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Original Article

A STUDY ON ANTIOXIDANT AND ANTI-AGING PROPERTIES OF FEW MEDICINAL PLANTS

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ABSTRACT

Objective: The present study was undertaken to appraise the antioxidant and antiaging properties of some important medicinal plants like *Syzygium cumini L.* (Jamun, the Indian Blackberry), *Tinospora cordifolia L.* (Giloy) and *Trigonella foenum-graecum L.* (Methi).

Methods: The methanolic extracts of these plants were prepared, incubated with Pheochromocytoma (PC-12) cells and total antioxidant potential of these plants was determined by 2, 2-diphenylpicrylhydrazyl (DPPH) and Ferric reducing ability of plasma (FRAP) assay. Lipid peroxidation assay was also performed to compare the free radical generating potential of these plants. Lipofuscin estimation was done in methanolic extracts of these plants to check which extract can reduce the lipofuscin accumulation in cells which is an anti-aging parameter.

Results: The highest inhibition of DPPH radical and ferric reducing ability (FRAP assay) was observed in methanolic extract of *Syzygium cumini* leaves followed by *Trigonella foenum-graecum* leaves and lowest inhibition was shown by *Tinospora cordifolia* leaf extracts. Lipid peroxidation assay done by determining the amount of Malondialdehyde(MDA) formed and highest levels of MDA was produced by *Tinospora cordifolia* leaf extracts followed by *Trigonella foenum-graecum* leaf extracts and lowest values were obtained were from *Syzygium cumini* leaf extracts. The anti-aging parameter which is a determination of lipofuscin pigments in PC-12 cells treated with methanolic extracts of different plants was also examined. Highest accumulation of lipofuscin pigment was observed in cells treated with *Tinospora cordifolia* leaf extracts followed by *Trigonella foenum-graecum* leaf extracts and lowest values were obtained were from *Syzygium cumini* leaf extracts.

Conclusion: The results of these findings revealed that the plants which have highest antioxidant potential could be a potent source of anti-aging drugs.

Keywords: Antioxidants, Antiaging, 2, 2-diphenyl1-picryl hydrazyl (DPPH), FRAP, Lipofuscin, Syzygium cumini, Trigonella foenum-graecum, Tinospora cordifolia.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide exert oxidative stress towards the cells of human body. This ROS overtakes the antioxidant defense of the cell, the free radicals start attacking the cell proteins, lipids, and carbohydrates and leads to a number of physiological disorders. Free radicals play a crucial role in the development of various ailments like diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, and neurological disorders and in the process of aging. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids, and tannins, etc. and can be utilized to scavenge the excess free radicals from the human body. Many herbs contain antioxidant compounds which protects the cells against the damaging effects of ROS. Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant-based drugs or formulations to treat various human ailments because they contain the components of therapeutic value [1]. In addition, plant-based drugs remain an important source of therapeutic agents because of the availability, relatively cheaper cost and non-toxic nature when compared to modern medicine [2]. Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free radicals [3]. They are well known as radical scavengers, metal chelator, reducing agents, hydrogen donors and singlet oxygen quenchers [4]. Free radicals produced in the body react with various biological molecules namely lipids, proteins and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants. Even though our body is safeguarded by the natural antioxidant defense, there is always a demand for antioxidants from natural sources [5]. Aging is an inevitable process for all living organisms. During this process reactive oxygen species generation is increased. According to free radical theory, mitochondria were identified as responsible for the initiation of most of the free radical reactions occurring in the cells. It was also postulated that the life span is determined by the rate of free radical damage to the mitochondria [6]. The increasing oxidative stress in aging seems to be a consequence of the imbalance between free radical production and antioxidant defenses with a higher production of the former [7]. The identification of free radical reactions as promoters of the aging process implies that interventions aimed at limiting or inhibiting them should be able to reduce the rate of formation of aging changes with a consequent reduction of the aging rate and disease pathogenesis [8].

Antioxidants from plants prevent the action of free radicals, so there is growing interest all over the world to identify the untapped reservoir of plants of medicinal importance. Although studies on *Syzygium cumini L.* (Jamun, the Indian Blackberry), *Tinospora cordifolia L.* (Giloy) and *Trigonella foenum-graecum L.* (Methi) have proved its efficacy as medicinal plant, but our studies will confirm the total antioxidant potential in methanolic extract of these plants by DPPH and FRAP assay *in vitro* by treating fibroblast cells with these extracts. Secondly, anti-aging parameter, lipofuscin accumulation will also be studied in cells treated with these extracts. Our results will demonstrate which plant can best act as anti-aging agent besides having best antioxidant potential.

MATERIALS AND METHODS

Collection of plant materials

All the plant samples were collected from the farms of Indian Agricultural Research Institute (IARI), New Delhi.

Preparation of methanolic extracts

Leaves of all the three plants were air-dried at room temperature to constant weights. The dried plant materials were separately ground to powders. Two hundred grams of powdered leaves were soaked in 1 l of methanol separately for 48 h in an orbital shaker. Extracts were filtered using a Buckner funnel and Whatman no. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at

40 °C using a rotary evaporator. The percentage yield for the leaves was 8.3% while that of the stems was 7.8%. Each extract was re-suspended in methanol to make a 50 mg/ml stock solution [9].

Cell culture

PC-12 cells treatment with Lycopene and other plant extracts

The purified lycopene was obtained in powdered form from SIGMA-ALDRICH. The extracts of different concentrations were made in the laboratory itself. The PC-12 cells were procured from the National Centre for Cell Sciences (NCCS), Pune and further processed in the laboratory itself. First set of $1x10^6$ cells/well were treated with6-Hydroxydopamine (6-OHDA) and the second set of $1x10^6$ cells/well were treated with 6-OHDA+lycopene extract and other plant extracts. After 48 h of incubation, cells were washed with Phosphate Buffered Saline (PBS) and lysed by using sonicator. Intact cells were used to estimate the total antioxidant potential by DPPH and FRAP assays. Lipid peroxidation measurements and Lipofuscin estimations were also done in cells treated with plant extracts and purified lycopene [10].

Cell cytotoxicity assay

Cytotoxicity Assay was performed using PBS (phosphate buffered saline), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) (in PBS), isopropanol and samples. Samples were incubated for 3 h at 37 °C after adding MTT to them. After adding acid-propanol, it was left in dark overnight, and the absorbance was recorded at 650 nm [11].

DPPH radical scavenging assay

The effect of extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi [12]. A solution of 0.135 mmol/l DPPH in methanol was prepared, and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid and Butylated hydroxytoluene (BHT) were used as references. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$ where $Abs_{control}$ is the absorbance of DPPH radical+methanol; Abs_{sample} is the absorbance of DPPH radical+sample extract/standard.

Total antioxidant activity (FRAP assay)

A modified method of Benzie and Strain [13] was adopted for the FRAP assay. The stock solutions included 300 mmol/l acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mmol/l2, 4, 6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mmol/l Hydrochloric Acid (HCl), and 20 mmol/l $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C before using. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyl triazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µ mol/l Ferrous Sulphate ($FeSO_4$) Results were expressed in µ mol/l Fe (II)/g dry mass and compared with that of ascorbic acid.

Lipid peroxidation

Lipid peroxidation was measured by estimating the amount of malondialdehyde: MDA (thiobarbituric acid reactive substances: TBARS) formed by using thiobarbituric acid (TBA) spectrophotometrically at 532 nm. The levels of lipid peroxides were expressed as Nanomoles of malondialdehyde (MDA) formed per mg of tissue [14].

4-Hydroxynonenal (4-HNE estimation)

To 0.5 ml of cell sample 1.875 ml of 1:2 (v/v) chloroform and methanol were added which was further centrifuged at 2000 rpm for 10 min. 0.625 ml of chloroform was added to the supernatant with an equal amount of distilled water. 1 ml of sample was withdrawn from the lower chloroform layer and mixed with 0.1 ml of methanol. Fluorescence was measured at an excitation wavelength of 360 nm and the emission wavelength was set at 430 nm. 0.1µg/ml in 0.05 mol/l Sulphuric Acid (H_2SO_4) was taken for making the standard curve [15].

Statistical evaluation

To assure the accuracy of the experimental data, each experiment was performed in triplicate and the result was expressed as mean±standard deviation of three replications. *P* value<0.05 was regarded as significant.

RESULTS

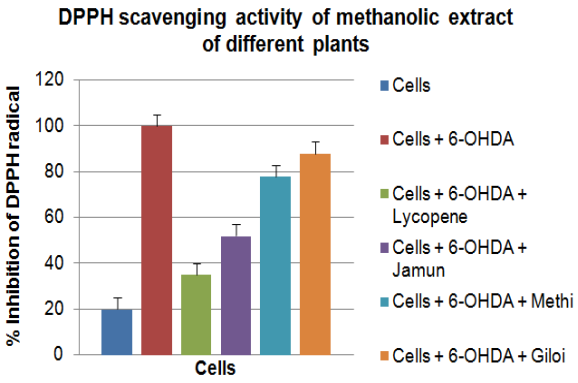

Fig. 1: DPPH scavenging activity of methanolic extracts of different plants

Methanolic extract of Jamun leaf showed maximum inhibition of DPPH radical (95%) followed by leaf extract of Methi (90%) and least percentage inhibition of DPPH radical was exhibited by Giloi leaf extract (80%) (fig. 1).

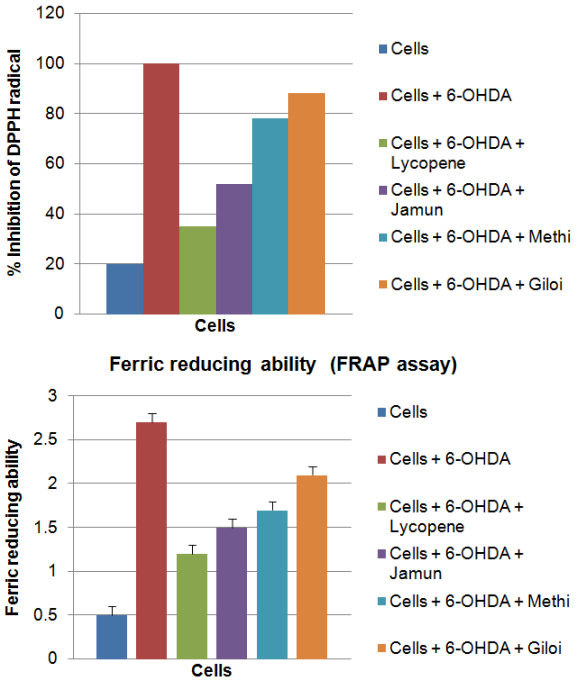

Fig. 2: Ferric reducing ability (FRAP assay)

Methanolic extract of Jamun leaf showed maximum ferric reducing ability (95%) followed by leaf extract of methi (85%). Minimum activity was shown by leaf extracts of Giloi (70%) (fig. 2).

Amongst the three medicinal plant extracts, leaf extract of Jamun showed a maximum decrease in TBARS formation followed by a methanolic extract of Methi and the minimum decrease was

observed by Giloi extract. The free radical formation was inhibited maximally by methanolic extract of Jamun plant. (fig. 3).

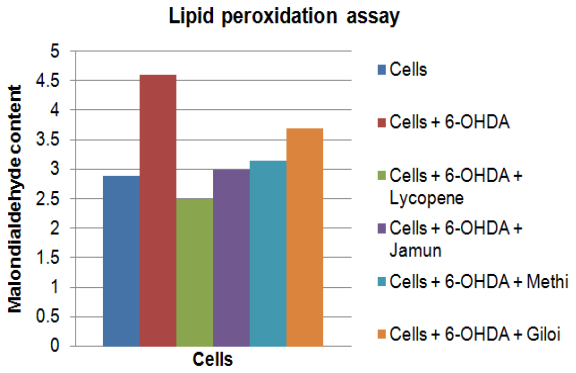

Fig. 3: Lipid peroxidation assay

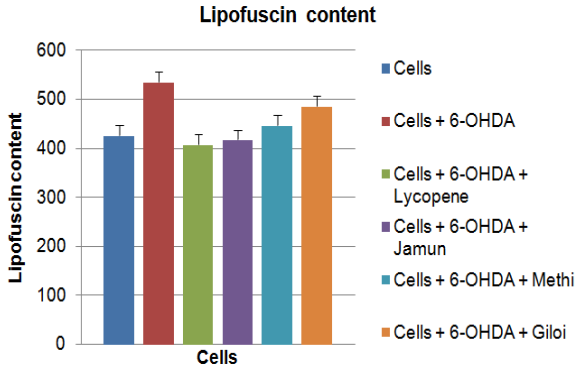

Fig. 4: Lipofuscin content in cells

4-HNE was measured spectrofluoro-photometrically and maximum concentration of 4-HNE was observed in cells treated with Giloi leaf extracts (90%) followed by methi leaf extract (80%) and minimum concentration of 4-HNE was observed in Jamun leaf extract (60%). (fig. 4). Maximum accumulation of Lipofuscin was observed in Giloi leaf extract followed by Methi and then Jamun Leaf extracts (Figure5). Lycopene showed minimum accumulation of Lipofuscin as well as 4-HNE concentration, so we took it as a control to compare our results.

DISCUSSION

DPPH scavenging assay was performed in methanolic leaf extracts of Jamun, Giloi and Methi using Lycopene as a control. DPPH is a protonated radical having absorption maxima at 517 nm which decreases with scavenging of the proton radical by natural plant extracts. Methanolic extract of Jamun leaf showed maximum inhibition of DPPH radical (95%) followed by leaf extract of Methi (90%) and least percentage inhibition of DPPH radical was exhibited by Giloi leaf extract (80%). Lycopene treatment was considered as control sample which showed 100% inhibition of DPPH radical. (fig. 1).

Free radical scavenging activity was also determined by FRAP (ferric reducing the ability of plasma) method, which depends upon the reduction of ferric tripyridyl triazine {Fe (III)-TPTZ} complex to ferrous tripyridyl triazine {Fe (II)-TPTZ}. Ferrous has an intensive blue color which can be monitored at 593 nm. Ferric reducing ability of the three medicinal plants was compared using TPTZ and cells treated with Lycopene as a control. Methanolic extract of Jamun leaf showed maximum ferric reducing ability (95%) followed by leaf extract of methi (85%). Minimum activity was shown by leaf extracts of Giloi (70%) (fig. 2). The free radical scavenging power of PC-12 cells+6-OHDA+Lycopene was significantly very high as compared to cells treated with 6-OHDA alone. This value reached to almost control level when cells were treated with Lycopene (fig. 2).

The antioxidant capacity of plant extract may be due to hydrogen donating ability of flavonoids present in it.

Levels of formation of Thiobarbituric acid reactive substance (TBARS), which was detected by measuring lipid peroxidation in the cells, were found to be increased in stressed cells (cells treated with 6-OHDA). But upon treatment with Lycopene, these levels were found to be significantly decreased. (fig. 3). Amongst the three medicinal plant extracts, leaf extract of Jamun showed a maximum decrease in TBARS formation followed by a methanolic extract of Methi and the minimum decrease was observed by Giloi extract. The free radical formation was inhibited maximally by methanolic extract of Jamun plant (fig. 3).

Cells produce defense against excessive free radicals by their preventive mechanisms, repair mechanisms, physical defenses, and antioxidant defenses [16]. Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. [17,18]. The oxidation of lipid, DNA, protein, carbohydrate and other biological molecules by toxic ROS may cause DNA mutation or serve damage to target cells or tissues; and this often results in cell senescence and death. Free radicals are involved in proteins, lipids, and carbohydrates, and this leads to a number of physiological disorders. Free radicals are also involved in the development of degenerative diseases; they have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, and neurological disorders and in the process of aging.

Aging is an inevitable process for all living organisms. During this process reactive oxygen species generation is increased which leads to mitochondrial damage in the cell. It was postulated that lifespan is determined by the rate of free radical damage to the mitochondria. Aging parameters which are determined by Lipofuscin accumulation in cells as well as the determination of 4-HNE concentration in cells was also monitored in our study to correlate between antioxidant as well as anti-aging capacity of the methanolic extracts of three plants.

4-HNE was measured spectro- fluoro-photometrically and maximum concentration of 4-HNE was observed in cells treated with Giloi leaf extracts (90%) followed by methi leaf extract (80%) and minimum concentration of 4-HNE was observed in Jamun leaf extract (60%). (fig. 4). Maximum accumulation of Lipofuscin was observed in Giloi leaf extract followed by Methi, and then Jamun Leaf extracts (Figure5). Lycopene showed minimum accumulation of Lipofuscin as well as 4-HNE concentration, so we took it as a control to compare our results. Thus our results suggest that plants which showed maximum antioxidant capacity showed minimum accumulation of Lipofuscin. The minimum concentration of 4-HNE was observed in Jamun plant which showed maximum antioxidant potential. (fig. 4).

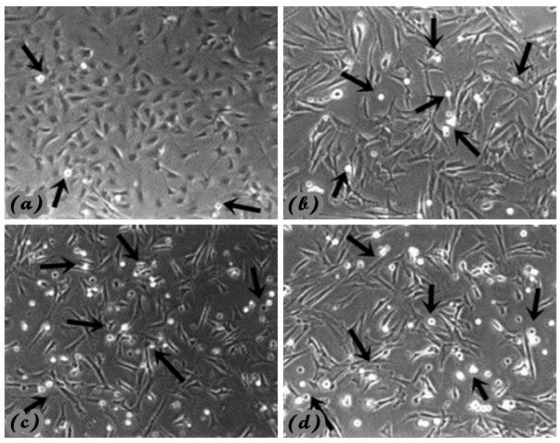

Fig. 5: Morphology of Cells after 72 h of incubation (Arrows indicate Lipofuscin accumulation) (a) Cells+Lycopene (Control), (b) Cells+Jamun leaf extract, (c)Cells+Giloi leaf extract, (d) Cells+Methi leaf extract

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress [19]. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process [20]. This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease [21]. Plants are a potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants [26]. Natural antioxidants present in the plants scavenge harmful free radicals from our body. DPPH and FRAP are widely used to the free radical scavenging effect of natural antioxidant. Antioxidants in different parts of plants such as ascorbic acid, vitamin E and phenolic compounds possess the ability to reduce the oxidative damage associated with many diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and aging.

Free radicals are casually to the basic aging process [22] is receiving growing acceptance as a possible explanation of the chemical reactions at the basis of aging [23]. The free radical theory of aging hypothesizes a single common process, modifiable by genetic and environmental factors, in which oxygen-derived free radicals are responsible (due to their high reactivity) for the age-associated damage at the cellular and tissue levels. In fact, the accumulation of endogenous oxygen radicals generated in cells and the consequent oxidative modification of biological molecules (lipids, proteins, and nucleic acids) have been indicated as responsible for the aging and death of all living beings [22, 24].

The most important source of antioxidants is provided by nutrition. The nutritional antioxidants act through different mechanisms and in different compartments, but are mainly free radical scavengers: (1) they directly neutralize free radicals, (2) they reduce the peroxide concentrations and repair oxidized membranes (3), they quench iron to decrease reactive oxygen species production (4) via lipid metabolism, short chain free fatty acids and cholesterol esters neutralize reactive oxygen species [25].

CONCLUSION

In our study, free radical scavenging activities of three different plants were investigated, out of which jamun plant showed the most promising activity. This plant showed maximum free radical scavenging activity as measured by DPPH and FRAP assay. The minimum concentration of TBA-reactive substance indicates free radical formation and lowest concentration of Lipofuscin in the aged cells. This plant can be subjected to further testing and isolation of the active compounds.

CONFLICT OF INTERESTS

The authors declare that we have no conflict of interest.

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