

Original Article

PHYTOCHEMICAL SCREENING AND EVALUATION OF *IN VITRO* ANTIOXIDANT POTENTIAL OF IMMATURE PALMYRA PALM (*BORASSUS FLABELLIFER* LINN.) FRUITS

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Received: 06 May 2018 Revised and Accepted: 11 Jul 2018

ABSTRACT

Objective: The present study was aimed to evaluate the *in vitro* antioxidant properties of immature Palmyra palm fruits which have been traditionally used for the treatment of diabetes. The qualitative phytochemical screening and quantitative estimation of total phenolic and flavonoids contents in the ethanolic extract were performed to substantiate the antioxidant and medicinal claims.

Methods: Immature palmyra palm fruits were collected, authenticated, dried and powdered in an electrical grinder. The powdered fruits were delipidated in petroleum ether and soxhilation using ethanol to extract the active ingredients. Qualitative phytochemical screening, total phenolic and flavonoid contents were carried out by established methods. The *in vitro* antioxidant potentials were performed by diphenyl-2-picrylhydrazyl, azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, nitric oxide and superoxide anion scavenging assays.

Results: The phytochemical screening showed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, phytosterols, triterpenoids and phenols in the immature palmyra palm fruits extract. The total phenolic and flavonoid contents in the fruits extract was found to be 104.00 ± 0.02 μ g gallic acid equivalents/100 mg of fruits extract and 98.45 ± 0.03 μ g quercetin equivalents/100 mg, respectively. The percentage inhibition of DPPH radicals range from 35 to 70% at a concentration ranges from 200-1000 μ g/ml. Similarly, the percentage of inhibition of ABTS radicals was found to be in the range of 40 to 75.5%. The nitric oxide scavenging activity of the fruits extract ranges from 45 to 76% whereas the superoxide radical scavenging activity ranges from 43 to 83%.

Conclusion: The observed significant free radical scavenging activity along with increased total phenolic as well as flavonoid contents suggest that the immature fruits may be considered as a potential source for the identification of pharmacologically active phytochemicals capable of controlling oxidative stress.

Keywords: *Borassus flabellifer* Linn. Phytochemical screening, Total phenolic content, Total flavonoid content, *In vitro* free radicals assays

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DOI: <http://dx.doi.org/10.22159/ijpps.2018v10i8.27162>

INTRODUCTION

Palmyra palm, botanically termed as *Borassus flabellifer* Linn. belongs to the family "*Arecaceae*" which is distributed widely in the tropical regions of Asian and African countries [1]. It is a slow-growing perennial capable of living more than 150 y and has no distinguishable features to identify the sex until flowering. The trunk with a canopy of large, fan-shaped grey-green leaves having petiole edges with hard horny spinescent serratures. Under marginal ecological conditions, the palm tree unfurls 12 leaves per year or occasionally one leaf per 28 d [2]. The palm tree is dioecious. Male palm produces 10-15 inflorescences each year which in turn bear 15-20 little flowers. On the other hand, each female inflorescence may have 2-3 branches which in turn possess 100-150 fruits. The palm tree starts flowering and fruiting 15-20 y after germination, during the dry season [3, 4].

Immature fruits are green and become dark brownish black when ripened. The fibrous, yellowish edible mesocarp pulp surrounds three and rarely one or two hard-coated seeds. The kernel of the nut contains galactomannan, sucrose, fructose, glucose and amino acids [5]. Immature endosperm is gelatinous and sweet which are consumed during the summer season to control the body heat [3]. The seeds which are sown in mound top and watered regularly germinate within two months [6]. The ripened fruit pulp is yellow-orange in colour, sweet in nature with abundant carotenoids which can be eaten fresh [7, 8]. Palmyra fruit pulp is rich in vitamin A and C and hence commercially utilized in food items and domestic feed [9, 10].

India stands first in the world in terms of its wealth of palmyra palms with a population of more than 125 million [11] and is commonly referred to as the "tree of life" with nearly 800

established uses including food, beverage, fibre, medicinal and timber [12]. Highly respected in Tamil culture, it is called "Karpaha Veruksham" (Celestial tree) because all its parts have a unique use [3]. Above all, due to the special contemporary significance, this tree is decreed as the official tree of Tamil Nadu in 1978 and it is being depicted in the state logo [6].

The various parts of the palmyra palm fruits have been widely used in the traditional medicine for the treatment of several ailments [13, 14]. The extract of immature Palmyra palm fruits has been widely used in the traditional medicine for the treatment of chronic diseases especially diabetes, cancer and gastrointestinal disorders [15, 16]. In the absence of systematic reports in the scientific literature regarding the pharmacological as well as beneficial properties of immature palmyra palm fruits, the present study was aimed to qualitatively screen the phytochemicals and to evaluate its antioxidant properties by *in vitro* free radicals scavenging assays. The total phenolic and flavonoids contents also have been determined to substantiate the antioxidant properties of the immature fruits.

MATERIALS AND METHODS

Chemicals

Gallic acid, quercetin, Griess reagent, DPPH and ABTS were purchased from Sigma-Aldrich, St. Louis, USA. All other chemicals and reagents procured for conducting present study were of analytical grade obtained from SRL, Mumbai.

Plant material

The immature (prior to the development of endosperms) palmyra palm fruits that have weighed between 30 to 50 gm were collected

from the trees near Chengalpet, Tamil Nadu during the month of December. The plant material was authenticated by a taxonomist at the Centre for Advanced Studies in Botany, University of Madras. A voucher specimen was deposited in the Herbarium (CAS-2017-07).

Preparation of the fruits extract

The immature fruits were washed thoroughly under running tap water and rinsed in distilled water. They were cut into slices and dried in an electric oven, powdered in an electrical grinder which was stored in an airtight container at 5 °C until further use. The powdered fruits were delipidated with petroleum ether (60-80 °C) for overnight to selectively remove the lipids. The delipidated fruits extract was subjected to soxhalation using ethanol. The ethanolic extract of the fruits was filtered, dried and weighed. The yield was around 42%.

Phytochemical screening

The ethanolic extract of immature palm fruits was subjected to phytochemical screening such as alkaloids, flavonoids, glycosides, saponins, tannins, phytosterols, triterpenoids, anthraquinones and phenols [17, 18]. The experiments were conducted in triplicates to substantiate the findings.

Determination of total phenolic content

Total phenolic content in the ethanolic extract of immature palm fruits was determined according to the Folin-Ciocalteu colourimetric method [19, 20]. A standard curve was drawn with gallic acid reference solutions. 2 to 10 ml of standard aqueous gallic acid solution (100 µg/ml) was pipetted into a 100 ml volumetric flask containing 70 ml of distilled water. Folin-Ciocalteu reagent (5 ml) and 10 ml of saturated sodium bicarbonate solution were added and the volume was made up to 100 ml with distilled water. The solution was thoroughly mixed. The blank was prepared in the same manner but without gallic acid. After 1 h of incubation at room temperature, the absorbance was measured at 760 nm. The samples were prepared in triplicates for each analysis and the mean value was calculated. For the determination of total phenolic content in the immature palm fruits extract, aqueous solutions at the final concentration of 20 µg/ml were used; proceeding in the same manner described for the reference solutions and the total phenolic content was expressed as mg per gram of gallic acid equivalents.

Determination of total flavonoid content

Total flavonoid content in the ethanolic extract of immature palm fruits was determined according to the method of Quettier-Deleu *et al.*, (2000) [21] with minor modifications. A standard curve was built with quercetin reference solutions. 2 to 8 ml of standard quercetin (50 µg/ml) were pipetted into 25 ml volumetric flasks containing 1 ml of 2% aluminium chloride dissolved in ethanol and the total volume was made up with ethanol. The blank was prepared by diluting 1 ml of 2% aluminium chloride dissolved in ethanol in a 25 ml volumetric flask with ethanol. After 1-hour incubation at room temperature, the absorbance was measured at 420 nm. Immature palm fruits extract were prepared at a final concentration of 20 µg/ml, proceeding in the same manner described for the reference solutions and the total flavonoid content was calculated as quercetin equivalents from a calibration curve. The experiments were performed in triplicate for each analysis and the mean value of absorbance was recorded to determine the total flavonoid content.

Free radical scavenging assays

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The free radical scavenging capacity of the ethanolic extract of immature palm fruits extract was determined using DPPH [22]. DPPH (200 µM) solution was prepared in 95% methanol. From the stock, fruits extract solution prepared in 0.1 mol L⁻¹ Tris HCl buffer (pH-7.9) 200, 400, 600, 800 and 1000 µg/ml were taken in five test tubes. 0.5 ml of freshly prepared DPPH solution was incubated with fruits extracts and kept under light protection for 20 min at room temperature. The decrease of absorbance at 517 nm was measured

using the spectrophotometer. Deionised water used as a blank experiment and standard ascorbic acid was used as positive control. The assay was performed in triplicate and the mean value of absorbance was calculated.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of the blank} - \text{Absorbance of the sample}}{\text{Absorbance of the blank}} \times 100$$

ABTS•+ (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging assay

ABTS•+ radical cation scavenging activity of ethanolic extract of immature palm fruits extract was determined according to the method of Re *et al.*, 1999 [23]. Briefly, ABTS radical cation (ABTS•+) in 20 mmol sodium acetate buffer (pH-4.5) was combined with 2.45 mmol potassium persulfate to generate a stable dark blue-green radical following 12-16 h of incubation at 4 °C in the dark. The reaction mixture is suitably diluted to an absorbance of 0.7±0.01 at 734 nm spectrophotometrically to form the test reagent. The reaction mixture containing different concentrations (200-1000 µg/ml) of fruits extract and 3.0 ml of test reagent were incubated in a water bath at 30 °C for 30 min. The test solution mixture turns colourless and the absorbance is reduced due to the sequestration of unpaired electrons in the test reagent by the antioxidants in the fruits extract. Standard ascorbic acid was used as a reference. The assay was performed in triplicate and the mean value of absorbance was calculated.

Assay for nitric oxide (NO) scavenging activity

Sodium nitroprusside (5 mmol) in phosphate buffer pH 7.7 was incubated with 200, 400, 600, 800 and 1000 µg/ml concentrations of fruits extract dissolved in a suitable solvent (alcohol) and tubes were incubated at 25 °C for 120 min. At intervals, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent. The assay was performed in triplicate and the mean value of absorbance was calculated. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent N-naphthyl ethylenediamine was measured at 546 nm. Standard ascorbic acid was used as a reference [24].

Superoxide anion (SO) radical scavenging assay

The Superoxide radical scavenging activity of immature palm fruits extract was measured by the method of Fontana *et al.* (2001) [25]. In this method, the activity is measured by reduction of riboflavin/light/NBT (Nitro blue tetrazolium). The 1 ml of reaction mixture contained phosphate buffer, NADH, NBT and various concentrations of the sample solution. The method is based on the generation of superoxide radical by autooxidation of riboflavin in the presence of light. The Superoxide radical reduces NBT to a blue coloured formazan that can be measured at 560 nm. Standard ascorbic acid was used as a reference. The assay was performed in triplicate and the mean value of absorbance was calculated.

Statistical analysis

The results were expressed as mean±SD and statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS (version 16) program followed by LSD.

RESULTS AND DISCUSSION

Table 1 shows the presence of biologically active phytochemicals such as alkaloids, flavonoids, glycosides, saponins, tannins, phytosterols, triterpenoids and phenols in the immature palmyra palm fruits extract. Phytochemicals are ecologically derived non-nutrient bioactive compounds that have the ability to exert pharmacological as well as several beneficial effects on human health. The phytochemical screening forms the basis for the quantitative estimation of bioactive constituents present in various parts of medicinal plants. The qualitative analysis of the fruits extracts evidenced that the immature fruits contain most of the important bioactive principles which readily accounts for its folklore medicinal claims. The results obtained are in accordance with the earlier reports on mesosperms [26, 27].

Table 1: Phytochemical screening of immature *Borassus flabellifer* fruits extract

Phytochemicals	Inference
Alkaloids	+
Flavonoids	+
Glycosides	+
Saponins	+
Tannins	+
Anthraquinones	-
Phytosterols	+
Triterpenoids	-
Phenols	+

+indicates presence; -indicates absence; n=3

The palm fruits are widely used to cure a series of diseases and disorders [28-33]. Palmyra fruit pulp (PFP) obtained either from unripened or ripened fruits are available in abundance. However, they are largely unutilized owing to the presence of a bitter steroidal tetraglycoside [34, 35]. If the bitter principle is absent, the PFP is a highly nutritious additive to various foods and feeds [8]. It has been reported that the moisture content of the palmyra fruits was around 80% and it can be reduced to 6% on a dry weight basis [36]. The palm fruits are highly fibrous and it is obvious that it may be a rich source of pectins which have the high potential for use in food, cosmetics and pharmaceutical industries [37]. However, the yield and quantity of the pectins vary greatly depending on the plant source. Similarly, the polyphenolic content of the immature fruits is responsible for the bitterness and astringency [38]. The beneficial effects of polyphenols are mainly attributed to their free radical scavenging action. The diets rich in polyphenols are closely correlated with reduced incidence of metabolic and inflammatory disorders. Further, the polyphenols are known to be responsible for the colour, astringency and taste of the phytoingredients.

Saponins are a structurally and biologically diverse class of glycosides of steroids and triterpenes that are widely distributed in terrestrial plants and intimately involved in our daily lives. The structural diversity of saponins lies mainly in their sugar moieties [39-41]. The inflorescence of palmyra fruits are reported to contain a bitter compound known as flabelliferins which belong to the class of steroidal saponins [42]. Additionally, the fruits also reported to contain flabelliferins which are steroidal saponins with many medicinal properties [43].

The presence of alkaloids in the fruits extract is of important significance in terms of its pharmacological and beneficial effects. Alkaloids constitute an important class of structurally diversified

compounds that are having a nitrogen atom in their heterocyclic ring and are derived from amino acids. Alkaloids form about 20% of plant derived secondary metabolites. They play a wide range of physiological actions on human health care such as antibiotics, anticancer and different degenerative diseases. Due to their immense pharmacological properties, alkaloids are in great demand for pharmaceutical formulations especially for the lethal diseases such as cancer and inflammatory disorders. Several synthetic and semisynthetic drugs are structural modifications of the alkaloids, which were designed to modulate the primary effect of the drugs to reduce the undesirable side effects [44, 45].

The presence of tannins in the fruits extract forms the basis for its medicinal value. Tannins are important plant secondary metabolites with anticarcinogenic and antimutagenic properties. The antinutritional and pharmacological effects of dietary tannins and their interactions with enzymes and other proteins have been extensively reported [46-51]. The pharmacological activity of tannins is mainly due to the presence of hydroxyl and other functional groups present in them.

Table 2 represents the total phenolic and flavonoid contents in the fruits extract. It was found to be 104.00±0.02 µg gallic acid equivalents/100 mg of fruits extract and 98.45±0.03 µg quercetin equivalents/100 mg of fruits extract, respectively. Phenols are very important plant constituents because of their free radicals scavenging ability which in turn due to the presence of hydroxyl groups in them [52]. Similarly, flavonoids are an important group of polyphenols widely distributed among the plant flora and containing a benzopyrone that is used as antioxidants or free radicals scavengers [53]. The significant levels of total phenolic and flavonoid contents further evidenced the presence of biologically active phytoingredients in the ethanolic extract of unripened palm fruits.

Table 2: Total flavonoid and phenolic content of immature *Borassus flabellifer* fruits extract

Constituents	Content
Phenolic content	104.00±0.02 µg gallic acid equivalent/100 mg extract
Flavonoid content	98.00±0.03 µg quercetin equivalent/100 mg extract

The values are expressed as mean±SD; n=3

The *in vitro* DPPH and ABTS radicals scavenging activity of immature palmyra palm fruits extract was presented in fig. 1 and 2, respectively. The fruits extract scavenges the DPPH free radicals in a dose-dependent manner. The percentage inhibition ranges from 35 to 70% at a concentration ranges from 200-1000µg/ml. Similarly, the percentage of inhibition of ABTS radicals was found to be in the range of 40 to 75.5%. Among the several *in vitro* antioxidant assays, DPPH* and ABTS* assays have been widely used as more reliable methods in determining the free radical scavenging efficacy of unknown

compounds [54, 55]. The antiradical activity assay is based on the reduction of DPPH* in methanolic solution. Due to the presence of an odd electron, DPPH gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured. It has been found that DPPH* can oxidize cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds [56].

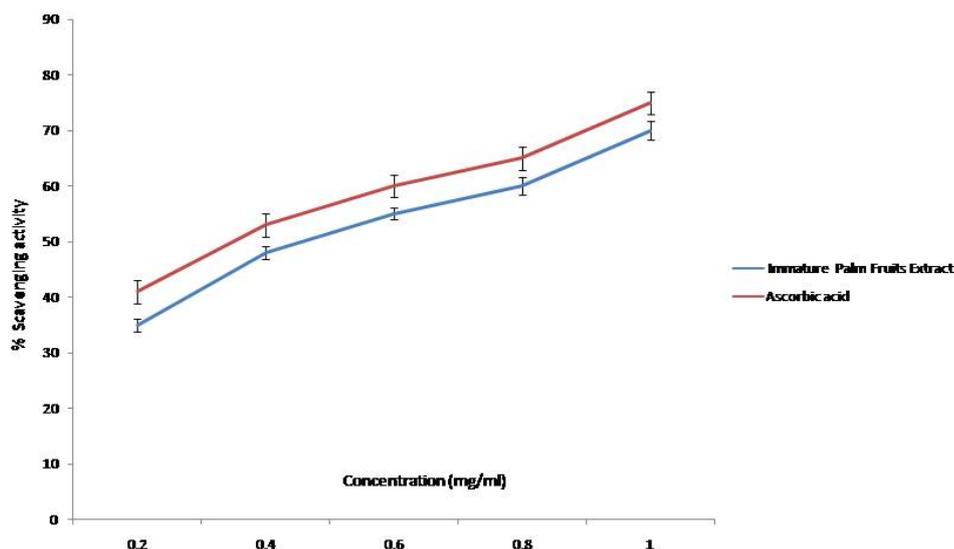


Fig. 1: DPPH radical scavenging assay of ethanolic extract of immature palm fruits. The values are expressed as mean \pm SD; $n=3$

ABTS⁺ produce more powerful free radicals than DPPH⁺ radicals and the reactions with ABTS⁺ radicals involve a single electron transfer process [57]. The principle of ABTS⁺ assay is that the

performed radical monocation of ABTS⁺ is generated by oxidation of ABTS⁺ with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants.

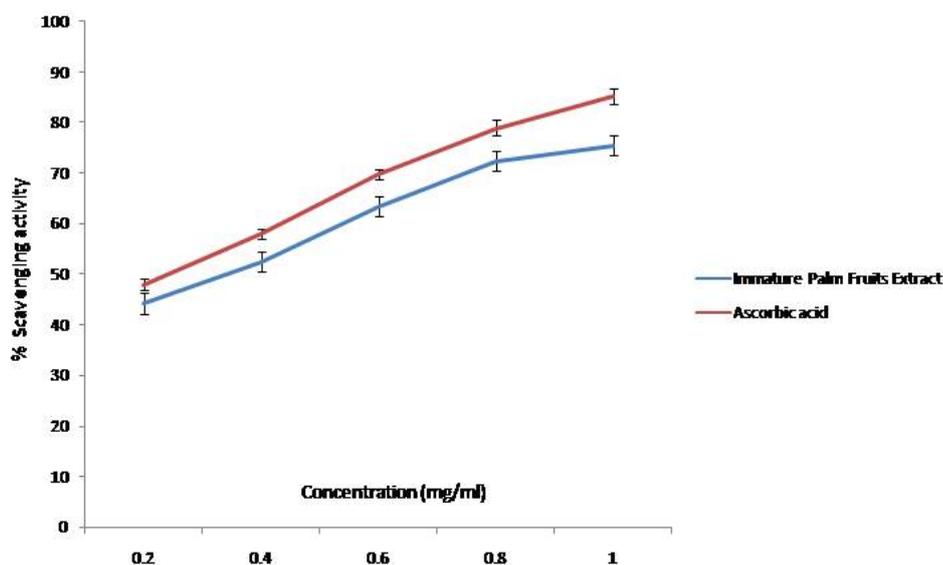


Fig. 2: ABTS radical scavenging assay of ethanolic extract of immature palm fruits. The values are expressed as mean \pm SD; $n=3$

The nitric oxide scavenging activity (fig. 3) of the fruits extract ranges from 45 to 76% whereas the superoxide radical scavenging activity (fig. 4) ranges from 43 to 83%. Nitric oxide radicals are derived from the interaction of NO with oxygen or reactive oxygen species. NO is a diffusible free radical that play many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilatation, antimicrobial and antitumor activities [58]. Chronic exposure to nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. The toxicity of NO increases greatly when it reacts with the superoxide radical, forming the highly reactive peroxynitrite anion (ONOO⁻). Nitric oxide has been shown to be directly scavenged by flavonoids [59].

In vitro quenching of NO radical is one of the methods that can be used to determine antioxidant activity [60]. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using the Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions [61].

Superoxides are formed from molecular oxygen by oxidative enzymes as well as via non-enzymatic reactions such as auto-oxidation by catecholamines [62]. It is extremely harmful to cellular components. Superoxide anions play an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which readily induce oxidative damage in lipids, protein and DNA [63].

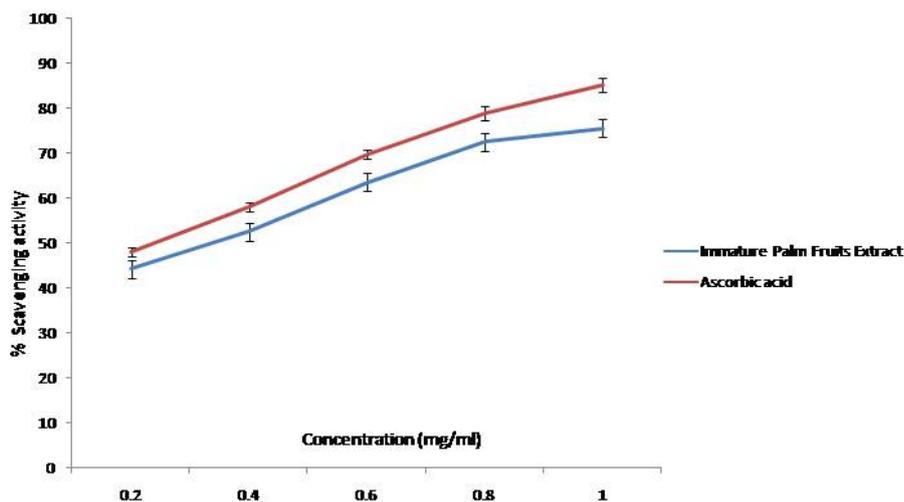


Fig. 3: Nitric oxide scavenging assay of ethanolic extract of immature palm fruits, the values are expressed as mean \pm SD; $n=3$

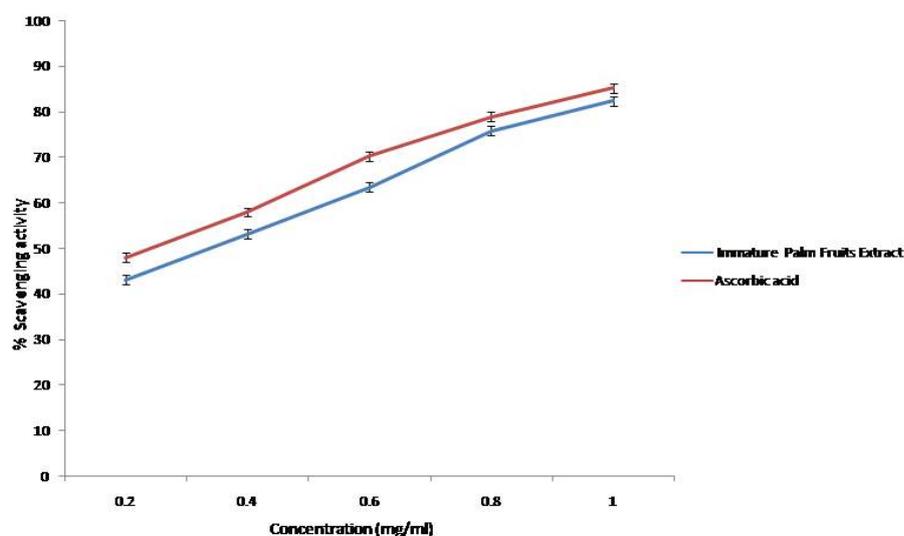


Fig. 4: Superoxide radical scavenging assay of ethanolic extract of immature palm fruits, the values are expressed as mean \pm SD; $n=3$

Superoxide, which is an anion radical, is produced by the one-electron reduction of molecular oxygen. In aqueous media, protonation of superoxide can form the uncharged hydroperoxyl radical ($\text{HOO}\bullet$), which exhibits a pKa of 4.8, meaning that the anion radical form is by far the predominant species at physiological pH ranges. A second reduction of superoxide would require the energetically disfavored compression of two full negative charges on a diatomic molecule. As a result, superoxide is generally a better reducing agent than the oxidizing agent. In addition, superoxide does not cross lipid membranes readily. Superoxide is relatively unreactive toward most biological molecules, although the low levels of superoxide permitted in cells and tissues indicate that limiting cell exposures to superoxide is a significant selector for survival. The levels of superoxide are kept low by effective compartmentalization of the sequential reductions of oxygen and by extensive expressions of superoxide dismutases with high affinities for superoxide [64]. The observed significant *in vitro* antioxidant properties of the fruits extract may be due to the increased levels of phenolic and flavonoids present in them [65].

CONCLUSION

A thorough literature survey revealed that the reports involving the pharmacological properties of immature palmyra palm fruits are sparse. The results of the present study evidenced the presence of pharmacologically active phytochemicals in the ethanolic extract of

immature palmyra palm fruits. The total phenolic and flavonoid contents readily account for the *in vitro* free radical scavenging activity of the fruits extract. Thus, it can be concluded that the immature fruits extract may be considered as a rich source for the identification of nutraceuticals with diversified medicinal values. From the data obtained, it can be concluded that the immature fruits extract may be considered as a potential source of antioxidants.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

All the authors have equal contribution in the manuscript and declare no conflict of interests.

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