.5 ± 0.03mg/ gm^{-1})** and minimum antioxidant capacity was found in ADVR **(1.5 ± 0.13mg/ gm^{-1})** gm-^{1} fresh weight.

3.3 Determination of percentage scavenging potential

The percentage inhibition/ discoloration of free parts of *Murraya koenigii* was measured against DPPH highest percentage of discoloration / inhibition was observed by the extracts of AXLR **(53.08 ± 0.149%)** which was followed by AXLS **(52.42 ± 0.415%)** and ADVS **(48.72 ± 0.196%)** whereas minimum discoloration of DPPH was exhibited by ADVR **(45.28 ± 0.248%)**.

3.4 Determination of DPPH radical scavenging activity (IC_{50})

The strongest DPPH radical scavenging activity was exhibited by the extracts of the AXLR with **IC_{50}= 0.0941mg/ml** followed by AXLS **IC_{50}=0.0948,** ADVS **IC_{50}=0.102** and minimum free radical scavenging activity was exhibited by ADVR **IC_{50}=0.11.**

Table: Determination of totalphenol, flavonoid and their antioxidant activity.

Parts of plants	Total phenol mg GAE 1g fresh mass (Mean ± SD)	Total flavonoids mg QE 1g fresh mass (Mean ± SD)	% of scavenging activity (Mean ± SD)	Total Antioxidant capacity determination (Mean ± SD)	IC_{50} value Mg/ml
ADVS	1.57 ± 0.101	0.750 ± 0.03	48.72 ± 0.196	2.5 ± 0.06	0.102
ADVR	1.38 ± 0.120	0.365 ± 0.01	45.28 ± 0.248	1.5 ± 0.13	0.11
AXLS	1.30 ± 0.070	0.710 ± 0.02	52.42 ± 0.415	3.0 ± 0.11	0.0948
AXLR	1.47 ± 0.10	0.70 ± 0.01	53.08 ± 0.149	2.5 ± 0.03	0.0941

4. Discussion

The natural antioxidants of plant origin and their importance in health, food and preventive medicine are well acknowledged. Besides the traditional use of antioxidant from tea, fruits, vegetables and spices, some natural antioxidants are already exploited commercially either as additives or a nutritional supplements 20. There has been an increased interest in the studies of antioxidants from plant materials a number of plant species have been studied in the search for new antioxidants. The Present investigation highlights a comprehensive profile of antioxidant activity of extracts of *in vitro* plant parts of an important aromatic plant, *M. koenigii* with respect to its containment of phenols and flavonoids. The data reported provides quantitative estimation of Phenols and flavonoids and their antioxidant activities. Many reports of natural antioxidants of plant origin have been published and their importance in health, food and preventive medicine has been well emphasized 21.Keeping this in the view, the data showed in present paper reports important observations on the antioxidant potential in different *in vitro* plant parts. In present investigation correlation was established between the Spectrophotometric estimation of antioxidant activity using DPPH free radical of plant extracts. The biochemical investigation of different plant parts showed maximum quantity of phenol **(1.57 ± 0.101mg)** and flavonoid **(0.750 ± 0.03mg)** in ADVS. The strongest antioxidant activity was exhibited by freshly prepared extract of **AXLS**. The Present

study reports first on Spectrophotometric analysis of methanolic extracts of different parts of *in vitro* plant of *Murraya koenigii*.Reports are also available on analysis and isolation of antioxidant vitamins and active carbazole alkaloids from fresh leaves of *M. koenigii* 22, 23, 24. But present investigation reports first on comparative analysis of antioxidant potential of extracts of *in vitro* plant parts of *M. koenigii.*

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6. Abbreviationss
In vitro adventitious shoot (ADVS), root of *in vitro* adventitious shoot (ADVR), *in vitro* axillary shoots (AXLS), root of *in vitro* axillary shoot (AXLR), 1,1 diphenyl-2 picryl hydrazyl (DPPH), Gallic acid equivalent (GAE), Quercetin equivalent per gram (QE/gm), Ascorbic acid equivalent (AAE), Standard Deviation (SD), Antioxidant capacity (AC), Desert Medicine Research Center (DMRC)

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