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Free radical scavenging and in vitro α-amylase inhibition activities of flavonoids from the fruit rind of *Terminalia bellirica*

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ABSTRACT

The treatment of oxidative stress by using antioxidants is a possible therapeutical strategy for the future. Nowadays continuous efforts are undertaken to identify new natural sources with antioxidant effects. Antioxidants can reduce oxidative stress and thus minimize the incidence of many diseases by various mechanisms. Natural antioxidants can protect cellular components from oxidative damage. Plant secondary metabolites, flavonoids are powerful natural antioxidants. The aim of this *in vitro* study was to assess the free radical scavenging and antidiabetic activity of flavonoids from Diethyl ether and Ethyl acetate fractions of *Terminalia bellirica* fruit rind. The extracted flavonoids displayed potent reducing activity and exhibited strong DPPH, Hydroxyl, Super Oxide and Nitric Oxide free radical scavenging activities. The IC_{50} values of both fractions are comparable with their respective standards. The antioxidant activities may be attributed to the polyphenolics present in the extracts. The flavonoids also possess antidiabetic activity, which shows a strong α-amylase inhibition which is also comparable to the standard acarbose.

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INTRODUCTION

In the developing countries like India herbal medicine play a vital role in the treatment of various illnesses even in this 21st century. Natural antioxidants gained from plants are widely used against oxidative stress-related diseases. These days cancer, neurodegenerative and cardiac diseases are the leading causes of death of Society, dietary antioxidants may decrease the risk of mortality to a certain extent (Minino, Smith, Ed, & Statistics, 2001). An imbalance between the Reactive Oxygen Species (ROS) and the rate at which antioxidants scavenge them is known as the oxidative stress. This ROS can react with and damage almost all biological molecules (Papas, 1996). Synthetic antioxidants are toxic and cause serious health risks and it should be replaced by natural antioxidants (Nampoothiri, Binil Raj, et al., 2011). Efforts are continuously made to identify new natural sources with active antioxidant components.

The administration of polyphenolic compounds with antioxidant effects can offer protection against various types of physiological malfunctions caused by oxidative stress (Ningappa, Dinesha, & Srinivas, 2008). Antioxidants are potential health benefactors and also used as food preservatives to prevent or delay oxidation of food initiated by free radicals which are formed during environmental exposure of food products. (Hraš, Hadolin, Knez, & Bauman, 2000).

Flavonoids are polyphenols, the most widespread class of secondary metabolites in nature, and their distribution is almost ubiquitous. They are categorized, according to their structural features, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones (Pal & Dubey, 2013). The flavonoids are considered as

potent antioxidants and the various biological activities of flavonoids are depend on their chemical structure. Quercetin, the most abundant dietary flavonol, considered as a powerful antioxidant because it possesses all the right structural features for free radical scavenging activity (Lakhanpal & Rai, 2007).

Terminalia bellirica, (Tb) from the Combretaceae family, widely known as 'bahera' is commonly found throughout Central Asia. The dried fruit rinds of *Terminalia bellirica* were selected for the present study. It mainly contains phenolic compounds such as gallic acid and ellagic acid. It is one of the constituents of Triphala, a popular Ayurvedic medicine used as a rejuvenating agent and is prescribed for liver-related diseases (Nampoothiri, Prathapan, et al., 2011). The aqueous extract of *Terminalia bellirica* fruits has antioxidant properties since these contain natural antioxidants, enzymatic and non-enzymatic and is very much effective against microbes causing various diseases (R. Kumar, Chauhan, Bhardwaj, Kumar, & Kumar, 2011).

Crude extracts of plants are used for the preparation of herbal medicines, but many unwanted compounds are also present in them. By fractionation method, we can obtain the polyphenolic rich part of the extract. The present study aimed to access the antioxidant and antidiabetic activities of flavonoids from the diethyl ether and ethyl acetate fractions of *Terminalia bellirica* fruit rind extract. Total phenol and flavonoid contents are determined. Different *in vitro* antioxidant assays like DPPH, Super Oxide, Hydroxyl radial, Nitric oxide, Reducing power assay was done for the samples. *In vitro* antidiabetic assay, α-amylase inhibition was also done for all the fractions.

MATERIALS AND METHODS

Sample Collection

The dried fruits of *Terminalia bellirica* were purchased from the local market, Payyannur, Kannur district, Kerala. The plant materials were identified by the taxonomist Dr. S.Sujanapal, KFRI, Thrissur. The fruit rind of the sample is thoroughly washed under running water and shade dried carefully. The well-dried samples were grinded to powdered form and keep in an airtight sample bottle.

Chemicals

Aluminum trichloride, FC reagent, Potassium ferricyanide, DPPH, 2-Deoxy-2-ribose, EDTA, Ethylenediamine tetraacetic acid, Ascorbic acid, Hydrogen peroxide, NBT, Nitro Blue Tetrazolium, Gallic acid, Quercetin, Riboflavin, Ammonium Molybdate, sodium phosphate, α-amylase, Dinitrosalicylic acid, Sodium nitroprusside, sulphanilamide, phosphoric acid, N-1-naphthyl ethylenediamine dihydrochloride. All the chemicals were purchased from Merck (India) and Sisco Research Laboratories (India). All the solvents used were of the standard analytical grade.

Extraction

The powdered sample was weighed and extracted in 80% methanol by Soxhlet extraction method. The extraction was continued until the solvent became colourless and then filtered. The filtrate obtained was washed thoroughly with petroleum ether and then subsequently extracted with diethyl ether and ethyl acetate (Subramanian et al., 1969). The petroleum ether fraction was discarded because it was rich in fatty matters. Both diethyl ether and ethyl acetate fractions were collected and used for free and bound flavonoids respectively. The collected fractions were dried in a desiccator for further analysis.

Total phenolic content

The amount of total phenols present in the extracts was determined by Folin-Ciocalteu's (FC) reagent using gallic acid as standard (Gupta, Sheth, Pandey, Yadav, & Joshi, 2015). Here 100µl of different concentration of DE and EA, 500µl of FC Reagent and 1 ml of 20% sodium carbonate were added and incubated at temperature 25 to 27^0 C. The absorbance was measured at 760 nm using a UV-Visible Spectrophotometer. The total phenolic content was expressed as mg of gallic acid equivalents/g of extract (GAEs).

Total flavonoid content

Total flavonoids were estimated quantitatively by Aluminium Chloride Method (Chang, Yang, Wen, & Chern, 2002). Quercetin was used to make the calibration curve. Different concentration of each extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The reaction mixture was incubated for 30 minutes at room temperature. The absorbance was measured at 415 nm with a Shimadzu UV-1700 Spectrophotometer. Measurements were done in triplicates.

Qualitative test for flavonoids

The qualitative tests for flavonoids were done by Shinoda test, Lead acetate test and Alkaline reagent test (H. D. Kumar, 2013).

Shinoda Test

The compound to be identified was mixed with small pieces of Magnesium ribbon and Con: HCl

was added dropwise into this mixture. Development of red colour after a few minutes indicates the presence of flavonoids.

Lead acetate Test

Few drops of 10% lead acetate solution were added to the compound, Formation of a yellow colour or creamy yellow precipitate indicates the presence of flavonoids.

Alkaline reagent Test

To the compound, NaOH solution was added. The intense yellow colour formed which loses the intensity of the addition of dilute acid indicates the presence of flavonoids**.**

***In vitro* antioxidant assays**

Reducing power assay

The reducing power of Ethyl acetate and Diethyl ether extracts of Tb was carried out by Potassium ferricyanide method (Yildirim, Mavi, & Kara, 2001). Different concentrations of the extracts were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was then incubated at 50° C for 20 minutes. 2.5 ml of 10% trichloroacetic acid was added to this mixture and then centrifuged at 3000 rpm for 30 minutes. 2.5 ml of the supernatant solution was collected and mixed with 2.5 ml of distilled water and 0.5 ml, ferric chloride(10%). The colour developed was measured at 700 nm. Here the standard and blank used were ascorbic acids and phosphate buffer respectively.

Free radical scavenging by DPPH

The radical scavenging activity of extracts was evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Duan, Zhang, Li, & Wang, 2006). Antioxidant react with DPPH, a stable free radical to form DPPH-H. On accepting hydrogen from a corresponding donor, the solution loses the characteristic deep purple colour and the discoloration was proportional to the concentration and scavenging power of the compound. Ascorbic acid was used as a standard. The reaction mixture contained 2.8ml of 100µM DPPH dissolved in methanol and various concentrations of the compound in methanol. The mixture was shaken well and incubated for 30 min in the dark at room temperature. Absorbance was read at 517nm to measure the reduction of DPPH radical. Every concentration was done in triplicate.

Super Oxide Free radical scavenging

Assay of Super Oxide was done by Valentao et al. method (Valentao et al.,2002). Superoxide is biologically important as it is a precursor of ROS and which damage cellular components. Overproduction of this radical leads to redox imbalance and associated with many physiological disorders. Superoxide anions are generated in the riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. Different concentration of Ethyl acetate and Diethyl ether extracts of Tb, 12.5 - 200µg/ml from a stock solution of 1mg/ml, 0.05ml of 0.12mM Riboflavin solution, 0.2 ml of 0.1M EDTA solution, and 0.1 ml 1.5 Mm NBT (Nitro-blue tetrazolium) solutions were mixed and it was diluted up to 2.64 ml with 0.067M phosphate buffer. A control without the test compound, but an equivalent amount of distilled water was taken. The absorbance of the solution was measured at 560 nm after illumination for 5 minutes in fluorescent light and again measured after illumination for 30 min at 560 nm on UV visible spectrophotometer. Here the standard used was ascorbic acid.

Hydroxyl Radical scavenging activity

Hydroxyl radical scavenging assay was done by a previously described method (Valentão et al., 2003). Hydroxyl radical was generated by Fenton reaction(The Fe^{3+} - ascorbate- EDTA – H_2O_2 system). The assay is based on the quantification of the degradation product of 2- deoxy-2- ribose by condensation with TBA. Different concentration of both the extracts 12.5-200µg/ml from a stock concentration of 1mg/ml mixed with 500µl reaction mixture (2 -deoxy -2- ribose (2.8mM), 100µm $FeCl_3$, 100µm EDTA, 1mM H_2O_2, 100µm ascorbic acid in 20 mM pH 7.4 KH_2PO_4 - KOH buffer) was made up to a final volume of 1 ml. A control without the test compound was taken. After incubation for 1 hour at 37° C, add 1ml of 2.8% TCA, then 1ml 1% aqueous TBA was added and incubated at 90° C for 15 minutes to develop the colour. After cooling the absorbance of the sample and the blank were measured at 532 nm. Gallic acid was used as the standard and all the measurements were done in triplicates.

Nitric Oxide Scavenging Activity

Sodium Nitro Prusside (SNP) generates nitric oxide which interacts with oxygen to produce nitrite ions which are measured using Griess reagent(1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Nitric oxide scavenging activity was measured spectrophotometrically by measuring the absorbance of chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphthyl ethylenediamine dihydrochloride at 540nm (Venkatachalam & Muthukrishnan, 2012). In short, the reaction mixture contains SNP(5mM)

Table 1: Total phenolic and flavonoid content of diethyl ether and ethyl acetate extract

Sample	TPC (mg GAE/g)	TFC (mg QE/g)
Diethyl ether	171.21± 0.73	41.20± 0.79
Ethyl acetate	158.46± 0.27	32± 0.18

Table 2: Reducing power activity of the extracts

Concentration (µg/ml)	Absorbance (nm)		
	Standard	DE	EA
125	1.036	1.297	0.765
250	1.656	1.314	1.364
500	2.231	1.406	1.451
1000	2.381	2.500	1.601
2000	2.437	2.532	1.799

in phosphate buffered saline (pH-7.4) with different concentration of the extracts (12.5-200µg /ml from a stock concentration of 1mg/ml) and was incubated at 25°C for 30 minutes. A control without the test compound but an equivalent amount of distilled water was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent and absorbance was taken. Here the standard used was gallic acid.

In vitro Anti-diabetic assay

α-Amylase Inhibition Assay

Inhibition of α-amylase by the extracts was determined by a modified dinitro salicylic acid (DNS) method (Bernfeld, 1955). The enzyme inhibition potential was expressed as a decrease in units of maltose liberated. 1ml of both the extracts of Tb were pre-incubated with α-amylase 1 U/ml for 30 min and then 1 ml (1% w/v) starch solution was added. The reaction mixture was again incubated for 10 minutes at room temperature. Finally, the reaction was stopped by adding 1 ml DNS reagent (12.0 g of sodium potassium tartrate tetrahydrate in 8 mL of 2M NaOH and 96 mM 3, 5-dinitro salicylic acid solution) and was heated in a boiling water bath for another 5 minutes. A blank was prepared without extracts and another without the amylase enzyme, replaced by equal quantities of buffer (20 mM Sodium phosphate buffer with 6.7 mM Sodium chloride, pH 6.9 at 20^{o} C). The absorbance was measured at 540 nm. The reducing sugar released from starch was estimated as maltose equivalent from a standard graph. Acarbose was used as the standard. The anti-diabetic activity was determined through the inhibition of α-amylase which was expressed as a percentage of inhibition.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Content

Total phenolic and flavonoid content of diethyl ether and ethyl acetate extract is shown in Table 1. The Phenolic compounds are one of the most effective antioxidative constituents that contribute to the antioxidant activity in medicinal plants. The total phenolic content of diethyl ether and ethyl acetate extract was calculated from the standard curve of gallic acid, y= 0.022x+0.052, R^2= 0.994. Whereas the estimation of the total flavonoid content of the same was calculated from the standard graph of Quercetin Y= 0.008x-0.026, R^2= 0.994. Diethyl ether extract of the sample possesses a higher amount of total phenolics and flavonoids when compared to the ethyl acetate fraction.

Qualitative test for flavonoids

Both the flavonoid fractions showed strong positive results on the qualitative test for the identification of flavonoids.

In vitro antioxidant assays

Reducing power assay

As illustrated in Table 2, both flavonoid fractions and the standard showed the same trend in the absorbance value, the reducing capability of standard and the flavonoids from the DE are almost the same. The reducing power of flavonoids may serve as a good indicator of its antioxidant activity. However, the antioxidant capacity of a compound may be attributed to various mechanisms like prevention of chain initiation, decomposition of peroxides, radical scavenging and reducing power (Hazra, Sarkar, Biswas, & Mandal, 2010)

DPPH Radical Scavenging Activity

Flavonoids from both DE and EA of *Terminalia bellirica* fruit rind were able to reduce the stable pink colour DPPH free radical to yellow coloured Diphenyl picryl hydrazine. As is evident from the Fig 1, the IC_{50} values are 10.21µg/ml and 16.47µg/ml in the case of DE and EA respectively, the value is comparable to the IC_{50} value of reference standard Ascorbic acid which is 11.33

µg/ml. The flavonoids from Diethyl ether showed a better result than the standard.

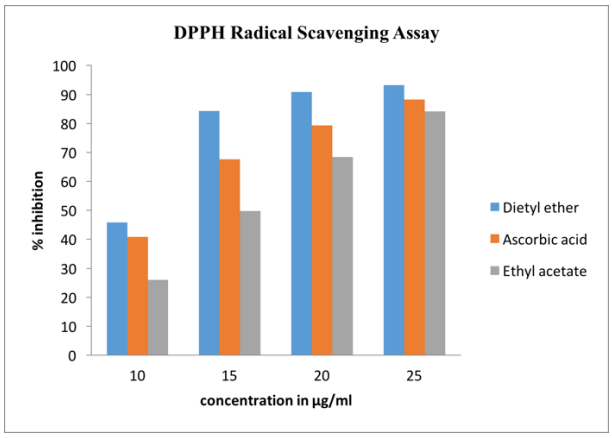

Figure 1: DPPH radical scavenging activity of the extract

Super Oxide Free Radical Scavenging Activity

Superoxide radicals are very harmful to cellular components as a precursor of highly reactive oxygen species. Fig 2 showed that the abilities flavonoids from DE and EA to quench the superoxide radicals in the reaction mixture. The result showed that the IC_{50} values of both DE and EA are comparable or even higher than that the standard. The IC_{50} value of the standard, EA and DE are 141.87 µg/ml, 142.96 µg/ml and 70.72 µg/ml respectively.

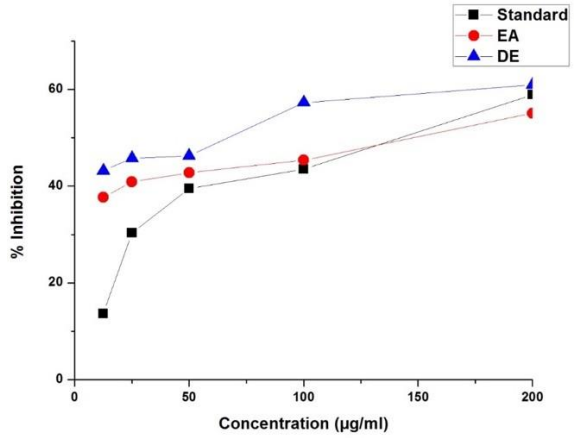

Figure 2: Superoxide radical scavenging activity

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals are the most reactive among free radicals and can react with almost all biological molecules which lead to serious tissue damage (Odunola, Olugbami, & Gbadegesin, 2015). The effects of flavonoids from DE and EA in scavenging hydroxyl radical to prevent the oxidative degradation of deoxyribose substrate was determined and compared with that of Gallic acid (IC_{50} value is 167.95 µg/ml). The IC_{50} values of EA and DE are 138.707 µg/ml and 68.211 µg/ml respectively which is clear from the Fig 3

Nitric Oxide Scavenging Activity

Large amounts of Nitric oxide, in addition to Reactive Oxygen species, may lead to tissue damage**.** Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The antioxidants from the plants can counteract with NO radical and thus be protecting the body from the adverse effects caused by the radical. The flavonoids from both DE and EA fractions of *Terminalia bellirica* showed strong NO inhibition when compared with the standard Gallic acid, which is understood from the Fig 4. The IC_{50} values of the standard, DE and EA are 15.07 µg/ml, 32.95 µg/ml, 78.38 µg/ml respectively.

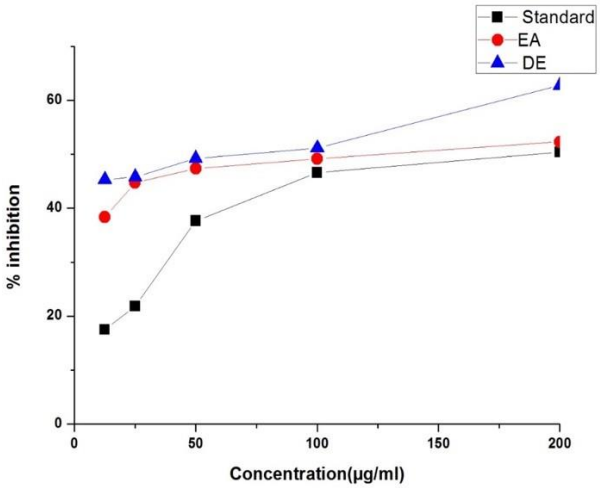

Figure 3: Hydroxyl radical scavenging activity

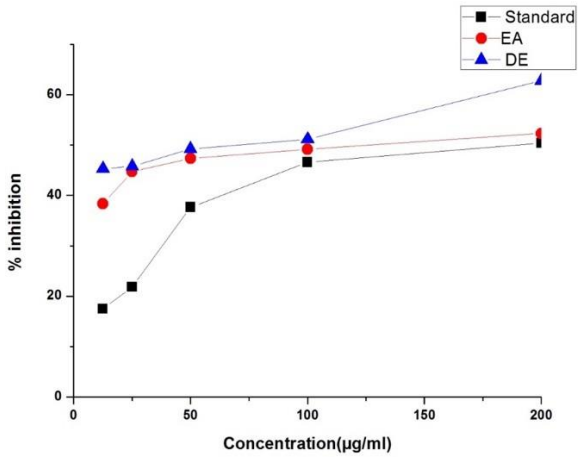

Figure 4: Nitric oxide radical scavenging activity

α- Amylase Inhibition Activity

Amylase inhibitors are important because they have a strong therapeutic potential against type 2 diabetes. Inhibition of pancreatic amylase is an effective strategy for the management of diabetes (Nampoothiri, Prathapan, et al., 2011). The results showed that the flavonoids from both DE and EA are useful inhibitors of α-Amylase, which is almost comparable to the standard Acarbose. The IC_{50} values of the standard, DE and EA are 201.07,

303.89 and 420.59 µg/ml respectively which is shown in figure 5.

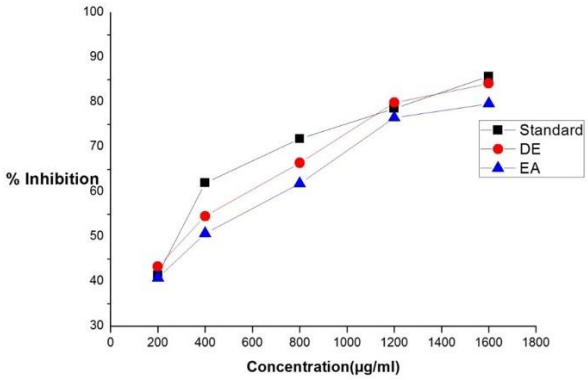

Figure 5: α- Amylase Inhibition Activity

CONCLUSION

The flavonoids from DE and EA fractions of fruit rind of *Terminalia bellirica* gave strong positive results on the qualitative tests for the identification of flavonoids. Reducing the power of the flavonoids proved that they are good antioxidants. The DPPH, Superoxide, Hydroxyl and Nitric oxide radical scavenging activity studies revealed that the extracted flavonoids are strong radical scavengers. The activities are compared with their respective standards. They also possess strong amylase inhibition potential. The positive results from the various assays revealed the high pharmacological value of T*erminalia bellirica* fruit rind. These flavonoids with high antioxidant potency may incorporate in modern drugs. However, there must be a further study on active component isolation is necessary for drug design.

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