Anti-oxidant activity of compounds and fractions of *Manilkara hexandra* (Roxb) bark

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Isolation of a protein mixture having IC_{50} of 60μg/mL against *Streptococcus mutans* from the methanol extract of bark of *Manilkara hexandra* (Roxb) along with six plants phenols Catechin, Catecol, Gallic acid, Phloroglucinol, Quercetin and Rutin has been reported. Where in phenolic constituents of the bark has been reported for the first time and the isolated compounds having moderate activity against *S.mutans*.In this work, the anti-oxidant activity of these pure compounds and fractions has been studied and its relevance in oral care is established. Comparative anti-oxidant activity of all the six compounds along with the crude extract and fractions have been assessed using eight different assay protocols. The results indicate that twice daily tooth cleaning with the bark can provide basic oral disease protection and thereby assuring several health benefits. Synergistic activity of the phenolic constituents along with the protein and other constituentsmight be a reasonable scientific explanation for the folklore use of the plant as one of the prescription for oral diseases.

Keywords: Anti-oxidant, *Manilkara hexandra*, Oral diseases, Plant phenols, *S.mutans*

Manilkara hexandra (Roxb) classified into a family of *sapotaceae*; it is widely distributed in south Asia,which is an ever green tree[1]. The bark of the plant has astringency, which is helpful in the treatment of gastric distress and gum diseases. *Manilkara hexandra* bark polysaccharide (MHPS) is constituted by sucrose, maltone, xylose and lactose each with 0.48%, 0.29%, 0.42% and 0.425%, respectively[1]. It stimulates one's own defence system optimally[2]. Bark is reported as useful natural anti-oxidant source[3]. Anti-diabetic and potent hypo-lipidemic potential of the bark 1:1 ethanol: water extract is known[4]. Flavanoids rich fraction of this plant part is reduce lipid peroxidation in *in-vivo* animal model studies there by inhibiting formation of gastric ulcers[5].

Streptococcus mutans and *Porphyromonas gingivalis* are the two harmful frequently encountered bacteria in buccal cavity. Enamel eroding acids produced by *Streptococcus mutans* causes tooth decay. Presence of *Porphyromonas gingivalis* associated with periodontitis (it is a disease developed gradually over long period of time, it damages supportive tissue and the hard tooth socket that hold the teeth), this organism is normally absent in healthy humans oral microbiome .Systemic diseases such as pre-term low birth weight, osteomyelitis in children, bacterial endocarditis, aspiration pneumonia, and cardiovascular disease are associated with specific oral bacterial species. It is widely reported that, the anti-oxidant level of saliva of patients with oral diseases significantly high compared to normal individuals. Total anti-oxidant capacity (TAC) of 100 high school students within the age of 15-17 years have been evaluated and found that TAC of saliva has higher in caries active group[6]. It means human body's natural defence mechanism is at work when oral hygiene has been challenged. It is very imperative to support the natural mechanism to overcome the assault. One of the best ways to support the natural defence system is to supplement phytochemicals and increase the usage of anti-oxidants[7]. Natural products are reliable and good source of such anti-oxidants and moreover drugs used for other ailments need to be tested for anti-cancer activity where metformin is now worked upon as a panacea drug[8]. A good oral hygiene is a dream which comes to any individual who is willing to pursue healthy life. We examined the plant *Manilkara hexandra* to validate its folklore use for oral care.

Experimental Section

Reagents required and common procedures

EDTA, $FeCl_2$, $FeCl_3$, potassium ferricyanide, potassium persulfate, sodium nitroprusside, sodium phosphate, sulphuric acid, trichloroacetic acid, tween 40, chloroform, methanol, distilled water were available as analytical grade reagents in the lab and used as such without further purification. Following reagents were purchased from Sigma: Ammonium molybdate, ascorbic acid, butylatedhydroxy toluene (BHT), curcumin, β-carotene, ferrozine, griess reagent, linoleic acid, NBT, riboflavin, DPPH radical (1,1-diphenyl-2-picrylhydrazyl), ABTS, Trolox and used as such. Sodium phosphate buffer, phosphate buffered saline of required pH were prepared by following standard procedures. UV: Shimadzu UV spectrophotometer (Model no: UV-1900) was used for sample analysis. Silica gel TLC aluminium sheets purchased from Merck were used for ensuring the purity of the compounds and authentication of bark fractions by TLC. TLC spots after development were detected using UV short and long wavelength as well by using sulphuric acid in methanol as spray reagent following standard protocols.

### Plant collection and authentication

In December 2017, all the aerial part of the plant *Manilkara hexandra (*Roxb) including bark (3.2 kg) were obtained from a tree at Chennai (Red-Hills) and shade dried. The part of the plant was authenticated by ataxonomist. A voucher specimen of the bark and other aerial part of the species were available in the Department of Chemistry, Ramakrishna Mission Vivekananda College, Chennai (Scheme 1).

Extraction and fractionation

Powder of bark1.5 kg was exhaustively extracted with methanol using soxhlet extractor. The extract was concentrated to obtain 0.2132kg of extract. The anti-microbial profile of the extract was studied against six microorganisms *Enterobacter aerogenes*, *Enterococcus faecalis*, *Micrococcus luteus*, *Streptococcus mutans, Staphylococcus aureus* and *Staphylococcus epidermidis*. The crude methanol extract was active against *S.mutans* showed an IC_{50} value of 4000 μg/ml. 115g of methanol was suspended in 25% methanol in water, liquid-liquid fractionation followed by concentration of organic layer yields chloroform fraction 16.09g, ethylacetate fraction 9.4g, n-butanol fraction 61.2g and an aqueous fraction of 27.14g. These four fractions were studied against *S.mutans*. Anti-microbial profile of chloroform and ethylacetate fractions were not very significant, showed$IC_{50}$of>10,000 μg/ml only, where as the other two fractions showed modest antimicrobial property,IC_{50} of 7500 μg/ml and 5000 μg/ml, respectively. The reasonably active *n*-butanol fraction was taken up for purification. It was passed through Sephadex (LH-20) column of length 36cm and diameter of 3.6cm utilizing water: MeOH solvent system with increasing concentration of methanol from 10% to 90% in a stepwise manner. Total 7 fractions each of 1200 ml volume were collected. Based on TLC similarity pooled the fraction obtained four fractions (Fr-1, Fr-2, Fr-3 and Fr-4). Further chromatographic purification of these four major fractions yielded more six known compounds. Fr-1, yields catechin (Compound-1), Fr-2, yields rutin (Compound-5), Fr-3, yields gallic acid & quercetin (Compounds-2&6) and Fr-4, yields phloroglucinol & catecol (Compounds-3&4). Detailed isolation procedure, structure elucidation details along with a unique identification of a protein mixture with IC_{50} value of 60μg/mL against *S. mutans* were reported by the authors[9].

The protein content of the bark is less significant and the bark is traditionally known as a very good source for oral care and hygiene. Anti-oxidant support is vital for oral health. Hence, we hypothesized that the plant material may encompass potential antioxidant principles. In this work eight different anti-oxidant assay methods were adopted to assess the presence of anti-oxidant actives and quantify their activity. The crude methanol extract, four fractions and six active principles isolated from the bark were tested using these assay systems.
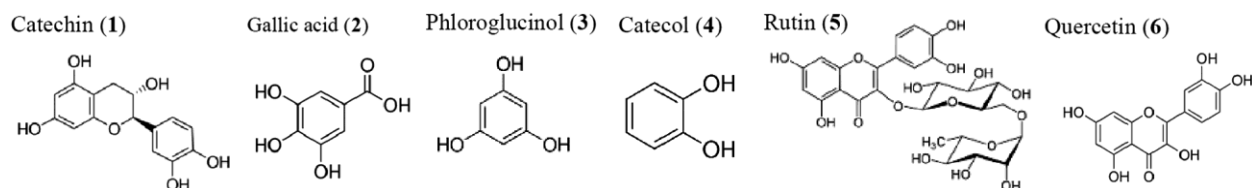
Scheme 1 — Compounds isolated and characterised from *Manilkara hexandra*-bark

Anti-oxidant assay

Eight different anti-oxidant assay methods are used in this study.

I. DPPH Radical scavenging activity
II. Assay of superoxide radical ($O_2^{.-}$) scavenging activity
III. Reducing power determination
IV. Assay of nitric oxide scavenging activity
V. Antioxidant assay using β–carotene linoleic acid model system
VI. ABTS cation radical scavenging activity
VII. Metal ion chelating activity
VIII. Determination of antioxidant capacity by phosphomolybdenum method

DPPH (2,2-diphenyl-1-picrylhydrazyl)radical scavenging activity procedure

This experiment was performed for the test samples and the standard was based on reported method with slight modifications as required[10]. Samples dissolved in methanol or water methanol mixture were placed in a 10 mL screw-capped culture tubes. Followed by addition of 3 mL of 0.004% solution of DPPH in methanol and mixed vigorously for 15 seconds. The solution stored at ambient temperature exactly for 30 min. Absorbance of the sample and standard were measured at 517 nm using UV-Vis spectrophotometer. A DPPH sample OD was used as control. A solvent (solvent mixture) without test samples and DPPH was used as blank (control). Absorbance value of the sample and control was obtained after correcting for blank absorbance. The radical (DPPH) scavenging % was computed as follows:

% Inhibition = $[(A_0 – A_1)/A_0] \times 100$ …(1)

where, A_0 is the absorbance value of the control, and A_1 is the absorbance value of the extract/standard at the given wavelength.

Superoxide radical ($O_2^{.-}$) scavenging activity (SORSA) procedure

SORSA of test samples and standard were determined based on reported method with required modifications. Experiments were performed using two set of assemblies with lining of aluminium foil, one set was maintained in dark throughout the process and it served as blank in the UV absorbance measurement at 590nm. In each set of assembly, in every sample vial, reaction mixture containing riboflavin 20μg, NBT 0.1mg, EDTA 12mM. The *p*H was maintained using Na_3PO_4buffer (*p*H 7.6). Total volume of this mixture was maintained at 3 mL,

mixed with 1mL sample solution, and the reaction was triggered in one of the assembly by illuminating the reaction mixture using 100W fluorescent lamp for 90 seconds. Absorbance at λ_{max} = 590nm was recorded immediately for the samples maintained in both assemblies. The inhibition percentage of superoxide anion generated was computed as follows:

% Inhibition = $[(A_o – A_1)/A_o] \times 100$ …(2)

where, A_o is the absorbance value of the control, and A_1 is the absorbance value of the sample/standard[11].

Reducing power (RP) determination procedure

RP of test samples and standard were determined based on reported method with slight modification. Samples 1 mL was mixed with 2.5 mL of phosphate buffer (pH 6.6) and 2.5mL of 1% $K_3[Fe (CN)_6]$ and incubated exactly for 10 min at ambient temperature. Then was added immediately 2.5 mL of trichloroacetic acid (10%in water), and shaken vigorously. The mixture was centrifuged; 2.5 mL of clear upper layer was diluted with 2.5 mL of DM water and mixed with 0.5 mL of 0.1% of $FeCl_3$ solution and was shaken well. Absorbance at 700nm was recorded. The % of reducing power was obtained utilizing the formula:

% Increase in reducing power = $[(A_1 – A_o)/A_o] \times 100$…(3)

where, A_o is the absorbance value of the control, and A_1 is the absorbance value of the sample/standard[12].

Nitric oxide (NO) scavenging activity procedure

NOscavenging activity of test samples and standard were determined based on the reported method with required modifications. Test samples at required concentrations were prepared and mixed with 1ml solution of sodium nitroprusside (10mM) in phosphate buffered saline and was mixed vigorously and incubated for 150 min at ambient temperature. Followed by addition of 0.5 mLof Griess reagent, the resulting mixture was shaken well. UV absorbance of the solution was recorded at 546nm. The inhibition percentage of nitric oxide generation was obtained based on the formula:

% Inhibition = $[(A_o – A_1)/A_o] \times 100$ …(4)

where, A_o is the absorbance value of the control, and A_1 is the absorbance value of the sample/standard[13].

β–Carotene linoleic acid system procedure

β-Carotene bleaching inhibition activity of test samples and standard were determined based on reported method with required modifications.0.02%

β-carotene was taken and dissolved in chloroform, 2 mL then was transferred into a 250 mL round bottom flask. Chloroform was removed completely by drying in rota vapour under vacuum. After this, 100 mL of aerated water containing 0.04% of linoleic acid 0.4% of tween 40 was added and vigorously shaken to form an emulsion. 4.8 mL emulsion was used to determine the efficacy of the test samples. A 4.8 mL of emulsion and 0.2 mL of test samples were mixed together and recorded the absorbance at 470nm. The sample vials incubated at 50°C for 2h and again recorded the absorbance. A blank experiment was performed using emulsion without β-carotene and was used as control solution for recording UV[14].Antioxidant activity was obtained based on the following expression:

Antioxidant activity = (β-carotene content after 2 h of assay/Initial β-carotene content) ×100…(5)

ABTS cation radical (ABTS$^{•+}$) scavenging activity procedure

The ABTS$^{•+}$ scavenging potential of the test samples and the standard were determined based on the reported method. ABTS cation stock solution preparation: Mix ABTS 7mM with 2.45mM$K_2S_2O_8$, the mixture was suitably diluted with 5mM phosphate buffered saline pH 7.4 to give absorbance of0.8-1.0 at 414nm. 100 – 500μL of test solutions of various concentrations was mixed with 3mL of ABTS stock solution. UV absorbance was measured immediately and after incubation at room temperature for 90 minutes so as to reach a plateau. If required the solution can be stored for longer period of duration to reach the plateau. The percentage of ABTS cation radical scavenging potential was calculated using the formula given below:

% Decrease in ABTS cation radical =
$$[(A_o – A_1)/A_0] × 100…(6)$$
where, A_o is the absorbance value of the control, and A_1 is the absorbance value of the sample/standard[15].

Metal Ion chelating activity procedure

Metal ion chelating potential of the test samples and the standard were determined based on the reported method. Ferrozine–Fe^{2+} complex formation: Various concentrations of test samples and standard were taken in 10mL screw capped vials. 50μL of 2mmol/L FeCl$_2$ mixed well with these solutions, followed by addition of 5 mmol/L ferrozine 200μL. The resulted solution was shaken vigorously and left at ambient temperature for 10 min. The UV absorbance of the solution was recorded at 562nm. Blank solution was prepared without test samples/standard and was used as control. The % inhibition of complex formation was obtained based on the formula:

% of inhibition of complex = [(A$_o$ – A$_1$)/A$_o$] × 100…(7)

where, A_0 is the absorbance value of the control, and A_1 is the absorbance value of the extract/standard[16].

Stimulation of formation of phosphomolybdenum complex procedure

The Mo^{6+}to Mo^{5+}reducing power of test samples and standard were determined based on reported method with slight modification. In a vial 28mM sodium phosphate in water, 0.6M sulphuric acid in water and 4mM ammonium molybdate in water were mixed together. 3ml of this reagent solution was combined with 300μl of test sample/standard solution[17]. Blank solution was prepared using distilled water instead of sample solution. The screw capped culture tubes were maintained at 95°C for 90 min. Then the solution was chilled to ambient temperature and UV-Vis measurement was made at 695nm for each solution and recorded. The percentage of reducing power was calculated using the formula given below:

% Increase in reducing power = [(A$_o$ – A$_1$)/A$_o$] × 100…(8)

where, A_o is the absorbance value of the control, and A_1 is the absorbance value of the sample/standard.

Results and Discussion

The research work was undertaken with an aim to understand and document the antioxidant role played by the constituents of the bark. Since the most active secondary metabolite isolated from the bark against *S. mutans* is a protein mixture which is a trace component of this plant part. The isolated components of the bark were showing only moderate activity against *S. mutans* i.e., 800-1000 μg/mL. Catechin (1) & Rutin (5) showed 800 μg/mL activity. Gallic acid (2), Phloroglucinol (3) and Catecol (4) showed 1000μg/mL activity, whereas Quercetin (6) showed >1000 μg/mL activity. The protein mixture with IC$_{50}$ value of 60μg/mL against *S. mutans* was the first report from our team[9]. The bark is traditionally known to provide oral care benefits. The moderate activity of the secondary metabolites along with the trace levels of the most active constituent provides a lead that, there are other mechanisms of action by the constituents of the plant. The afterthought of identifying the protein mixture as an active component is that, other organic and inorganic

components of the bark must support the active ingredient synergistically or additively. Plant metabolites, particularly phenols and polyphenols are known to possess appreciable anti-oxidant activity. Since the isolated compounds are of phenolic in nature, the anti-oxidant property of them plays a role in the biological application of the bark. A brief survey of literature reveals that, anti-oxidant support is vital to maintain healthy oral hygiene, moreover supplementation of several natural chemicals are proved as anti-microbial and anti-oxidants[18-20].

As mentioned earlier, TAC of saliva was higher in caries active group[6]. Difference between the efficiency of inbuilt anti-oxidant defence arrangement of an individual and level of various ROS (reactive oxygen species) formed with their body is a characteristic feature of many diseases conditions including dental caries. Progression of dental caries can be tracked based on total antioxidant status. Significant difference in TAC of saliva as well as serum of adults with active caries and individuals without dental caries were measured, compared and documented. There is a linear relationship between TAC and severity of level of caries, i.e., the TAC level increases as the severity of caries increases. Mean TAC level of saliva as well as serum increased significantly ($P<0.001$) with the decayed, missing, filled and total teeth index[16]. Total antioxidant capacity and severity of inflammation in periodontitis is inversely proportional. Type-2 diabetic patient's (with and without periodontal disease) saliva TAC level was compared with healthy subjects and the results showed that the mean salivary TAC was lowest in diabetic patients with periodontitis[21].

Melatonin a hormone secreted by the human has powerful anti-oxidant effects. It protects the oral cavity from tissue damage, modulates osteoblastic and osteoclastic activity. It stimulates type-I collagen fiber production there by it may help in regeneration of alveolar bone. Degree of periodontal disease and salivary melatonin levels is inversely proportional. When there is a reduced salivary melatonin levels, the severity of periodontal disease is more. This indicates that this hormone protects the body from external bacterial attacks. Plasma as well as saliva melatonin levels of diabetic patients were significantly lower than controls[22-25] ($P<0.001$).

Thus, it can be concluded that, the anti-oxidant support is very much essential to overcome periodontal disease. To understand the free radical quenching role of extract of the bark its fractions and isolated compounds, we have selected eight anti-oxidant assay systems. Four of them are ET based systems, two of them are HAT based assay systems and the remaining two belongs to general anti-oxidant assay systems. The general working principle of these eight assay systems are briefed below for the better appreciation of the perspective of selecting these assays.

(i) DPPH radical scavenging activity: In presence of antioxidants the rate of decrease in yellow colour was studied. i.e., Diphenylpicrylhydrazine formation from the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was studied. The absorbance value decreases at 517nm indicating the reaction kinetics.

(ii) Superoxide anion radical (O_2^{-}) scavenging activity: The assay method records the inhibition of blue formazan colour formation. It was achieved by scavenging the superoxide radicals formed in test system. The difference in absorbance at 590nm indicates the radical scavenging potential.

(iii) Reducing power determination: The measurement of the anti-oxidative ability (reductive ability) of test samples was examined based on Fe^{3+} reduction to Fe^{2+}. Anti-oxidants increase the conversion of $Fe3^{+}$ to Fe^{2+}. The Fe^{2+} formation was examined by recording the formation of Prussian blue (Perl's) colour at 700 nm.

(iv) Nitric oxide scavenging activity: Nitrite ions (NO_2^{-}) formation in the presence of anti-oxidants, from phosphate buffered saline solution of sodium nitroprusside can be estimated using Greiss reagent. Nitrite ions (NO_2^{-}) production was reduced by the extracts/antioxidant compounds, it can be observed by measuring the absorbance at 546 nm.

(v) Inhibition of β – carotene bleaching: Anti-oxidant in the experimental mixture delays the extent of β-carotene decomposition by ''deactivating'' the linoleate and any other free radicals formed within the system. The absorbance was measured at 470 nm immediately after adding of anti-oxidants, and at a predetermined time intervals for 2 hrs.

(vi) ABTS cation radical scavenging activity: Oxidation of ABTS (2,2'-azinobis(3-ethylbenzothiaziline-6-sulfonicacid) generates stable radical cation $ABTS^{\bullet+}$. In the presence of H-atom donors, such as plant phenols, the $ABTS^{\bullet+}$ converted into a non-coloured

form of ABTS. $ABTS^{•+}$ had high molar absorptivity at 414 nm. Antioxidant potential of test samples were measured by measuring decrease in the UV absorbance.

(vii) Metal ions chelating activity: The assay estimates extend to chelation of Fe^{2+} ions by ferrozine reagent indicated by a red coloured complex formation. The presence of other chelating reagent such as test samples with anti-oxidant potential disturbs complex formation. Resulting in the reduced formation of the red coloured complex was measured at 562 nm UV absorbance.

(viii) Reduction of Mo^{6+} to Mo^{5+} in the experimental setup (at acidic pH) in the presence of anti-oxidants results in green-phosphate Mo (V) complex formation. The capacity of the test samples was measured based on increase in UV absorbance at 695 nm.

The results of anti-oxidant assay of the crude methanol extract(CME), aqueous fraction(WF), n-butanol fraction(nBF), ethyl acetate fraction(EAF), chloroform fraction(CF),and all the six compounds was isolated using above described eight protocols as is summarised in Table 1. As a representative example the IC_{50} value estimation based on DPPH activity of all the test samples is discussed in detail here. The DPPH activity (OD values) of the crude extract and fractions were determined from

1500µg/mL to 28000µg/ml, (in case of nBF concentration changed from 3000µg/ml to 45000µg/mL to enable determination of IC_{50} value). Then % of inhibition of DPPH radical by the anti-oxidants was determined. Based on this % inhibition versus concentration graph was drawn. From the graph, regression equation and R^2 (correlation coefficient) value for the test samples was estimated. Using the regression equation IC_{50} values were calculated.

Regression equation of Crude methanol extract: $Y = 0.0028\ X + 6.0651$; R2 = 0.9903.Regression equation of Chloroform Fraction:$Y = 0.0031\ X + 2.9337$; R2 = 0.9865. Regression equation of Ethyl acetate Fraction:$Y = 0.0034\ X\ -0.7956$; R2 = 0.9808. Regression equation of n-Butanol Fraction:$Y = 0.0019\ X + 2.5373$; R2 = 0.9918.

Regression equation of Aqueous Fraction: $Y = 0.003\ X+3.6304$; R2 = 0.9842.

Based on the regression equation IC_{50} value of DPPH activity of the crude extract &fractions were calculated. The IC_{50} value of the CME, CF, EAF, nBF and WF are 15,725µg/mL, 15,185µg/mL, 14,950µg/mL, 25,000µg/mL and 15,475µg/mL, respectively. The calculated values approximated to nearly thousand are given in Table 1. Similarly IC_{50} values of pure compounds were calculated, approximated to nearest tens and reported in Table 1.

Table 1 — Comprehensive anti-oxidant activity data.

S. No	Description	(1)DPPH Radical scavenging activity	(2)Assay of Superoxide Radical Scavenging	(3)Reducing power determination	(4)Assay of Nitric oxide scavenging activity	(5)β-carotene Linoleic acid model system	(6)ABTS cation Radical scavenging activity	(7)Metal Ion chelating activity	(8)Phosphomo lybdenum method
		Anti-oxidant activity of actives from bark of *Manilkara hexandra*							
		TYPE OF METHOD							
		ET BASED			HAT BASED			OTHER MECHANISMS	
		Concentration µg/mL							
1	Crude Methanol Extract	15,000	25,000	25,000	15,000	25,000	15,000	20,000	25,000
2	Chloroform Fraction	15,000	25,000	15,000	25,000	15,000	25,000	25,000	25,000
3	Ethylacetate Fraction	15,000	20,000	25,000	15,000	25,000	15,000	15,000	25,000
4	n-butanol Fraction	25,000	15,000	20,000	25,000	20,000	20,000	25,000	>45,000
5	Aqueous Fraction	15,000	15,000	12,500	25,000	20,000	15,000	10,000	>28,000
6	Catechin (1)	5	125	125	500	125	5	50	50
7	Gallic acid (2)	10	150	125	500	150	10	25	25
8	Phloroglucinol (3)	50	250	50	250	500	50	25	50
9	Catecol (4)	25	200	50	250	500	25	125	50
10	Rutin (5)	2	125	125	250	500	2	15	50
11	Quercetin (6)	2	250	125	125	500	2	10	50

The overlay diagram of % inhibition versus concentration graph of the crude methanol extract and fractions are given in Fig. 1. The % inhibition versus concentration graph of the *n*-butanol fraction is given in Fig. 2.

The anti-oxidant assay protocol followed by us signifies that, all the test samples, crude methanol extract, organic and aqueous fractions and the six pure compounds were displaying relevant anti-oxidant profile. Surprisingly, the *n*-butanol and aqueous extract are more active compared to other fractions and crude extract. Since this will enable and ensure the *in-vivo* delivery of anti-oxidant actives in the oral cavity. These extracts are more active in metal ion chelation and in reducing the metal ions (reducing power and metal ion chelation assay) a most desirable property for oral care. This will enable binding of the actives from the bark with certain metal ions present in the enzymes of harmful bacteria. Simultaneously, this property guarantees, chelation and inactivation of reactive oxygen species (ROS) generated under oxidative stress within the oral cavity of anti-oxidant deprived patients. These two fractions also has good radical scavenging (DPPH, ABTS, superoxide scavenging) properties. This is interesting because, it facilitates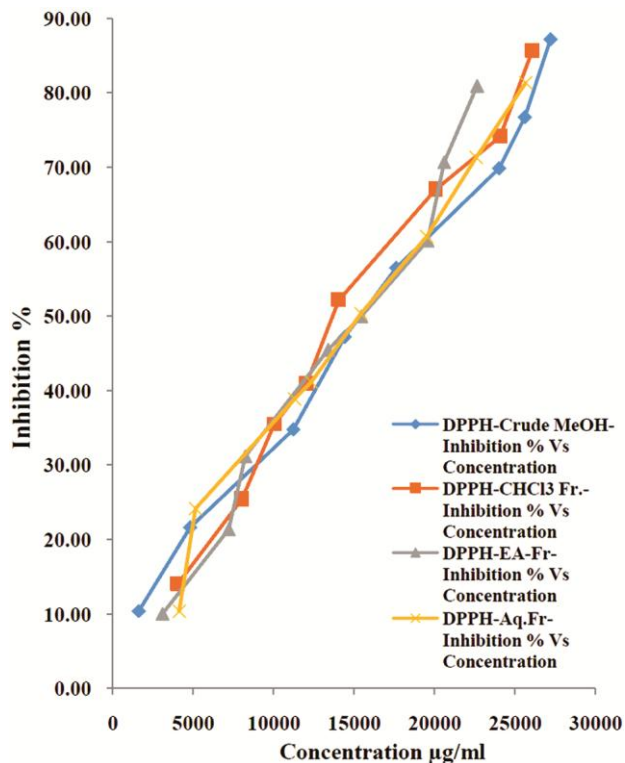 efficient ROS quenching. Particularly, these extracts are moderately active in nitric oxide inhibition assay which is most desirable, since NO generation is a desirable benefit from certain oral bacteria, prominent inhibition of NO within the oral cavity is not desirable. Thus all put together, the *n*-butanol and aqueous fractions were more relevant in delivering anti-oxidant support to the vulnerable group of patients who depend on supplements to overcome the challenges posted by dental caries, and periodontal disease so as to safeguard them from tooth loss. Catechin(1) & gallic acid(2)were the major components of the isolated compound. Catechin (1) constitutes 29.33% and gallic acid (2) constitutes 33.33%of the isolated yield of all pure compounds. The other components phloroglucinol, catecol, rutin and, quercetin collectively yielded 37.33% of the isolated yield of all pure compounds. Hence, it can be stated that, catechin and gallic acid are the major constituent of the plant bark. However, with regard to anti-oxidant efficiency, rutin (5) and quercetin (6) were the most efficient components. These two components of the bark are the most efficient in five of the eight assay protocols followed. In summary catechin (1) and gallic acid (2) can be stated as more abundant and efficient anti-oxidant constituent of the bark.Rutin (5) and quercetin (6) can be regarded as minor constituents with most efficient anti-oxidant activity.

The water soluble extractive of the bark was 12.5%. The experimental details revealed that 1450g of bark of the plant provides 213.16g of methanol extract. Fractionation of 115g of crude methanol extract provides, 61.2g, 27.14g of *n*-butanol and
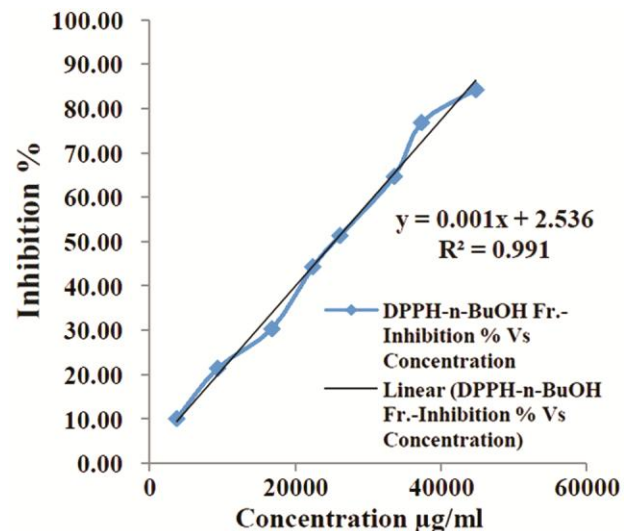

Fig. 1 — DPPH -Inhibition % versus concentration overlay diagram.

Fig. 2 — Inhibition % versus concentration of n - butanol fraction.

aqueous extract. Thus it can be derived that 213.16g of methanol extract will yield 113.44g of *n*-butanol extract and 50.31g of aqueous extract. Technically, the *n*-butanol fraction can also be considered as water soluble portion of the bark, thus total water soluble part obtained from the 213.16g of methanol extract amounts to 163.74g. This amounts to 11.3% of water soluble part of crude methanol extract. This value and the reported water soluble extractives value of 12.5% (of the bark) are in good agreement. These water soluble extractives can be assumed to be released under physiological conditions of usage of plant bark for oral cleaning as an abrasive or as a toothpowder. The antioxidant potential of the aqueous fraction of plant bark is in the range of 10000-25000 μg/mL excluding the Phosphomolybdenum assay, which is specific for identifying lipid soluble anti-oxidants. In fact tocopheryl acetate (Vitamin-E) is the standard compound used in this assay system. Hence, it is wise to exclude it while evaluating the anti-oxidant potential of the aqueous soluble extractives. If we assume that an individual is using 1g of bark powder for tooth brushing along with 10ml of oral secretions such as saliva during usage, the above calculations makes it clear that, approximately 1g of bark usage for daily tooth cleaning may provide 113mg to 125mg of saliva soluble extractives. This amount is almost equal to anti-oxidant activity range displayed by the aqueous fraction of the bark extract 10000-25000 μg/mL.

Thus our earlier research work[9] together with the current findings supports our hypothesis that the bark of *Manilkara hexandra* is suitable for daily oral care. Daily usage of this bark can deliver problem free oral status.

Conclusions

Earlier isolation and characterisation of six phenolic constituents from the bark of *Manilkara hexandra (Roxb)* along with a mixture of protein with MIC value of 60 μg/mL has been reported active against *Streptococcus mutans* as a first time report[9]. In this study, the crude extract, fractions and the six secondary metabolites have been assessed using eight anti-oxidant assay protocols. The *n*-butanol and aqueous fraction of the crude methanol extract displays all the desirable anti-oxidant profile. The anti-oxidant activity of these fractions is within the range of 10000-25000 μg/mL. 1g of bark is found to deliver 113–125mg of aqueous soluble extractive.

Hence, usage of 1g of plant bark powder can generate required amount of extractives (100 – 250mg) within the oral cavity during brushing. Thus one can safely state that, twice daily usage of the plant bark for tooth cleaning, can deliver the desirable protection from oral diseases.All these data put together shows that synergistic and or additive activity of the phenolic constituents along with the protein and other constituents might be a reasonable scientific explanation for the folklore use of the plant as one of the prescription for oral diseases.Our collective finding of the presence of a superior active protein mixture along with delivery of required amount of anti-oxidant extractives from the bark of this plant can make anyone to appreciate the wisdom and patient care, the fundamental elements of the Indian system of medicine, which prescribes *Manilkara hexandra* bark as one of the treatment choices for oral care.

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