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Evaluation of total phenol, total flavonoid content and antioxidant activity of *Careya Arborea* Roxb. Bark and leaves

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Abstract
The medicinal plants have been investigated since last few decades to evaluate their potential antioxidant activities. Many researchers are involved in search of powerful nontoxic antioxidants from natural sources and to evaluate their free radical scavenging properties. *Careya arborea* Roxb. belonging to family Lecythidaceae, is an important medicinal plant and traditionally the plant have been found to be effective in the treatment of tumours, skin diseases, toothache, wounds, colic, ulcers, cough and cold, etc. The present research work was designed to determine total phenol, total flavonoid content and the antioxidant activity of 50% hydro alcoholic extract of stem bark and leaves of *C. arborea*. Total phenolic content of leaves was found to be higher than bark. The antioxidant activity was assessed by Total Antioxidant Capacity (TAC), Reducing Power, FRAP (Ferric ion reducing antioxidant power), DPPH (1, 1- diphenyl-1, 2- picryl hydrazyl), Nitric Oxide and Superoxide Radical Scavenging assays. Leaves extract showed better antioxidant activity as compared to bark in terms of IC_{50} in radical scavenging assays and in FRAP assay. The ferric reducing power of bark and leaves was increasing with the increase in concentration of extracts. The findings of this study suggest that *C. arborea* could be a potential source of natural antioxidant.

Keywords: *Careya arborea*, antioxidant activity, bark, leaves

1. Introduction
Free radicals, commonly known as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are highly reactive species. They are generated *in vivo* by living cells from the normal cell metabolic activities [1]. The ROS includes superoxide anionic radical (O_2^-), hydrogen peroxide (O_2^{-2}), peroxyl (ROO^-) and hydroxyl radicals (·OH) and the nitrogen derived free radicals are nitric oxide and peroxynitrite anion ($ONOO^-$) [2].
Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deadly effects [3]. They are controlled by endogenous enzymes such as glutathione peroxidase, superoxide dismutase, catalase or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione [4]. An imbalance between production of free radicals and antioxidant defences is called oxidative stress [5]. Excess production of free radicals or oxidative stress cause oxidative damage to biomolecules such as lipids, proteins and DNA, eventually leading to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [6,7].
Antioxidants may protect the body against free radicals generated toxicity either by preventing the formation of free radicals, by bringing interruption in free radicals attack, by scavenging the reactive metabolites or by converting them t o less reactive molecules [8]. Currently, synthetic Antioxidants, such as Butylated Hydroxytoluene (BHT), Butylated Hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are widely used in food, health and pharmaceutical industries. However, restriction on the synthetic antioxidants is being imposed because of their toxicity to liver and carcinogenicity [9, 10]. Therefore, the development and utilization of more effective, less toxic and economically viable antioxidants are desired. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical induced tissue damages.
Careya arborea Roxb. (Family: Lecythidaceae) is one of the important medicinal plant known as Sthala kumbhi in Hindi and wild guava in English [11, 12]. It is small to medium sized deciduous tree [13], distributed in different tropical regions of the world like India, Sri Lanka and Malay Peninsula [14]. *C. arborea* Roxb. as a whole plant and its different parts has a long history of utilization for a variety of medicinal uses [15]. It is traditionally used in treatment of sores, ear pain, snake bite, inflammation, piles, tumours, cough and cold, toothache, wounds, bronchitis, colic, intestinal worms, haemorrhoids, dyspepsia, dysentery, spermatorrhoea, leukoderma, epileptic fits, abscesses, ulcers and eruptive fevers particularly smallpox [13, 14, 16, 17].
The use of antioxidants in treatment of oxidative stress-related ailments is a possible therapeutically strategy for the future. Natural product with antioxidant properties could trigger this goal. Therefore, the aim of this *in vitro* study was to assess the antioxidant potential and free radical scavenging activity of the 50% hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves.

2. Materials and Method

2.1 Plant materials

Stem bark and leaves were collected from the forest area of Badlapur, Thane (Maharashtra) and authenticated from Agharkar Research Institute, Pune (Maharashtra, India). Leaves were washed with running tap water to remove any foreign matter and air dried. Bark was also air dried. After drying, both the plant parts were ground into fine powder and stored in airtight container at room temperature.

2.2 Extract preparation

1.0 g of *Careya arborea* Roxb. bark and leaves powder were soaked separately overnight in 10.0 ml of 50% hydro alcohol to get hydro alcoholic extracts. The extracts were filtered through Whatmann filter paper no. 1.

2.3 Chemicals

All the chemicals and reagents including the solvents were of analytical grade and used without further purification. Quercetin, Pyrocatechol, Folin- Colicalteau reagent, Ascorbic acid, Aluminium chloride, Ferrous Sulphate, Potassium ferricyanide, 2,4,6- Tripyridyl-s-Triazine (TPTZ), Trichloroacetic acid, Ferric chloride ($FeCl_3$), Sodium phosphate, Ammonium molybdate [$(NH4)_2MoO_4$], 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Naphthyl ethylene diamine dihydrochloride (NEDD), Sodium nitroprusside ($Na_2[Fe(CN)_5NO$), Sulphanillic acid, Nitro blue tetrazolium (NBT), Phenazine Methosulphate (PMS) and Reduced NADH were purchased from HiMedia, Mumbai (India).

2.4 Determination of total phenolic content

The amount of total phenols in extracts of the plant materials was determined by Folin-Ciocalteau method [18]. An aliquot of the extract and standard (300ul) was added to test tubes followed by 1.5 ml of Folin-Ciocalteau reagent (10 times dilution) and 1.2 ml of 7.5% sodium carbonate. The tubes were vortexed for few seconds and allowed to stand for 30 min for colour development. The absorbance of the resulting blue colour was measured at 765 nm against blank. TPC was expressed as mg pyrocatechol per gram dry weight of the plant material. A calibration curve was also prepared using different concentrations of the standard pyrocatechol.

2.5 Determination of total flavonoid content

Total flavonoid was measured by aluminium chloride colorimetric assay [19]. 1.0 ml of extract and different dilutions of 0.1% standard quercetin were added to 10.0 ml of volumetric flask containing 4.0 ml of water. To the above mixture, 0.3 ml of 5% sodium nitrite was added. After 5 minutes, 0.3 ml of 10% aluminium chloride was added. After 6 minutes, 2.0 ml of 1M sodium hydroxide was added and the total volume was made up to 10.0 ml with distilled water. Then the solution was mixed well and the absorbance was measured against blank at 510 nm. Total flavonoid content of the extracts was expressed as mg Quercetin equivalent per gram dry weight of the plant material. A calibration curve was also prepared using different concentrations of the standard quercetin.

2.6 Determination of antioxidant activity

Total antioxidant capacity (TAC)

The total antioxidant capacity was evaluated by the phosphomolybdenum method [20]. 0.3 ml of extracts and standard ascorbic acid was added to 3.0 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM Ammonium molybdate) in the test tubes. The tubes were capped and incubated at 95ºC for 90 minutes. After cooling at room temperature, the absorbance of the solution was measured at 695 nm against blank. The results of TAC assay were expressed as ascorbic acid equivalent antioxidant capacity (AEAC). A calibration curve was also prepared using different concentrations of the standard ascorbic acid.

Reducing power (Fe^{3+}-Fe^{2+} transformation ability)

The reducing power ability of the extracts was evaluated by the method described by Oyaizu [21]. The reaction mixture contained 1.0 ml of various concentrations of extracts or standard, 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M phosphate buffer (pH 6.6). The mixture was incubated at 50º C for 30 minutes and the reaction was terminated by the addition of 2.5 ml of 10 % trichloro acetic acid, followed by centrifugation at 3000 rpm for 10 minutes. 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blank prepared in the same conditions by replacing sample with 1.0 ml of distilled water. Increase in absorbance indicates increased reducing power of the extracts. Ascorbic acid was used as standard.

Ferric ion reducing antioxidant power (FRAP)

The FRAP assay was performed according to a modified method described by Benzie and Strain [22]. 100 µl of extracts or standard were mixed with 3 ml of FRAP reagent. The reaction mixture was then incubated at 37 ºC for 30 minutes and the absorbance was recorded at 595 nm against the blank that was prepared using distilled water. A calibration curve was generated using ferrous sulphate. FRAP values were expressed as mM of ferrous equivalent per gram of the dry weight of the sample.

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of extracts was examined using DPPH radicals as described by Chen *et al.* [23]. 2.0 ml of various concentrations of sample and standard were mixed with 2 ml of 0.02 M phosphate buffer (pH 6) and 2 ml of 0.2 mM DPPH solution. The mixture was shaken vigorously and left to stand for 30 min at room temperature and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The inhibition percentage for DPPH radical was calculated according to the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control–Absorbance of sample)/Absorbance of control] × 100

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity of the extracts was carried out using sodium nitroprusside. This can be determined by the use of Griess Illosvoy reaction [19]. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 1 ml of extracts and standard at various concentrations and the mixture was incubated at 25° C for 150 minutes. From the incubated mixture, 0.5 ml was taken out and added into 1.0 ml of sulphanillic acid reagent (0.33% in 20% glacial acetic acid)

and further incubated at room temperature for 5 minutes. Finally, 1.0 ml of NEDD was added and maintained at room temperature for 30 minutes. The absorbance was measured at 546 nm. A control solution contained the same solution mixture without plant extract or standard. The percentage inhibition was calculated according to the following equation:

Nitric oxide radical scavenging activity (%) = [(Absorbance of control –Absorbance of sample)/Absorbance of control] × 100

Superoxide anion scavenging assay

1 ml of NBT solution (144µM in 100 mM phosphate buffer, pH 7.4), 1 ml of 677 µM reduced NADH and 0.5 ml of sample and standard were mixed and the reaction mixture was initiated by adding 100 µl of 60 µM PMS solution. The reaction mixture was incubated at 25º C for 5 minutes and the absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture indicated increasing superoxide anion scavenging activity [24]. The percent inhibition was calculated according to the following equation:

Superoxide radical scavenging activity (%) = [(Absorbance of control –Absorbance of sample)/Absorbance of control] × 100

2.7 Statistical Analysis

All the experiments were performed in triplicates using bark and leaves extract and the data were expressed as mean ± SD. Linear regression analysis was used to calculate IC_{50} (the concentration required to scavenge 50% of radicals) for bark and leaves extract.

3. Results and Discussion

3.1 Total phenol content

Many environmental stresses that cause oxidative stress often induce the synthesis of phenolic metabolites in the plants. These phenolic compounds are excellent oxygen radical scavengers. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm [25]. The total phenol content of 50% hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves were found to be 5.79 ± 0.49 and 8.32 ± 0.12 mg Pyrocatechol equivalent per gram dry weight of the plant material, respectively by using the calibration curve of standard pyrocatechol (fig 1).

According to a study conducted by Wadje *et al.* [26], phenolic content of the *Careya arborea* Roxb. leaf extracts were found to diminish in the subsequent order ethyl alcohol >ethyl acetate >chloroform> Petroleum ether. The ethanolic extract exhibited the highest total phenolics content (33.03 ± 1.39 mg gallic acid equivalent/g of extract), followed by ethyl acetate extract (26.36 ± 2.40 mg gallic acid equivalent/g of extract). According to Senthil Kumar *et al.* [27] methanol and aqueous extracts of *C. arborea* Roxb. bark possessed high amount of total phenol content.

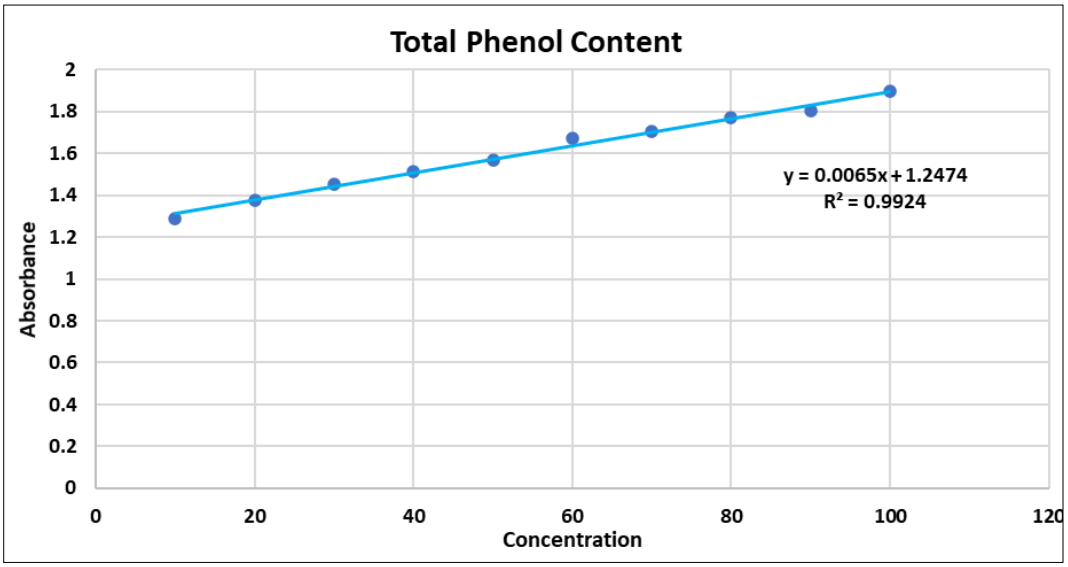

Fig 1: Calibration curve of standard pyrocatechol for determination of total phenol content of *C. arborea* bark and leaves

3.2 Total flavonoid content

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable [28]. The flavonoid content of 50% hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves were found to be 510.2 ± 1.3 mg and 347.7 ± 2.5 mg quercetin equivalent per gram dry weight of the plant material, respectively by using the calibration curve of standard quercetin (fig 2).

According to Wadje *et al.* [26], flavonoid content of the *Careya arborea* Roxb. leaf extracts were found to diminish in the subsequent order ethyl acetate extract >ethyl alcohol >chloroform> Petroleum ether. The content of total flavonoids in all the four extracts varied from 3.33 ± 0.82 to 33.33 ± 2.18 mg as quercetin equivalent/g of extract. According to Wadkar and Magdum [29], aqueous extract and alcoholic extract of *Careya arborea* Roxb. bark showed high amount of phenolic and flavonoid content and hence possessed high antioxidant activity. Petroleum ether extract contain very less amount of phenols and flavonoids so, did not showed any antioxidant activity.

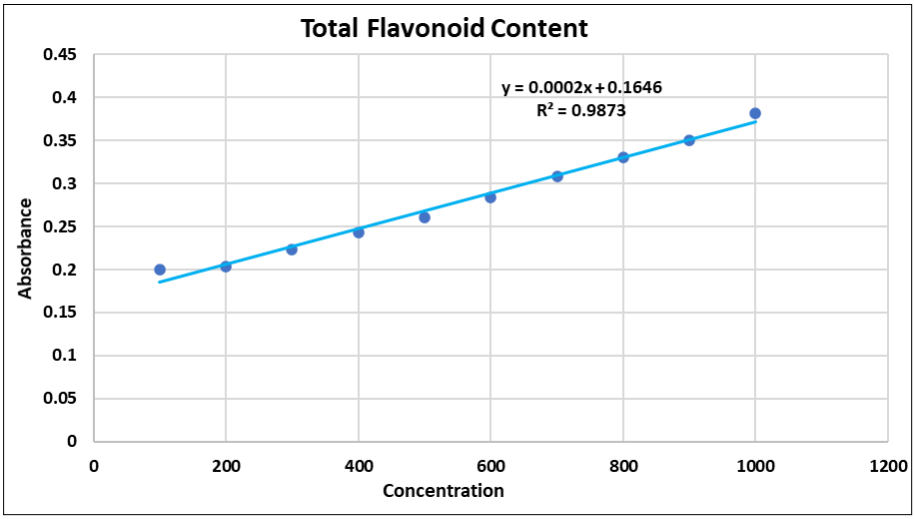

Fig 2: Calibration curve of standard quercetin for determination of total flavonoid content of *C. arborea* bark and leaves

3.3 Total antioxidant capacity (TAC)

The phosphormolybdenum method is an important antioxidant assay based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate/Mo (V) complex with a maximal absorption at 695 nm [30]. The total antioxidant capacity of 50% hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves were found to be 397.07 ± 11.21 and 325.28 ± 9.47 mg Ascorbic acid equivalent per gram dry weight of the plant material, respectively by using the calibration curve of standard ascorbic acid (fig 3). Result suggests that bark has more total antioxidant capacity than leaves.

According to Wadje *et al.* [26], *Careya arborea* Roxb. ethyl acetate leaves extract showed highest total antioxidant capacity (193.11 ± 1.02 mg/g ascorbic acid equivalent) when compared with petroleum ether, chloroform and ethanol extracts. Ramanathan *et al.* [31] studied the antioxidative activity of the methanol extract of *Careya arborea* Roxb. bark by ammonium thiocyanate method. They found the IC_{50} value of methanol extract 36.58 g/ml.

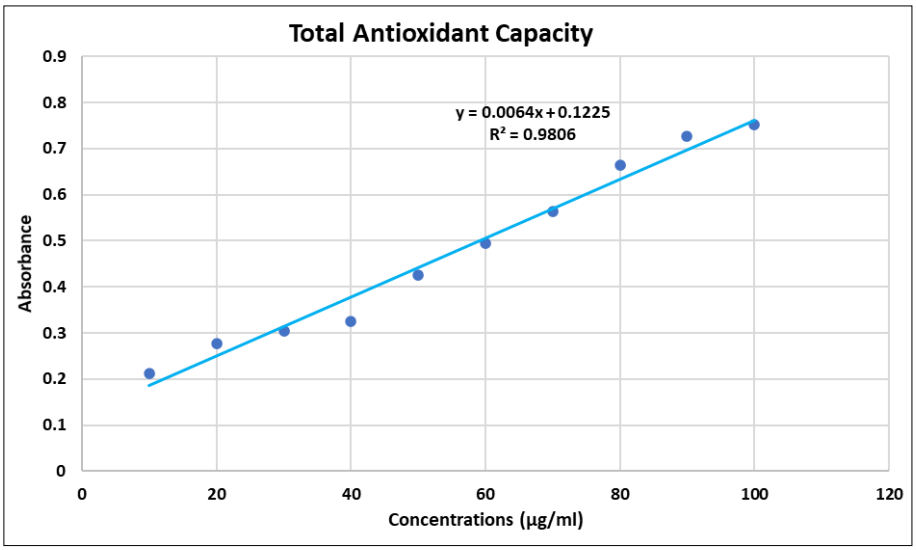

Fig 3: Calibration curve of standard ascorbic acid for determination of total antioxidant capacity of *C. arborea* bark and leaves

3.4 Reducing power (Fe^{3+}-Fe^{2+} transformation ability)

The ferric reducing power of *Careya arborea* Roxb. bark and leaves were increasing with the increase in concentration of extracts (fig 4). Venkatachalam and Muthukrishnan [28] and Babu *et al.* [32] also found the same trend of increasing absorbance with increase in concentration of ethanolic extract of *Desmodium gangeticum* and ethanolic extract of Triphala respectively, at 700 nm and indicated an increase in reductive ability.

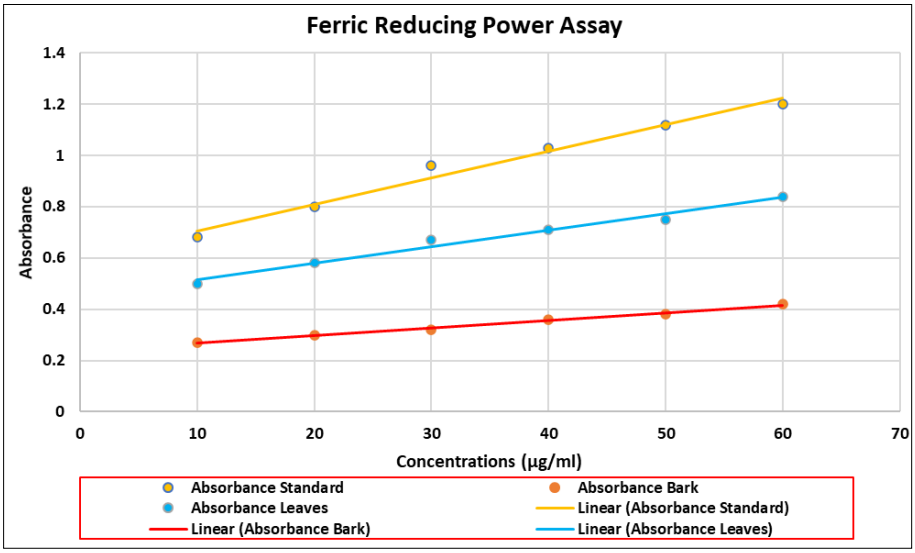

Fig 4: Ferric reducing power of *Careya arborea* Roxb. bark and leaves

3.5 Ferric ion reducing antioxidant power (FRAP)

The FRAP value for the bark and leaves extracts were found to be 1.70 ± 0.02 and 2.51 ± 0.01 mM Ferrous sulphate equivalent per gram dry weight of the plant material respectively by using the calibration curve of standard ferrous sulphate (fig 5), which are lower than that of standard ascorbic acid.

Attanayake *et al.* [33] found the reducing power of *Gmelina arborea* 8.98 ± 0.09 µM by FRAP assay. Shah *et al.* [34] found the FRAP value for aqueous extract of *Moringa pterigospermagaertn*. leaf as 0.28 mM Ferrous sulphate equivalent per gram dry weight of the plant material.

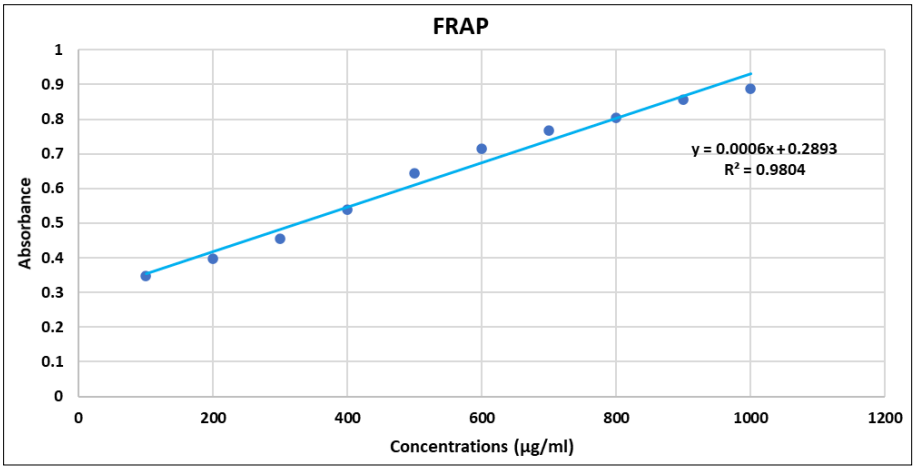

Fig 5: Calibration curve of standard ferrous sulphate for determination of Ferric ion reducing power of Standard ascorbic acid, *C. arborea* bark and leaves

3.6 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

DPPH scavenging assay is widely used method to evaluate the free radical scavenging ability of plant extracts. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with DPPH (deep violet colour) and convert it to yellow coloured α, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant [35, 36]. The DPPH radical scavenging activity of hydro alcoholic extract of *C. arborea* bark and leaves is shown in fig 6. The IC_{50} values of bark, leaves extract and standard ascorbic acid was found to be 73.66 ± 1.60, 47.14 ± 0.50 and 16.96 ± 2.16 µg/ml, respectively.

Wadje *et al.* [26] studied DPPH scavenging effect of four extracts (ethyl alcohol, ethyl acetate, chloroform and petroleum ether) of *Careya arborea* Roxb. leaves. They found ethanol extracts had the highest DPPH radical scavenging activity, shown by the lowest value of IC_{50} values (78.10 µg/ml). According to Ramanathan *et al.* [31], IC_{50} value of methanol extract of *Careya arborea* Roxb. bark on DPPH radical scavenging assay was found to be 132.25 g/ml.

Kaur *et al.* [37] studied different extracts (ethanolic, hydroalcoholic and aqueous extracts) of *C. arborea* stem bark for anti-oxidant activity by DPPH analysis and concluded that hydro alcoholic and aqueous extracts of stem bark showed dose dependent response of percentage inhibition whereas the alcoholic extract does not show any trends in comparison to standard Rutin.

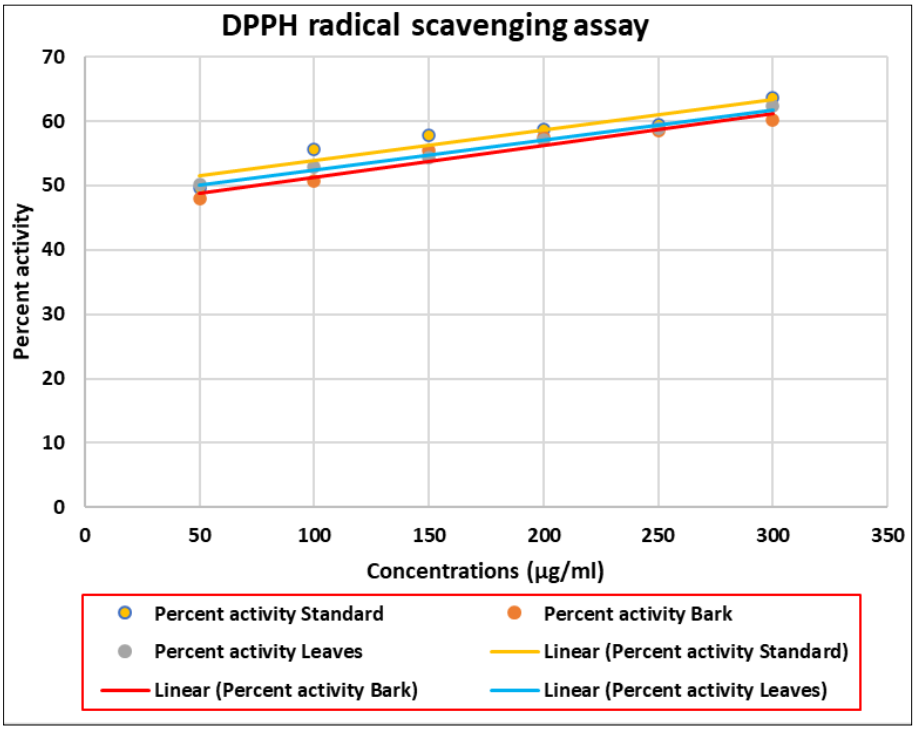

Fig 6: Percentage DPPH radical scavenging activity of *C. arborea* Roxb. bark and leaves

3.7 Nitric oxide radical scavenging assay

Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 150 min resulted in the generation of NO. *Careya arborea* Roxb. bark and leaves effectively reduced the generation of NO. The nitric oxide radical scavenging activity of hydro alcoholic extract of *C. arborea* bark and leaves is shown in fig 7. The IC_{50} values of bark, leaves extract and standard ascorbic acid was found to be 197.48 ± 1.65, 144.47 ± 2.34 and 33.58 ± 0.87 µg/ml, respectively.

Patel *et al.* [19] investigated leaves of *Tephrosia purpurea* Linn. and found IC_{50} value by nitric oxide radical scavenging assay as 938.92 µg/ml and 805.85 µg/ml for aqueous and ethanolic extract, respectively.

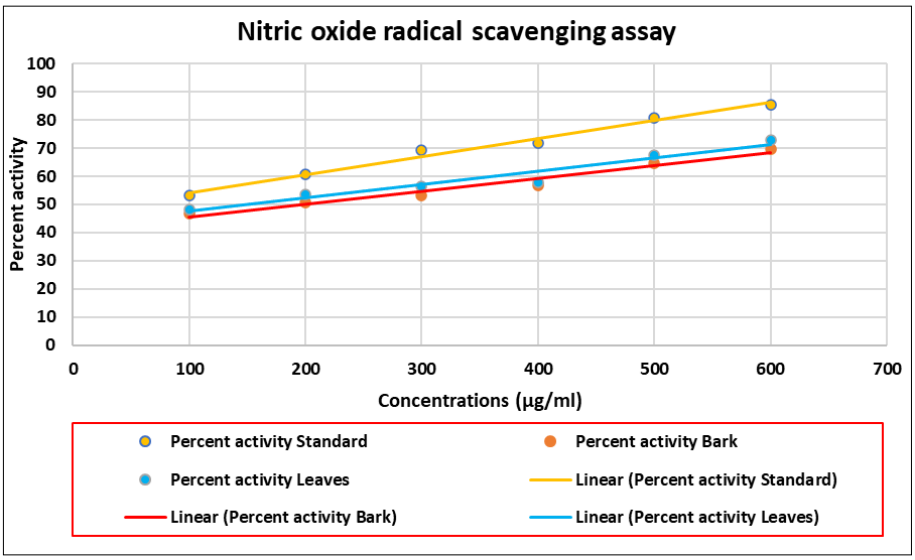

Fig 7: Percentage Nitric oxide radical scavenging activity of *C. arborea* Roxb. Bark and leaves

3.8 Superoxide anion scavenging assay

This assay was based on the reduction of nitro blue tetrazolium (NBT) to purple formazan in the presence of NADH and phenazine methosulphate under aerobic condition. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The superoxide radical scavenging activity of hydro alcoholic extract of *C. arborea* bark and leaves is shown in fig 8. In this assay, the IC_{50} values of bark, leaves extract and standard ascorbic acid was found to be 591.34 ± 1.17, 473.43 ± 2.34 and 96.03 ± 0.98 µg/ml, respectively.

According to Ramanathan *et al.* [31], the methanol extract of stem bark of *C. arborea* at concentrations ranging from 10 to 100 µg/ml inhibited the production of superoxide anion radicals by 15.55 to 71.32 %. The IC_{50} value of this extract on superoxide radical scavenging activity was found to be 94.17 µg/ml, whereas the IC_{50} value of standard BHT and quercetin were found to be 22.77 and 31.58 µg/ml, respectively.

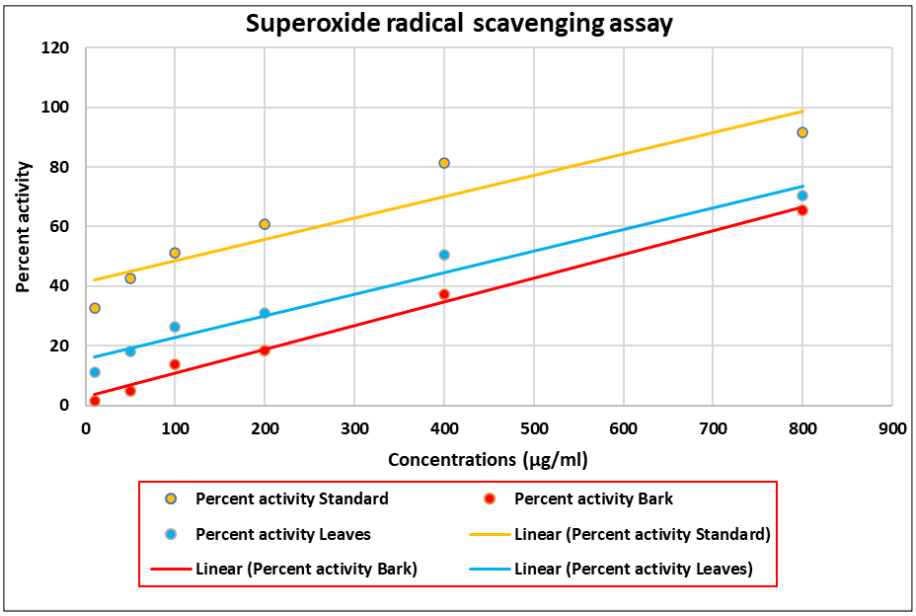

Fig 8: Percentage Superoxide radical scavenging activity of *C. arborea* Roxb. Bark and leaves

4. Conclusion

The results of this study showed that hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves can be of use as a source of natural antioxidants as a possible food supplement or in pharmaceutical industry. However, the components responsible for the antioxidative activity of hydro alcoholic extract of *Careya arborea* Roxb. bark and leaves are currently unclear. Therefore, it is suggested that further work could be done on the isolation and identification of the active antioxidative components in *Careya arborea* Roxb.

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