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Original Research Article

Antioxidant Activity of *Terminalia bellirica* (Gaertn.) Roxb. of Tawang, Arunachal Pradesh, India

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Abstract: The fruits of *Terminalia bellirica* are sold in plenty in the local market of Tawang. The local Monpa community uses it as a part of their dietary component, in making pickles and also used by the herbalist in the treatment of various diseases like conjunctivitis, kidney diseases, and constipation. Often diseases induce oxidative stresses but can be counteracted by antioxidants. The present investigation was designed to evaluate the amount of antioxidant percentage present in the fruit of *T. belliricaI*. The methanol extract of the fruit was prepared and diluted to a working concentration of 1mg/ml, which is further diluted serially to (1000, 500, 250, 125, 62.5, 31.2 and 15.625) g/ml fractions in methanol. 100 µL of each of the fractions were reacted with 200µl each of 0.1 mM DPPH solution prepared in methanol. After 30 minutes' incubation in the dark, at room temperature, the absorbance was taken at 517 nm in Multiskan™ spectrophotometer. Ascorbic acid was used as the standard reference. Free radical scavenging activity (% Inhibition) was calculated from the absorbance values using the control as a reference. Logarithmic graphs were drawn between %Inhibition against the concentrations using excel and calibrate the IC_{50} of both the sample and standard. The result shows a high antioxidant percentage close to the standard. The % inhibition shows a strong positive correlation with the concentration at a low level of significance and with low Pearson's correlation coefficient (r) and high coefficient of determination (R^2) values. The qualitative evaluation of the samples for secondary metabolites (flavonoids and phenolics that normally favors antioxidant properties) also shows high positive results.

Keywords: Antioxidant activity, DPPH scavenging activity, Flavonoid, Phenolic compounds, Superoxide radical

Introduction

The birth of free radical biochemistry was an outcome of the events of World War II (1939-1945). The two atom bombs (Hiroshima and Nagasaki, 6th and 9th August 1945, respectively) led to massive deaths to the entire population and shortened life-span of the survivors. However, followed with the tragedy, in 1954, Gershman and Gilbert speculated that the lethal effects of ionizing radiation might be due to the formation of reactive oxygen species (ROS). Since then free radicals (atoms with an unpaired electron) such as ROS and reactive nitrogen species (RNS) have gained notoriety (Gilbert, 1981). Free radicals and other reactive oxygen species (ROS), the superoxide radical ($O\cdot^{2-}$) and hydrogen peroxide (H_2O_2) are constantly generated in the brain, nervous system and several areas of the body and stimulate free-radical reactions. During injury and diseased conditions (viz. cancer, atherosclerosis, malaria, rheumatoid arthritis and neurodegenerative diseases etc.), ROS generated, causes more damages of the cells and body (Aruoma, 1998). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage (Hayyan, 2016). Free radicals and related species

are mainly derived not only from ROS but also from RNS, and are produced endogenously in our body, when exposing to different physicochemical or pathophysiological conditions and can alter DNA, lipids, and proteins which can lead to aging and a number of human diseases. Free radical damage to DNA, can result in mutagenesis and carcinogenesis, while damage to lipid and protein causes lipid peroxidation and loss of enzyme activity respectively (Devasagayam *et al.*, 2004). Most of the damage induced by ionizing radiations in biological systems is indirect and is mediated by products of radiolysis of water including hydrogen radical (•H), •OH, hydrated electron (e_{aq}^-), H_2O_2, peroxyl radical (ROO*), O_2^{*-}, $1O_2$ etc. (Von, 1987; Devasagayam and Kesavan, 1996). Antioxidants are substances capable of neutralizing the actions of free radicals (Sies, 1996), by preventing the formation of ROS, and acts at different levels (viz. prevention, interception, and repair). The action of antioxidants is supplemented by the enzyme, Superoxide dismutase (SOD), carry out partitioning of the superoxide (O_2^-) radical into either O_2 or H_2O_2. H_2O_2 which are converted by catalase into water and oxygen (Cadenas and Packer, 1996).

Vitamin E and tocotrienols (such as those from palm oil) are efficient lipid soluble antioxidants that function as a 'chain breaker' during lipid peroxidation in cell membranes and various lipid particles including LDL (Packer and Ong, 1998). Vitamin C (ascorbic acid) is a water-soluble free radical scavenger. Apart from these carotenoids such as b-carotene, lycopene, lutein and other carotenoids function as important antioxidants and they quench 1O2 and ROO•. Flavonoids, mainly present as colouring pigments in plants also function as potent antioxidants at various levels (Kagan *et al.*, 2002). Apart from the above mention antioxidants, nutritional components like selenium, phenols, flavonoids, isoflavones, isothiocyanates, diterpenes, methylxanthines, dithiols, and coumarins are believed to inhibit tumor formation (Krishnaswami, 1996). Fruits are an important source of minerals, and vitamins. They are also a good source of energy and have medicinal value, therefore, essential for a balanced diet and good health. Fruits have antioxidant activity and keep many deadly diseases such as cancer, cardiovascular diseases and free-radical induced oxidative stress at bay (Ames, 1983; Steinberg *et al.*, 1989, Steinberg, 1991; Gey, 1990; Mahantesh, *et al.*, 2012).

Among the family *Combretaceae*, the genus *Terminalia* (in Latin, terminus = ending), referring to the crowded leaves on the shoot tips, and consists of 514 species of these 54 are accepted species names. In India *T. chebula, T. citrina and T. bellirica* are reported as major related species and along with them, *T. arjuna* are commonly distributed in every part of India (Pankaj and Robert, 2008). Fruits of some of the Terminalia species are reported to use as fodder and food such as *T. arjuna, T. citrina*, *T. chebula, T. catappa*, *T. bellirica, T. glabra* etc. (Singh, 2008; George, 2014) and herbal medicine (*T. arjuna*) (Maulik *et al.*, 2012). Fruits of *T. chebula* and *T. bellirica* with the stem bark of *T. arjuna (stem bark)* were used in Europe as traditional herbal medicine. Fruits of *T. bellerica* are reported to have anti-microorganisms and used to treat piles, dropsy, (Elizabeth, 2005; Alam *et al.,* 2011), fever, cough, skin diseases, oral thrush, diarrhea (Kumar, *et al.*, 2010), hypertension (Chaudhary, 2012), ulcer (Jawanjal, 2012), antidiabetic (Latha, 2010) and anthelmintic (Kumar, 2010), anticancer (Kumudhavalli, 2010). Even stem bark of *T. bellirica* and *T. chebula* along with *Terminalia tomentosa* are reported as adulterants of *T. arjuna*. (Sharma and Srivastava, 2016) The fruits of *T. bellirica, T. chebula and Emblica officinales* were used in several herbal treatments by varying the proportions by Monpa local herbalist of Tawang district of Arunachal Pradesh, similar to the report made by William (1890) about the synergistic use of these three fruits, way back in Ayurvedic medicine. This species is also used locally for its mind-altering qualities by smoking dried kernels which on higher dosages, cause nausea and vomiting.

The fruits of *T. bellirica*, variously known as "Bahera" or Beleric or bastard myrobalan, a large deciduous tree of up to 40 m height, 3m diameter, frequently buttressed at the base and branchless up to 20 m, found between 900m-1900m

elevation in the state. The bark is grey covered with numerous cracks, with a yellowish inner bark. Leaves large, glabrous, alternate, broadly elliptic to elliptic-obovate, leathery, dotted, entire, narrow- pointed or rounded leaf tip. Leaves 6-22 cm length, 5-13 cm wide, on a 2.5-9 cm long petiole, base rounded to cuneate, glabrescent, with 6-9 pairs of secondary veins. Secondary and tertiary venation prominent on both surfaces. Flowers, small, solitary, honey-scented, greenish yellow, 4-15 cm × 5-6 mm dimensions on spikes in leaf axils, upper flowers, stamens 3-4 mm length, lower flowers, bisexual. Fruit is obovoid, sub-globular to broadly ellipsoid, 2-4 cm×1.5-2.5 cm dimensions, densely velutinous or sericeous, light-yellow, obscurely 5-angled and minutely brown tomentosa. In spite of its local popularity as food and medicine, there is a scanty report on the use of *T. bellirica* from the state and Monpa community of Tawang district in particular. Therefore, the study on the antioxidant activity of *T. bellirica* was conducted and compared with the that of the pure ascorbic acid standard antioxidant.

Materials and methods

i. Sample collection & preparation

The fruit of *Terminalia bellirica* (Gaertn.) Roxb. (Fig. 1) was collected from the local market of Tawang district, Arunachal Pradesh, India during the month of February 2017 and brought to the Department of Botany, Rajiv Gandhi University for the analysis. The sample was cleaned, chopped into pieces (removing seeds), shade-dried under the fan for (4-5) days at room temperature. The dried sample was grounded into powder using mixer grinder. The dried powder sample 20 g was extracted in 100mL MeOH for 10 days by cold maceration method. The extract was filtered using Whatman filter paper No. 1 and the filtrate was concentrated using rotary vacuum evaporator and finally dried completely. The final dried extract was then used for the following experiments.

ii. Qualitative test for antioxidant components

The qualitative evaluation of the antioxidant related secondary metabolites such as flavonoids and phenolic compounds, of all the fractions were carried out following standard methods (Ramaan, 2006).

Fig. 1. *Terminalia bellirica* (Gaertn.) Roxb.

a) **Detection of Flavonoids**: by Alkaline reagent test- An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids

b) **Detection of Phenolic compounds**: by Ferric chloride test (Mace, 1963) - 50 mg of the extract was dissolved in 5 mL of distilled water. Then few drops of 5% neutral ferric chloride solution were added to it. A dark green color indicated the presence of phenolic compounds.

iii. Quantitative Antioxidant test

Free radical scavenging activity of the sample was carried out following DPPH method (2,2-Diphenyl-1-picrylhydrazyl; Sigma Aldrich) of Blios (1958) using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, 96-well plate readers, Germany, instead of using simple cuvette UV-VIS spectrophotometer. For this method, the dry powder of *T. bellirica* (20 g) was extracted in 100 mL methanol (MeOH) by cold maceration for about 10 days. The extract was then filtered using Whatman filter paper No. 1 and the filtrate was concentrated by rotary evaporator and later dried completely to yield a dry extract of 3.77 g. Dry extract (10 mg) was diluted in 10ml MeOH solvent making a working extract of 1mg/ml (=1000µg/ml) concentration. A serial dilution of the above working extract of the order (1000, 500, 250, 125, 62.5, 31.2 and 15.625 µg/ml) fractions were prepared in the same solvent. To a 100 µL of each fraction, it was reacted with 200µL each of 0.1

mM DPPH solution prepared in MeOH. After half-an-hour incubation in dark, at room temperature, the absorbance was taken at 517 nm using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, 96-well plate reader, German. For the accuracy, six replications per concentration were made both for the sample as well as the standard. Pure ascorbic acid was used as the standard reference. A control solution (a similar mixture of solution without the sample/standard) was used as the basis for calculating the free radical scavenging activity of each replica. Free radical scavenging activity of the sample was calculated using the formula:

DPPH scavenging activity (% of free radicle Inhibition)
$= (A_0 - A_1) / A_0 \times 100$
Where A_0 is the absorbance of control and A_1 is the absorbance of the fruit sample.

Graphs were drawn between the concentration (C-coordinates) vs Inhibition % (Y-coordinates) by the Logarithmic plotting of the points using excel and calibrate the IC_{50} of both the sample and standard replicates. The IC_{50} is calculated from the formula $Y = a\ln(x) \pm c$. Where Y is the 50% inhibition, x is the concentration of the sample extract at which 50% inhibition of the DPPH radicles takes place.

iv. Statistical analysis

Correlation and regression between the concentrations and the free radical inhibition percentage of both the sample and standard were calculated using Excel.

Results

The preliminary qualitative analysis for flavonoid and phenolic compounds of the methanolic extract of the sample is shown in Table 1.

Table 1. The preliminary tests for flavonoid and phenolic compounds

Sl. No.	Experiments performed	Detections
1	Flavonoid detection (Alkaline reagent test)	++
2	Phenolic compounds detection (Ferric chloride test)	+++

++ = presence detected; +++ = high presence detected

The free radical inhibition percentage of the MeOH extracts for the fruits of *T. bellirica* at seven different concentrations (1.56, 3.12, 6.25, 12.5, 25, 50 and 100 μg/ml) in six replicates were shown in Table 2. The table also shows the IC_{50} of the sample replicates obtained, from the logarithmic plotting of the coordinates and their average and standard deviation values.

Table 2. The concentration wise individual and average inhibition percentage and IC_{50} of *T. bellirica*

Concentration ($\mu g ml^{-1}$)	Free radical inhibition % of six replicates obtained per concentration						Average Inhibition % (± SD)
	1st	2nd	3rd	4th	5th	6th	
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.563	7.757	8.176	7.967	6.709	7.757	7.338	7.617 ± 0.53
3.125	16.772	16.352	17.191	17.191	15.723	14.885	16.352 ± 0.91
6.250	25.157	24.738	24.738	25.996	23.690	26.415	25.122 ± 0.98
12.500	48.637	51.782	45.283	49.266	49.057	49.266	48.882 ± 2.33
25.000	72.746	72.117	73.585	72.537	74.004	74.633	73.270 ± 0.96
50.000	87.002	87.631	87.631	87.421	87.212	87.212	87.352 ± 0.25
100.000	87.212	87.002	87.212	87.002	87.421	87.002	87.142 ± 0.17
IC_{50}	9.635	9.433	9.746	9.545	9.741	9.531	9.641 ± 0.13

The percentage inhibition of the DPPH free radicals and the IC_{50} values, at the different concentrations of the standard ascorbic acid was shown in Table 3.

The average inhibition percentages of the sample (7.62, 16.35, 25.12, 48.88, 73.27, 87.35 and 87.14%) and the standard (15.82, 22.13, 38.30, 68.47, 88.94, 89.27 and 90.91%) when plotted against the concentrations using excel a logarithmic graph/plot was obtained and helps to calculate the average IC_{50} value accurately (Fig. 2).

Table. 3. The concentration wise individual and average inhibition percentage and IC_{50} of Ascorbic acid (standard).

Concentration (μgml-1)	Free radical inhibition % of six replicates obtained per concentration						Average Inhibition % (± SD)
	1st	2nd	3rd	4th	5th	6th	
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.563	15.061	15.712	15.549	15.223	16.362	17.013	15.820 ± 0.74
3.125	23.034	21.569	22.383	22.871	21.407	21.569	22.139 ± 0.72
6.250	38.167	38.329	38.329	37.841	37.679	39.468	38.302 ± 0.63
12.500	71.361	60.361	69.409	69.409	69.409	70.873	68.470 ± 4.06
25.000	85.237	84.237	89.749	89.749	89.749	89.911	88.939 ± 2.31
50.000	89.749	85.911	89.911	90.074	89.911	90.074	89.272 ± 1.65
100.000	90.400	95.237	89.586	90.725	89.586	89.911	90.907 ± 2.17
IC_{50}	6.468	7.919	6.283	6.457	6.320	6.140	6.598 ± 0.66

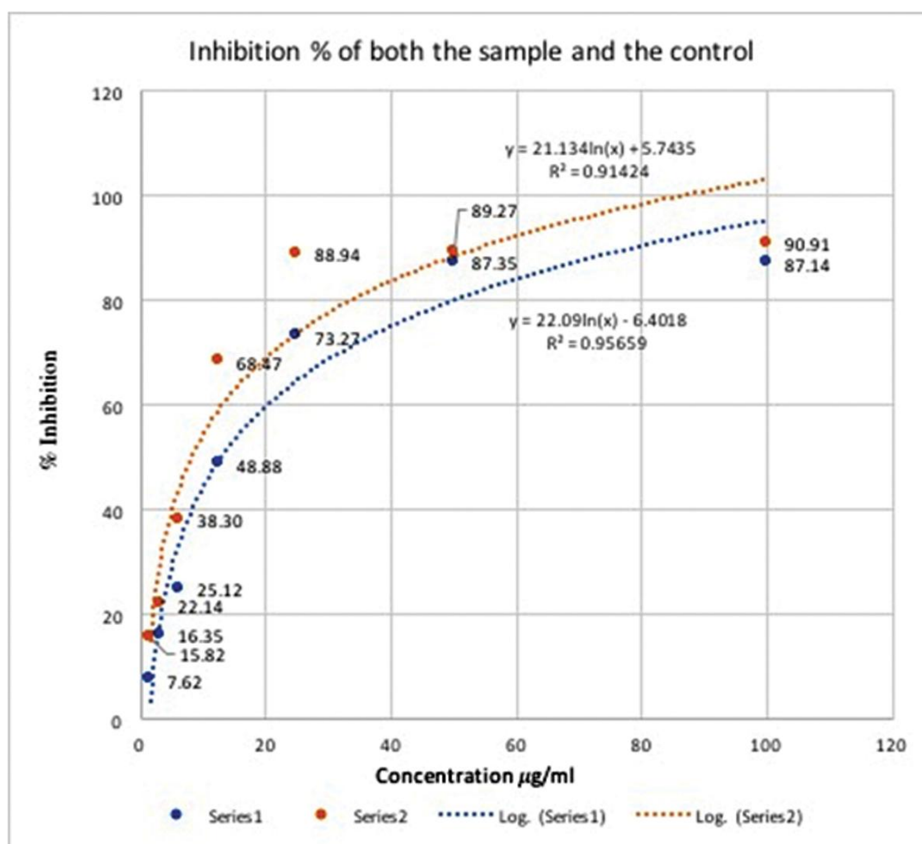

Fig. 2. Average inhibition percentage for ascorbic acid and *Terminalia bellirica*.

Discussion

The preliminary qualitative test helps to find the chemical constituents present (Bhandary *et al.*, 2012). *In vitro* detection of flavonoid and phenolic compounds with better detection for phenolic compounds (Table 1), gives the clues of possible high antioxidant activity in the sample material, because Flavonoids are known to be part of overall antioxidant of the immune system and protect us from many kinds of diseases (Pal and Verma, 2013) and are categorized under polyphenols (King and Young, 1999). Also, flavonoids and phenolic compounds are known to impart antioxidant activity (Banjarnahor and Artanti, 2014 and Rice-Evans *et al.*, 1997). DPPH (2, 2 -diphenyl-1-picrylhydrazyl) is composed of the stable free radical molecule. It has deep violet color. When it is reacted with suitable antioxidant, it accepts an electron or hydrogen and changes into its neutral form. On completion of the reaction with an antioxidant compound, the color gets changed into light yellow color. The discoloration is associated with the presence of antioxidants (Blois, 1958; Huang *et al.*, 2005). The coordinates for concentration when plotted on X-axis and percentage inhibition on Y-axis shows most fitting in the logarithmic plot, hence logarithmic plot was used in the graph, where most of the points fall on the graph. The sample, *T. bellirica* fruits shows IC_{50} value of (9.64 ± 0.13 µg ml^{-1}) (Table 2), while the IC_{50} value of the standard pure ascorbic acid was (6.60 ± 0.66 µg ml^{-1}) (Table 3). The IC_{50} values denote the concentration of the sample required to scavenge 50% of the free radicals present in DPPH. The lower the value of IC_{50}, the greater is the antioxidant activity. According to the obtained data, the inhibition percentage of the *T. bellirica* is very close the inhibition percentage of the ascorbic acid. The standard being a pure compound while the sample being a crude extract, it is possible for the fruits of *T. bellirica* to have a higher antioxidant activity in its pure form higher than the standard, which suggests for a possible strong medicinal plant as reported as

antibacterial, antiviral and anti-inflammatory effects (Nampoothiri *et al.*, 2011). A strong positive correlation between the concentration and percentage inhibition was observed for both the sample and the standard. The correlation value obtained was supported by the significance level, multiple $r = 0.89$, and $p < 0.05$, $R^2 = 0.78$ in both the cases of the sample as well as the standard. The antioxidant activity of *T. bellirica* was reported from the aqueous extract and ethanolic fraction, by Kumar *et al.* (2011). However, no report is on record till date from *T. bellirica* from Arunachal Pradesh and also from the methanolic extract.

Conclusion

Studies have shown that antioxidants are required to keep human body healthy for a long term as it protects us from many degenerative diseases. From this experiment, it can be inferred that *Terminalia bellirica* can also be a great source of antioxidant with a mere difference from the pure ascorbic acid. Which indirectly shows the possibility of having compounds with antioxidant property higher than the pure antioxidant, ascorbic acid. Therefore, *T. bellirica* fruit is fit to recommend as a great source of antioxidant. The present study enabled us to suggest *T. bellirica* as one of the fittest candidates for searching novel, bioactive compounds in future research.

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