Assessment of antioxidant enzymes and free radical scavenging activity of selected medicinal plants

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ABSTRACT

Indian medicinal plants (*Cassia fistula*, *Cinnamomum cassia*, *Acacia catechu* and *Citrus limon*) were analyzed to study their antioxidant activity. Preliminary phytochemical screening revealed that *C. fistula* and *C. cassia* are fairly richer source of alkaloids and flavonoids than the others plants while least amount of Saponins, tannins and terpenoids were observed in all studied plants. The highest free radical scavenging activity was observed in *Cassia fistula* (91.66 ± 4.33) whereas the lowest was noted in *Citrus limon* (67.33 ± 3.33). The activities of enzymatic antioxidants: superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and polyphenol oxidase were assayed and found significantly higher ($P < 0.05$) i.e. 2.29, 1.196, 0.746, 0.951 (units/g dry tissues) and 15.15 (min/g dry tissue) respectively in *Cassia fistula* than the others, where *C. cassia* was recorded next plant in order to be better-off in the entire enzyme activity assessed except ascorbate peroxidase. The results provided the evidence that the studied medicinal plants are to be potent source of natural antioxidant and medicinally important compounds.

Keywords: Antioxidant activity, ascorbate peroxidase, catalase, superoxide dismutase, phytochemical screening.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from medicinal plants. Phytochemical compounds are found in plants that are not required for normal functioning of the body, but have a beneficial effect on health or play an active role in amelioration of diseases. The effectiveness phytochemicals in the treatment of various diseases may lie in their antioxidant effects.[1] Oxygen is an element obligatory for life; living systems have evolved to survive in the presence of molecular oxygen and for most biological systems. Oxidative properties of oxygen play a vital role in diverse biological phenomena. Oxygen has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events.[2]

A free radical may defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital and are capable of independent existence.[3] Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are describes free radicals and other non-radical reactive derivatives. The reactivity of radicals is generally stronger than non-radical species though radicals are less stable.[4] Free radicals are formed from molecules by the homolytic cleavage of a chemical bond and via redox reactions, once formed these highly reactive radicals can start a chain reaction.[5–6] Oxidative stress can cause damage to all molecular targets; DNA, proteins and lipids often it is not clear which is the first point of attack, since injury mechanisms overlap

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widely. The primary cellular target of oxidative stress can vary; DNA is an important early target of damage.[7]

Oxidative stress plays a role in inflammation, accelerates aging and contributes in variety of degenerative conditions as cardiovascular diseases, atherosclerosis, cancer, cataract, central nervous system disorders, rheumatoid arthritis, diabetes, liver diseases and AIDS.[8]

Cells manifest potent antioxidant defenses against oxidative stress, counting detoxifying enzymes and exogenous free radical scavengers. The major enzymes that convert reactive oxygen species to less reactive molecules are superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase.[9] The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2, and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. For example, when CAT activity was reduced in plants, scavenging enzymes such as APX and GPX were upregulated. Unexpected effects can also occur. When compared to plants with suppressed CAT, plants lacking both APX and CAT were less sensitive to oxidative stress.[10] *Cassia fistula* Linn. (Family-Leguminosae) is of great therapeutic value in treating diseases such as liver disorder, gout, dysentery, leprosy and diabetes. It also helps in shrinking engorged veins and has a powerful antioxidant and anti-inflammatory effect.[11] *Acacia catechu* is highly effective in a wide spectrum of diseases and reported to posses antibacterial, antifungal, anti-inflammatory and antioxidant properties.[12] *Cinnamomum cassia* (Family-Lauraceae) bark is attributed with numerous medicinal uses such as diaphoretic, antipyretic and analgesic. It is used as a carminative, cardiac stimulant, antioxidant, refrigerant and diuretic.[13] *Citrus limon* (Family-Rutaceae) its therapeutic effects have been attributed to its vitamin C rich fruit pulp. The seeds and bark are used in the treatment of asthma, bronchitis, scurvy, rheumatism, dysentery and diarrhea, it also posses bactericidal and antioxidant properties.

MATERIAL AND METHODS

Plant material

The bark of four medicinal plants namely *Cassia fistula, Cinnamomum cassia, Acacia catechu* and *Citrus limon* were collected from Auraon Research Center, National Botanical Research Institute, Lucknow. The plant materials were cleaned and powdered.

Preparation of extract

The plant materials were air-dried in shed at room temperature (26°C) for 2 weeks, after which it was grinded to a uniform powder. Methanol extracts were prepared by soaking 100 g each of the dry powdered plant materials in 1 litre of methanol at room temperature for 48 h. The extracts were filtered first through a Whatmann filter paper No. 42 (125 mm) and then through cotton wool. The extracts were concentrated using a rotary evaporator with the hot water bath set at 40°C. The percentage yield of extracts ranged from 5–20% (w/w).

Preliminary phytochemical screening

The preliminary phytochemical screening was carried out on the methanolic extract of bark of studied plants for qualitative identification. The tests for common phytochemicals were carried out by standard methods described in practical pharmacognosy by Khandelwal.[14]

Free radical scavenging activity

The free radical scavenging activity by different plant extracts was done according to the method reported by Gyamfi et al.[15] 50 µl of the plant extract in methanol, yielding 100 µg/ml respectively in each reaction was mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µl) only was used as control of experiment. After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured reading the absorbance at 517 nm. L-Ascorbic acid and BHT used as controls.

The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = [\text{Absorbance of control} - \text{Absorbance of test sample}/\text{Absorbance of control}] \times 100$$

Enzymatic antioxidants

The enzymatic antioxidants assayed were superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and polyphenol oxidase.

Superoxide dismutase

Assay of Superoxide dismutase was carried according to the method of Beauchamp and Fedovich.[16] To 0.5 ml of plant extract, 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 µM NBT and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1 mM Hydroxylamine hydrochloride and the absorbance

was read at 560 nm using spectrophotometer at 1 min intervals. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

Catalase

Catalase activity was determined by the titrimetric method described by Chance and Maehly.[17] To 1 ml plant extract, 5 ml of 300 µM phosphate buffer (pH 6.8) containing 100 µM hydrogen peroxide (H_2O_2) was added and left at 25°C for 1 min. The reaction was arrested by adding 10 ml of 2% H_2SO_4, and residual H_2O_2 was titrated with potassium permanganate (0.01 N) till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of µM H_2O_2 per min per mg protein.

Ascorbate peroxidase

Ascorbate oxidase activity was measured according to Diallinas et al.[18] In 1.0 ml of reaction mixture contained 20 mM potassium phosphate buffer (pH 7.0) and 2.5 mM ascorbic acid. The reaction was initiated with the addition of 10 µl plant extract. The decreased in absorbance was observed for 3 min at 265 nm due to ascorbate oxidation and calculated using extinction coefficient, 14 $mM^{-1}cm^{-1}$.

Glutathione reductase

The activity of glutathione reductase (GR) was assayed using the method that was described by Carlberg and Mannervik.[19] The GR assay was performed in a cuvette that contained 1M Tris-HCl buffer + 5mM EDTA (pH 8.0), 0.033 M GSSG, 2 mM NADPH, and a 20 µl plant extract in a final volume of 1.0 mL. The decrease in absorbance, which reflects the oxidation of NADPH during reduction of GSSG by GR present in the sample, was monitored spectrophotometrically at 340 nm. Results were expressed as units of GR activity/mg cell protein.

Polyphenol oxidase

The polyphenol oxidase activity was measured by the method of Mahadevan and Sridhar[20] using catechol as a substrate. The reaction mixture contained 3.0 ml of phosphate buffer, 1.0 ml of 0.01 M catechol in phosphate buffer and 2.0 ml of the plant extract. Changes in absorbance were recorded in a UV Vis spectrophotometer at 495 nm for 3 min at an interval of 1 min. The enzyme activity was expressed as changes in absorbance in min/g dry tissue.

RESULTS

Multifarious antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. These include antioxidant enzymes, non enzymatic antioxidants and secondary metabolites.

Preliminary phytochemical screening of the studied plants (*Cassia fistula*, *Cinnamomum cassia*, *Acacia catechu*, and *Citrus limon*) revealed the presence of phytochemicals: alkaloids, saponins, flavonoids, tannins, steroids, terpenoids and terpenoids (Table 1). *Cassia fistula* and *Cinnamomum cassia* showed comparatively higher concentration of Alkaloids and Flavanoids than the *Acacia catechu* and *Citrus limon* plants. Saponins, Tannins and Terpenoids were seen in almost same concentration in all tested plants. Steroid was found to be absent in *Cinnamomum cassia* and *Acacia catechu* and Glycosides in *Acacia catechu* and *Citrus limon*. Among all four medicinal plants used in the study, the maximum free radical scavenging activity 91.66 ± 4.33% (DPPH percentage inhibition) was observed in *Cassia fistula* followed by c*innamomum cassia* (86.6 ± 4.33% DW), *Acacia catechu* (76 ± 1.66% DW) and the least activity was noted in *Citrus limon* with the activity 67.33 ± 3.33% DW (Table 2). The enzymatic antioxidants evaluated in the plants are represented in Table 3. Superoxide dismutase (SOD) activity was found to be significantly higher ($P < 0.05$) in *Cassia fistula* (2.29 ± 0.325 units/g

Table 1 Phytochemical screening of Indian medicinal plants

Plants name Phytochemical constituents	Cassia fistula	Cinnamomum cassia	Acacia catechu	Citrus limon
Alkaloids	+++	+++	++	+
Flavanoids	++++	+++	+	++
Saponins	++	++	+	+
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Steroid	+	-	-	+
Glycosides	+	+	-	-

Table 2 Free radical scavenging activity in the bark of Indian medicinal plants

Plant names	Antioxidant activity (% DW)
Casia fistula	91.66 ± 4.33
Acacia catechu	76 ± 1.66
Cinnamomum casia	86.66 ± 4.33
Citrus limon	67.33 ± 3.33
BHT	78.30 ± 1.422
Ascorbic acid	76.71 ± 1.830

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

dry tissues) as compared to other studied plants whereas least SOD activity was assessed in *Citrus limon* (1.24 ± 0.070 units/g dry tissue). However, C*innamomum cassia* had possessed significantly higher SOD activity (1.72 ± 0.211 units/g dry tissue) than the *Acacia catechu* (1.34 ± 0.094 units/g dry tissue). Catalase activity was also observed to be significantly higher (P < 0.05) in *Cassia fistula* (1.196 ± 0.065 units/g dry tissue) followed by C*innamomum cassia* (0.872 ± 0.013 units/g dry tissue), *Acacia catechu* (0.545 ± 0.029 units/g dry tissue) and *Citrus limon* (0.348 ± 0.025 units/g dry tissue). The variable activity of Glutathione reductase (GR) was observed in the bark of studied plants. The *Cassia fistula* showed highest activity (0.951 ± 0.047 units/g dry tissues) as compared to other three medicinal plants and lowest activity was examined to be 0.445 ± 0.031 units/g dry tissue in *Acacia catechu*. C*innamomum cassia* displayed a bit higher GR activity (0.774 ± 0.030 units/g dry tissue) than *Citrus limon* (0.610 ± 0.009 units/g dry tissue). Ascorbate peroxidase (APX) activity, on the other hand, was recorded to be significantly higher (P < 0.05) in *Cassia fistula* (0.746 ± 0.009 units/g dry tissue) followed by *Citrus limon* (0.661 ± 0.045 units/g dry tissue), C*innamomum cassia* (0.545 ± 0.033 units/g dry tissue) and *Acacia catechu* (0.349 ± 0.024 units/g dry tissue). Polyphenol oxidase (PPO) was also found to significantly higher (P < 0.05) in *Cassia fistula* (15.15±1.64 min/g dry tissue) and lowest activity was observed to be in *Citrus limon* (4.17 ± 0.241 min/g dry tissue). *Cinnamomum cassia* (10.42 ± 0.905 min/g dry tissue) had approximately two fold PPO activities than *Acacia catechu* (6.77 ± 0.644 min/g dry tissue).

DISCUSSION

In the present study, four traditionally used medicinal plants, *Cassia fistula, Cinnamomum cassia, Acacia catechu and Citrus limon*, were evaluated for their antioxidant potential to support the traditional claims of their medicinal uses. The plants were tested for preliminary phytochemical screening, free radical scavenging activity and antioxidant enzymatic potentials.

Free radicals, oxidative stress and damage are implicated in many diseases such as ageing, stroke, asthma, cancer, atherosclerosis, diabetes and arthritis. Antioxidants can be given as food supplements to regulate such conditions.[21–22] Due to adverse effects associated with synthetic antioxidants, there is a quest for the search of a potent natural antioxidant.[23] Natural antioxidants can be classified as primary (chain-breaking) antioxidants, which can react directly with lipid radicals and convert them into stable products, or as secondary (preventive) antioxidants, which can lower the rate of oxidation by different mechanisms.[24]

The present study shows that alkaloids saponins, flavonoids, tannins, terpenoids glycosides and steroids were observed in the bark extract of the medicinal plants. These phytochemicals exhibit various pharmacological and biochemical actions when ingested by animals. Alkaloids are beneficial chemicals to plants. They affect glucagon, thyroid stimulating hormone and inhibit certain mammalian enzymatic activities.[25] Steroidal saponins and alkaloids such as ergot alkaloids have been reported to elicit uterine muscle activity.[26] Saponin is a known anti-nutritional factor that can reduce the uptake of certain nutrients including cholesterol and glucose at the gut through intra luminal physicochemical interaction or other yet unidentified activity.[27] The plants bark extract also contain flavonoids, which are phenolic compounds that serve as flavouring ingredients of spices and vegetables.[28] Flavonoids and other phenolic derivatives have been identified in *S. mombin* leaves with anti-herpes, antioxidant and anti-aging properties.[29] Furthermore, flavonoids, alkaloids and tannins, terpenoids, glycosides

Table 3 Specific activity of antioxidant enzymes in dry bark extract of Indian medicinal plants

Plants name	Superoxide dismutase (SOD) (units/g dry tissues)	Catalase (CAT) (units/g dry tissues)	Glutathione reductase (GR) (units/g dry tissues)	Ascorbate peroxidase (APX) (units/g dry tissues)	Polyphenol (oxidase (min/g dry tissue)
Casia Fistula	2.29 ± 0.325	1.196 ± 0.065	0.951 ± 0.047	0.746 ± 0.014	15.15 ± 1.64
Acacia catechu	1.34 ± 0.094	0.545 ± 0.029	0.445 ± 0.031	0.349 ± 0.024	6.77 ± 0.644
Cinammomum cassia	1.72 ± 0.211	0.872 ± 0.013	0.774 ± 0.030	0.545 ± 0.033	10.42 ± 0.905
Citrus limon	1.24 ± 0.070	0.348 ± 0.025	0.610 ± 0.009	0.661 ± 0.045	4.17 ± 0.241

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

and steroids observed in the plant had lower concentrations and have been associated with the observed antioxidant effects and antimicrobial effects in various studies involving plant extracts.[30]

The DPPH radical has been used widely to test the potential of the compounds as free radical scavengers of hydrogen donors and to investigate the antioxidant activity of plant extracts. The DPPH free radical scavenging activity is due to the neutralization of DPPH free radical by extract either by transfer of hydrogen or of an electron.[31] Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit the lipid oxidation.[32] *Cassia fistula* (91.66 ± 4.33%) showed high DPPH radical scavenging activity than other studied medicinal plants and standard antioxidants, BHT and ascorbic acid showed 78.30±1.422% and 76.71±1.830% of inhibition respectively (Figure 1).

Reactive oxygen species (ROS) generates due to many factors such as drought, cold, heat, herbicides and heavy metals, all of these factors lead to increasing number and accumulation of ROS in plant cells.[33] Scientific research shows that ROS are harmful to the cell because they can raise the oxidative level through loss of cellular structure and function.[34] ROS detoxification agents in cells include antioxidative enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and polyphenol oxidase. Enzymatic antioxidants serve as an intrinsic defense tool to resist oxidative damage in plants.[35] One of the mechanisms *in vivo* is improving the endogenous cellular antioxidants mechanisms, such as up-regulation of the activity of superoxide dismutase.[36]

SOD is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species that are generated during aerobic respiration for energy production; it converts more toxic superoxide anion radicals to less toxic hydrogen peroxide.[37]

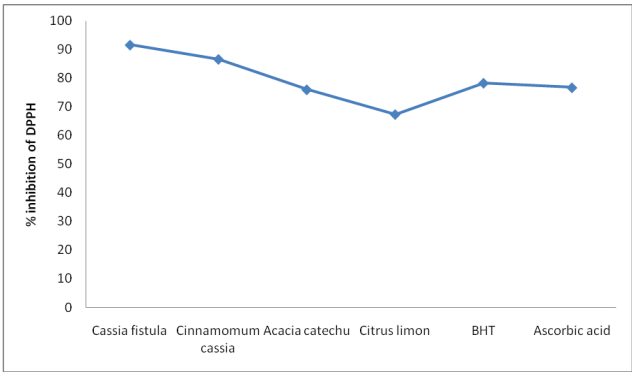

Figure 1. DPPH % inhibition of medicinal plant extracts.

Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen.[38] In the studied medicinal plants *Cassia fistula* showed higher catalase activity to other plants, it protects plant from biotic and abiotic stress. Ascorbate peroxidase functions as hydrogen peroxide detoxification and gluthathione regeneration via ascorbate-gluthathione pathway. Ascorbate peroxidase is able to scavenge hydrogen peroxide produced by superoxide dismutase using ascorbate as an electron donor.[33] Glutathione reductase and glutathione (GSH, γ-Glu-Cys-Gly) are important components of the cell's scavenging system for reactive oxygen compounds in plants. GSH is a major reservoir of non protein reduced sulfur. In addition, GSH plays a crucial role in cellular defense, where it gets oxidized to glutathione disulfide (GSSG). GR mediates the reduction of GSSG to GSH by using NADPH as an electron donor, and thus a highly reduced state of GSH/GSSG and ASA/DHA ratios is maintained at the intracellular level by this reaction during oxidative stress.[39]

CONCLUSION

The present study demonstrate that bark extracts of *Cassia fistula*, *Cinnamomum cassia*, *Acacia catechu* and *Citrus limon* have promising antioxidants and free radical scavenging activities and the difference in their antioxidant activities can be attributed to their difference in phenolic content. From the observations it can be concluded that the bark of *C. fistula*, *C. cassia*, *A. catechu* and *C. limon* are the good sources of natural antioxidants and might be useful in treating the diseases associated with oxidative stress.

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