# *In vitro* study of antioxidant activity *of Aegel marmelos,* Cassia *fistula* and *Psida cordifolia*

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**Abstract**

Traditional pharmacopoeias included the discoveries of novel compounds isolated from plants with pharmaceutical value. Several medicinal plants extracts were screened for antibacterial, anti-inflammatory, antioxidant, anthelmintic, anti-amoebic, antischistosomal and antimalarial activity, as well as psychotropic and neurotropic properties. Medicinal plants and their products are used as antioxidants in traditional and modern medicines with prior scientific authentication.In the present study, attempts have been made to elucidate and validate the antioxidant activity of the knowledge related to the comparative bioprospection of medicinal plants *Aegel marmelos,* Cassia *fistula* and *Psida cordifolia*. Total phenolic and flavonoids content, free radical scavenging activity, superoxide dismutase, catalase activities estimated and comparatively evaluated in plants selected.

**Introduction**

Renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. Instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Gordon and David, 2001).

Already an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Nadkarni, 2006). Various assays can be used to test for biological activity, firstly *in vitro* and later, for promising natural products, *in vivo*. Crude or fractionated extracts and sometimes individual compounds were screened for antibacterial, anti-inflammatory, antioxidant, anthelmintic, anti-amoebic, antischistosomal and antimalarial activity, as well as psychotropic and neurotropic properties. In testing for biological activity *in vitro*, a standard drug is always included in the test system to ensure that the assay is working effectively. The activity of an extract can then also be compared between different assays, although not with pure standards, as crude extracts contain a myriad of compounds that may be acting synergistically (Murthy and Pandey, 1983). The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also cause for alarm. Nowadays, medicinal plants and their products are used as antioxidants in

traditional and modern medicines with prior scientific authentication. The claimed therapeutic properties of these plants could be due in part to their capacity for scavenging oxygen free radicals which may be involved in many diseases. For example, in the case of plants used to treat inflammatory diseases and gastric ulcers.

Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an efficient antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases (Guyton and Hall, 1998).Oxygen is absolutely necessary for the life processes, in particular cell respiration. However, the metabolism of oxygen may generate reactive elements called free radicals, in particular the superoxide ion ($O^{2-}$) and the hydroxyl ion ($OH^{-}$). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms, while damage to proteins causes enzyme inhibition, denaturation and protein degradation, which may lead to cell death. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Valko et al, 2004).

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants to minimize this ill effect. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. As oxidative stress appears to 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. Moreover, oxidative stress is both the cause and the consequence of disease. In so-called “physiological conditions” there is a balance between the production of free radicals and antioxidant endogenous defense mechanisms. These mechanisms mainly involve specific enzymes viz, superoxide dismutase or SOD, catalase, gluthation peroxidase or Gpx as well radical scavengers such as antioxidant vitamins A, C, E, thiols and ß-carotene that trap free radicals (Vouldoukis, 2004).

The important role of oxidative stress is well known in a great many diseases: neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease), atherosclerosis, rheumatoid arthritis, Cohn’s disease and even certain cancers. Free radicals are also known to contribute to the aging process. For this reason, we are currently witnessing the development of a great many antioxidant products (functional food and drugs). However, their bioactivity with oral administration is often low, thereby limiting their efficacy. In addition, the products available on the market are made to correct a possible deficiency and do not specifically stimulate the antioxidant endogenous defenses.

The determinant role of superoxide dismutase (SOD) in the antioxidant defense systems has been known since 1968. It is well known that superoxide ion ($O_2^-$) is the starting point in the chain production of free radicals. At this early stage, superoxide dismutase inactivates the superoxide ion by transforming it into hydrogen peroxide ($H_2O_2$). The latter is then quickly catabolised by catalase and peroxidases into dioxygen ($O_2$) and water ($H_2O$).Superoxide dismutase enzymes contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the cytosol, while manganese SOD is present in the mitochondrion (Zelko et al, 2002). There also exists a third form of SOD in extracellular fluids, which contains copper and zinc in its active sites. The mitochondrial isozyme seems to be the most biologically important of these three, since mice lacking this enzyme die soon after birth. In contrast, the mice lacking copper/zinc SOD (Sod1) are viable but have numerous pathologies and a reduced lifespan, while mice without the extracellular SOD have minimal defects (sensitive to hyperoxia).

Catalases are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase - "acatalasemia"- or mice genetically engineered to lack catalase completely, suffer few ill effects (Ogata, 1991; Mueller, 1997; Ravishankar et al, 2002).

In the present study, attempts have been made to enrich the knowledge related to the comparative bioprospection of medicinal plants *Aegel marmelos,* Cassia *fistula* and *Psida cordifolia* for its antioxidant potential.

## Methods and materials

### Collection of plant material

Plants materials of *Aegel marmelos* Linn. (Rutaceae local name Bel), *Cassia fistula* Linn. (Caesalapiniaceae, local name Bahava) and *Psida cordifolia* Linn. (Tiliceae, local name Chikna) (Fig 1.,a,b,c) collected from botanic gardon of the MGSM's Institute, Chopda to avoid any conflict with Forest officers in Satpuda ranges so far exploitation and conservation of Biodiversity is concerned. The plant materials cleaned with distilled water and shade dried at room temperature as per routine herbarium methods and they were authenticated by Dr.R.M.Bagul, Head, Department of Botany and species specific voucher numbers were given to each specimen and they were deposited in the PG research center, Department of Zoology, MGSM's ASC College, Chopda for further study .

(a) (b) (c)

Figure 1 Photographs of (a)*Aegel marmelos,* (b) *Cassia fistula* & (c) *Psida cordifolia*

All the experimental processes and protocols to assess the activities of plants on model animals used in this study were reviewed by the Committee for purpose of Control and Supervison on Experiments on Animals (CPCSEA) and Institutional Animal Ethical Committee (IAEC) established in College of Pharmacy, the sister institute present in the campus of M.G.S. Mandal, Chopda (District. Jalgaon, Maharashtra India) and the experiments with animals were conducted according to the guideline of the Animal Ethical Committee (LAEC/CPD/415/6 dated 25.2.2018).

**Methods Used For Evaluating Antioxidant Potential of Plants**

**Extraction procedure:** Air shade dried and pulverized material (60.0 g) of leaves of each plant under study was used. It was extracted successfully with methanol (360 ml, 1:6 w/v) by keeping it for 72 hours at room temperature. It was filtered using Whatman no.1 filter paper. The solvent was evaporated to dryness in vacuum using a rotary evaporator to yield crude methanol extract (5.8%). The methanol extracts of individual plants species was used for total content of phenols, flavonoids and for the assessment of antioxidant capacity.

**Estimation of total phenolic content:** The total phenolic contents of methanol extract of each plant species were determined according to the method developed by Malik and Singh (1980). The Folin Ciocalteau reagent and sodium carbonate were added in alkaline solution of test sample. A blue coloured complex was developed due to phosphomolybdic acid, which is present in Folin-Ciocalteu reagent. Calibration plot was expressed as pyrrocatechol (2-10 µg/ml) equivalent of phenol per gram of sample. Experiments were performed in triplicates and results were recorded as mean ± SEM.

**Estimation of total flavonoid content:** Aluminum chloride colorimetric method was used for flavonoids determination (Chang et al, 2002). Each extract of the plant material (0.5 ml of 1:10 g/ml) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a

double beam spectrophotometer (BioEra, Pune). The calibration plot was generated by using quercetin solution at concentrations 12.5 to 100 µg/ml in methanol.

**Evaluation of *in vitro* antioxidant activity :** *In vitro* antioxidant activity of the plant material Cassia *fistula*, *Psida cordifolia,* and *Aegel marmelos* was carried out the process as explained by Lobo et al (2010).

The DPPH (2, 2-Diphenyl -1- picrylhydrazyl) assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet colour.

DPPH (4.3 mg) dissolved in methanol (6.6 ml); it was protected from light by covering the test tubes with aluminum foil. DPPH solution (150 µl) was added to 3ml methanol and absorbance was noticed immediately at 516nm for control reading. A different volume of test samples i.e. 50 µl, 100 µl, 150 µl, 200 µl, 250 µl and 300 µl taken. Each of the sample was diluted with methanol up to 3ml and to it 150 µl DPPH was added. Absorbance was observed after 15 minutes at 516 nm using methanol as blank. $IC_{50}$ values for the samples were calculated and compared with Ascorbic acid as a positive control. The % reduction and $IC_{50}$ values were calculated. The free radical scavenging activity (% antiradical activity) was calculated using the equation:

% Antiradical Activity = Control Absorbance - Sample Absorbance/Control Absorbance × 100

**Enzymatic Methods for Evaluation of Antioxidant Potential of Plants under study:**

**Assay of Superoxide Dismutase (Sod) :** SOD was assayed according to the method of Poonam *et al.* (1984). The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon (NBT-complex) by the enzyme. The colour formed at the end of the reaction can be extracted into butanol and measured at 550 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-complex formation. The extent of reduction in the appearance of NBT-complex is a measure of SOD activity present in an experimental sample

**Preparation of Enzyme Extract**

The different samples, namely leaves and seeds (0.5g) of each plant under study were ground with 3.0ml of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were used for the assay. Prior to experimentations, the standard curve for SOD activity was prepared using SOD

(1 unit/µL, Himedia) as a positive control. The SOD has a concentration of 1 unit/µL (1 unit is that amount of SOD which inhibits the rate of increase in absorbance due to NBT-complex formation by 50%). A typical standard curve preparation included the following SOD concentrations: 0.1 unit, 1 unit, 2 units, 5 units, and 10 units. For the 0.1 unit point, diluted 1 µL of SOD to 10 µL with 1X Reaction Buffer and 1 µL. Add 1 µL, 2µL, 5 µL, and 10µL of undiluted SOD were used for the 1 unit, 2 unit, 5 unit, and 10 unit activity points, respectively.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation (for evaluating the potential of plant extract for SOD enzyme, the concentration of enzyme preparation was kept constant in each estimate) and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 550 nm in a spectrophotometer (BioEra, Pune). One unit of enzyme activity (IU) is defined as the amount of enzyme that gave % inhibition of NBT reduction in unit time.

**Assay of catalase (CAT)** Catalase activity was assayed following the method of Antony et al (2001). The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

**PREPARATION OF ENZYME EXTRACT**

A 20% homogenate of the leaves and seeds of *Aegel marmelos*, Cassia *fistula* and *Psida cordifolia* was prepared in phosphate buffer separately. The homogenate was centrifuged and the supernatant was used for the enzyme assay. $H_2O_2$-phosphate buffer (3.0 ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer (BioEra, Pune). The enzyme solution containing $H_2O_2$-free phosphate buffer served as control. One unit of enzyme activity (IU) was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

**Statistical methods adopted:**

Each experiment was carried out in triplicates and results were recorded as mean % antiradical activity ± SEM. The observed data was analyzed by an analysis of variance i.e. one way ANOVA and student 't' test using GraphPad Prism 6.0. The two-tailed $P< 0.05$ the difference was considered to be statistically significant and if $P< 0.001$ the difference was considered to be extremely statistically significant. The $IC_{50}$ values were calculated from linear regression analysis.

**Results and Discussion:**

**Total Phenolic and Flavonoid content:**

The amount of total phenolic and flavonoids for the test samples prepared from leaves and seeds of *Aegel marmelos*, Cassia *fistula* and *Psida cordifolia* are given in Table 1. The observed data indicated that the total phenolic content in leaves and seeds of *A.marmelos* was 35.45± 2.3 mg/g and 24.65±3.3 mg/g respectively. These estimated values implied that leaves had higher phenolic compounds than seeds. The total phenlic content in leaves and seeds of *C.fistula* was 46.68±4.1 mg/g and 28.89±3.2 mg/g respectively, indicating that leaves had higher phenols. The total phenolic content in leaves of *P. cordifolia* was 56.79± 5.4. Evaluation of plants for its total phenolic content revealed that leaves of *P. cordifolia* had higher potential as compared to that of *A. marmelos* and *C.fistula.*

Table 1 Total Phenolic and Falvonoid content (mg/g ± SEM) in *Aegel marmelos*, Cassia *fistula* and *Psida cordifolia*

| | *Aegel marmelos* | Cassia *fistula* | *Psida cordifolia* |
|---|---|---|---|
| Total Phenolic content | | | |
| Leaves | 35.45± 2.3 | 46.68±4.1 | 56.79±5.4 |
| Seeds | 24.65±3.3 | 28.89±3.2 | - |
| Total Flavonoid content | | | |
| Leaves | 68.56±5.4 | 54.65±5.5 | 74.35±6.4 |
| Seeds | 31.87±2.5 | 39.33±4.3 | - |

Each value represents mean± SEM (n=3)

The total phenolic content of standard catechol vs. absorbance is given in figure 2 with its regression equation and $R^2$ value.

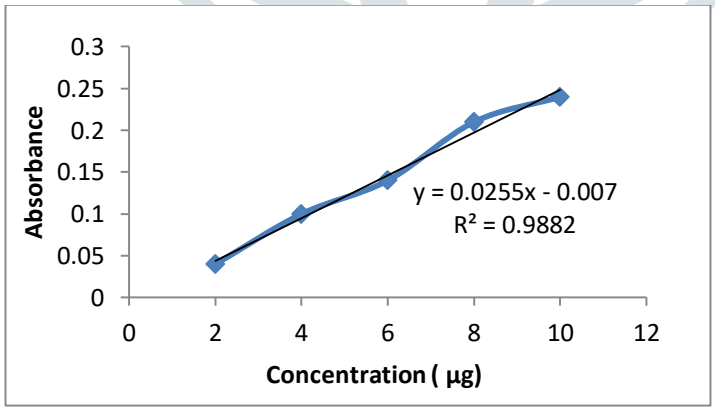


Figure 2 Total Phenolic content of standard catechol

During evaluation of plant potential for their total flavonoid content, it was found that in leaves and seed of *Aegel marmelos*, total flavonoids were 68.56±5.4 and 31.87±2.5 mg/g respectively and thus leaves had higher potential for its flavonoid content (Figure 3). The leaves and seeds of Cassia *fistula* had total flavonoids 54.65±5.5 mg/g and 39.33±4.3 mg/g respectively. These figures also indicated that leaves of *C. fistula* had higher potential for falconoid content than its seeds. Whereas

the leaves of *P. carodifolia* had 74.35±6.4 mg/g of total flavonoids. The estimated values of total flavonoid content in leaves and seeds of three plants under study revealed that leaves of *P. carodifolia* had higher potential than that of *A. marmelos* and *C.fistula.* The total flavonoid content of standard quercetin vs. absorbance is given in figure 4.4 with its regression equation and $R^2$ value. Kamble et al (2011) noted resembling results during *in-vitro* evaluation of antioxidative activity of leaves of *Ehretia laevis*.

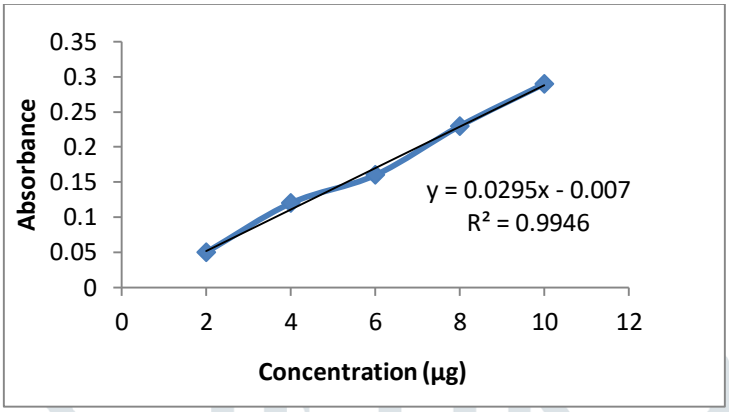


Figure 3 Total Falvonoid content of standard quercetin

**Free radical scavenging activity**

DPPH radical scavenging activity has been widely used to evaluate the antioxidant activity of plant extracts and foods (Upadhya *et al*., 1997). The presence of antioxidant in the plant extract reacts with DPPH, which is a stable free radical, and convert it to 1,1-diphenyl-2-(2,4,6- tri nitrophenyl) hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant compounds which was be detected at 516 nm.The antioxidant activities due to ascorbic acid and methanolic extracts of plants under study by DPPH free radical method are given in Table 2. Estimated values of free radical scavenging activity in leaves and seeds of *A. marmelos* were 28.44 ± 0.92µg/ml and 21.88 ± 0.13µg/ml respectively. Antioxidant activity in leaves and seeds of *C.fistula* was 25.02 ± 0.72 µg/ml and 16.48 ± 0.17 µg/ml respectively. Whereas methanolic extract of leaves of *P.cardifolia* showed 6.25 ± 0.41 µg/ml free radical scavenging activity.

Table 2: $IC_{50}$ values (µg/ml) ascorbic acid and methanolic extracts of leaves and seeds of plants determined by DPPH method with regression equation and $R^2$

| Parameters | | $IC_{50}$ (µg/ml) | Y= ax +b | $R^2$ |
|---|---|---|---|---|
| Ascorbic acid | | 3.03 ± 0.06 | y = 5.147 x - 0.82 | 0.997* |
| *Aegel marmelos* | Leaves | 28.44 ± 0.92 | y = 0.46 x + 28.8 | 0.987* |
| | Seeds | 21.88 ± 0.13 | y = 1.11 x + 20.1 | 0.897** |
| Cassia *fistula* | Leaves | 25.02 ± 0.72 | y = 2.31 x + 30.2 | 0.893** |
| | Seeds | 16.48 ± 0.17 | y = 3.05 x + 19.6 | 0.942* |
| *Psida cardifolia* | Leaves | 6.25 ± 0.41 | y = 1.33 x + 7.3 | 0.995* |

$IC_{50}$ values are mean of three replicate determinations (n=3) ± SD.
$IC_{50}$ values compared with Ascorbic acid. * P< 0.001 and **P< 0.05.

Concentration of the sample necessary to decrease initial concentration of DPPH by 50% ($IC_{50}$) under the experimental condition using ascorbic acid and plant eaxtract was calculated. The lower $IC_{50}$value indicates higher antioxidant activity. Methanolic extract of seeds of *A.marmelos* and *C.fistula* showed higher antioxidant activity that their leaves. Experimental data also revealed that methanolic extract of leaves of *P.cardifolia* displayed the highest DPPH scavenging effect followed by the *C. fistula* and *A. marmelos.* The radical scavenging activity of the extracts could be related to the nature of phenolic compounds and flavonoids and their hydrogen donating ability (Shimada *et al*., 1992, Kelm et al, 2000; Lahvale, et al, 2007; Edeoga et al, 2008; Jain et al, 2009).

**SOD and CAT potential of plants**

The enzyme preparations of leaves and seeds of *Aegel marmelos,* Cassia *fistula* and *Psida cordifolia* were evaluated for their super oxide dismutase (SOD) and catalase (CAT) activity. Figure 4 displays a plot of standard SOD (positive control) concentration vs. % inhibition of the rate of increase of absorbance at 560 nm due to the reduction of NBT to NBT-complex by the superoxide radical ($O_2$). While figure 5 displays CAT activity noted as reduction in absorbance per minute in a control condition.

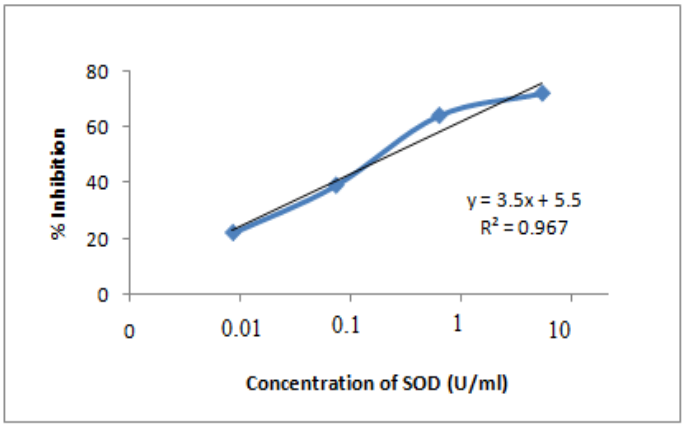


Figure 4. Plot of standard SOD (positive control) concentration vs. % Inhibition of the rate of increase of absorbance at 560 nm due to the reduction of NBT to NBT-complex by the superoxide radical ($O_2$).

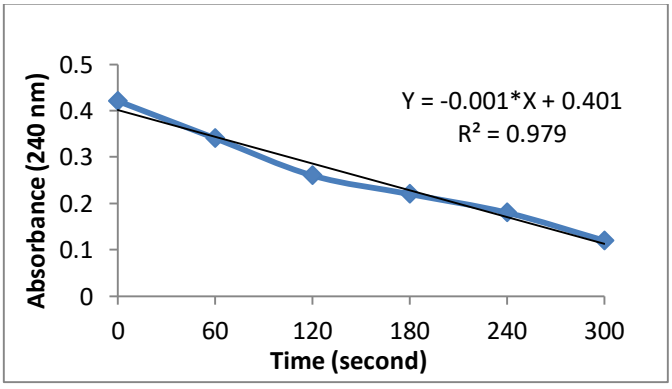


Figure 5. CAT activity noted as reduction in absorbance per minute in a control condition

The *in vitro* assay for SOD and CAT was well recognized and allows for the screening of a large number of plant extracts for their antioxidant activities at a time (Cao et al, 1996; Antony et al, 2010). The improper balance between reactive oxygen intermediates and antioxidants defense results in 'oxidative stress' and it cause damage to the cells. Cellular antioxidant enzymes such as SOD and CAT normally challenge oxidative stress. SOD and CAT activity in leaves and seeds of plants under study is given in table 3.

Table 3. SOD and CAT activity of plant extract

| Plants | | SOD (IU) | CAT (IU) |
|---|---|---|---|
| *Aegel marmelos,* | Leaves | 0.24 ± 0.08 | 0.63 ± 0.12 |
| | Seeds | 0.18 ± 0.05 | 0.37± 0.09 |
| Cassia *fistula* | Leaves | 0.41 ± 0.06 | 0.24 ± 0.16 |
| | Seeds | 0.34 ± 0.04 | 0.29 ± 0.07 |
| *Psida cardifolia* | Leaves | 0.45 ± 0.05 | 0.38 ± 0.11 |

Each value represents mean± SEM (n=3). IU is unit of enzyme.

Pertaining to recorded data, the leaves of *A.marmelos* showed higher SOD and CAT activity than the samples of enzyme preparation made from its seeds. SOD activity in leaves of *C.fistula* was higher than its seeds whereas CAT activity was less in leaves than its seeds. Comparatively higher SOD activity was noted in leaves of *P.cardifolia* than that of *A.marmelos* and *C.fistula* whereas CAT activity was highest in leaves of *A.marmelos* than *C.fistula* and *P.cardifolia.* (Kamalakannan and Mainzen, 2005; Hu et al, 2009).

**Conclusion:**

In an extensive screening programme of plants used in traditional medicine, researchers provided scientific evidence for their rational use in treating infections, inflammation, and human diseases. Using the ethnobotanical approach and bioassay-guided fractionation, several compounds having biological activity were isolated and identified in the past. In the present part of investigation, the leaves and seed extract of *Cassia fistula, Psida cordifolia,* and *Aegel marmelos* exhibited the significant antioxidant activity, which is attributed to presence of phenolic compounds and flavonoids as well enzymes showed promising free radical scavenging activity. The high scavenging activity may also be due to hydroxyl groups existing in the phenolic compounds and chemical structure that can provide the necessary component as a radical scavenger. Further studies to unravel novel treatment strategies for disorders associated with free radicals induced tissue damage or diseases caused due to oxidative stress.

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