Research Article

In vitro antioxidant activities of isolated bioactive flavonoid apigenin-7-O-β-D-glucuronide methyl ester from ethyl acetate leaf extract of *Manilkara zapota*

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ABSTRACT

Introduction: Apigenin-7-O-β-D-glucuronide methyl ester is a bioactive flavonoid, has been isolated from the ethyl acetate leaf extract of *Manilkara zapota*, and shown both *in vitro* and *in vivo* anti-inflammatory activities. **Objective:** The present study was aimed to evaluate the *in vitro* antioxidant activities of bioactive flavonoid apigenin-7-O-β-D-glucuronide methyl ester from the ethyl acetate leaf extract by *in vitro* antioxidant assays such as 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) assays. **Materials and Methods:** The *in vitro* antioxidant assays such as DPPH and NO were carried out using standard methods. **Results:** The results showed that the apigenin-7-O-β-D-glucuronide methyl ester (25, 50, and 100 µg/mL) has significant DPPH and NO free radical inhibiting activity at 100 µg/ml was 83.26%, 86.19% with half maximal inhibitory concentration (IC_{50}) values of 36.38 and 29.74, whereas the standards, ascorbic acid, and 3,5-di-tert-butyl-4-hydroxytoluene at 10 µg/ml, exhibited 54.21% and 56.75% with IC_{50} values of standards 4.49 µg/ml and 3.93 µg/ml, respectively. **Conclusion:** From this study, it can be concluded that the apigenin-7-O-β-D-glucuronide methyl ester, exhibited significant scavenging activity on DPPH and NO free radical inhibiting activity, and could serve as a potential source of natural antioxidants, for the development of therapeutic antioxidant drugs.

KEY WORDS: 1, 1-diphenyl-2-picrylhydrazyl, Antioxidants, Apigenin-7-O-β-D-glucuronide methyl ester, *Manilkara zapota*, Nitric oxide

INTRODUCTION

Oxidative stress is a most important phenomenon with clinical significance in a wide variety of disease conditions such as cancer, muscle hypertrophy, Parkinson disease, and diabetes, Alzheimer's disease, and cardiovascular disease.[1] Oxidative stress is featured in the production of oxygen-derived free radicals such as hydrogen peroxide, superoxide, nitric oxide (NO), and hydroxyl radicals, these free radicals are collectively termed as reactive oxygen species (ROS), apart from oxygenated species, nitric species such as nitrous oxide (NO^-) and peroxynitrite ($ONOO^-$) act as a significant part in causing oxidative stress. These nitric species are collectively termed as reactive nitrogen species (RNS). Both ROS and RNS exert oxidative stress making each human cell to undergo 10,000 oxidative hits per second,[2] which results in alteration of redox potential, leading to irreparable damage to biomolecules such as lipids, nucleic acid, and proteins, leading to cellular dysfunction.

Both enzymatic and non-enzymatic systems are responsible for the removal of free radical and maintain cellular integrity. Several synthetic antioxidant medications are used for the treatment of oxidative stress and inflammatory diseases, which are associated with adverse side effects on various organ systems.[3] In recent years, the usage of natural antioxidants from plants has gained attraction and interest due to lack of side effects and high therapeutic value.[4] Natural antioxidants such as flavonoids, terpenoids, tannins, and alkaloids are preventing oxidative stress by activating antioxidant enzymes such as catalase and glutathione peroxidase.[3]

Access this article online	
Website: jprsolutions.info	ISSN: 0975-7619

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Received on: 12-03-2018; Revised on: 19-04-2018; Accepted on: 27-05-2018

Manilkara zapota is a large, evergreen forest tree belongs to family Sapotaceae. *M. zapota* is a species of lowland rainforest. Various research groups worked on *M. zapota* plant to explore its therapeutic value of assessing its pharmacological activities such as antibacterial, antioxidant, anti-inflammatory, antipyretic, analgesic, antiarthritic, and antiproliferative activities.[5] Hence, the present study has been undertaken to evaluate the antioxidant effects of isolated compound (apigenin-7-O-β-D-glucuronide methyl ester) from ethyl acetate leaf extract of *M. zapota* by *in vitro* antioxidant assays such as 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and NO assays.

MATERIALS AND METHODS

DPPH purchased from Sigma, USA. 3,5-di-tert-butyl-4-hydroxytoluene (BHT), ascorbic acid, sodium carbonate, sodium azide, dimethyl sulfoxide, and sodium hydroxide obtained from Hi-Media Laboratory Ltd., Mumbai, India.

Plant Material Collection

Fresh leaf material of *M. zapota* plant was collected from Vizag steel plant area, Visakhapatnam district, Andhra Pradesh, during May 2011. Plant leaf material was authenticated by Dr. S. B. Padal, Associate Professor, Department of Botany, Andhra University. A specimen of this plant was deposited in Botany Department Herbarium, Andhra University with Accession Number AU (BDH) 21913.

Preparation of Ethyl Acetate Extract

About 500 g of dried powder was taken and extracted with solvent ethyl acetate by a hot percolation method using a Soxhlet apparatus. The obtained ethyl acetate extract was rotavaporized to obtain a crude ethyl acetate leaf extract weighing about 35 g.

Extraction of Flavonoids

The *M. zapota* ethyl acetate leaf extract is adsorbed on a modest quantity of silica gel and the solvent is left to evaporate. The 100–200 mesh silica gel was mixed with hexane and made into slurry. This slurry is packed into the column up to 40 cm. The settled stationary phase was run thrice with hexane. After running, silica adsorbed leaf extract was applied along the height of the bed and covered with absorbent cotton to avoid disturbance in the plant extract, then column ran with different solvents from non-polar to polar, i.e. hexane to methanol. This gradient elution method was very efficient for elution of individual compounds from mixtures. The flow rate was adjusted to 10 ml/min. Collected plant extracts Rf value was checked on thin-layer chromatography plates and similar Rf value extract samples were pooled up and condensed by distillation of solvent. These condensed samples were sorted separately and the residues were freeze dried to yield the flavonoids.

DPPH Radical Scavenging Activity

DPPH free radical scavenging assay is an established assay and is widely used to assess the radical scavenging activity of antioxidant natural and chemical compounds. This assay is based on the reduction of DPPH in methanol in the presence of hydrogen-donating antioxidant constituents of plant extracts, due to the formation of the non-radical form of DPPH.[6] This transformation results in color change from purple to yellow which is measured spectrophotometrically. The disappearance of purple color is monitored at 517 nm. Briefly, an aliquot of 1 ml, 0.3 mM DPPH ethanolic solution was added to various concentrations (25–100 µg/ml) of 2.5 ml of apigenin-7-O-β-D-glucuronide methyl ester and allowed to incubate at room temperature in dark conditions. After 30 min, the absorbance values were measured at 517 nm. Ethanol was used as a blank. DPPH solution (1 ml, 0.3 mM) plus ethanol (2.5 ml) serves as negative control. Ascorbic acid (1–5 µg/ml) was used as standard. The percentage of DPPH inhibition was calculated using the formula:

$$\text{Percentage of inhibition} = \left(\frac{\text{O.D.of control} - \text{O.D.of test}}{\text{O.D.of control}}\right) \times 100$$

Determination of NO Radical Scavenging Activity

NO is a major secretory product of mammalian cells that initiate host defense, homeostatic, and development functions by either direct effect or intercellular signaling. NO is formed from amino acid L-arginine by NO synthase.[7] NO assay was measured by spectrophotometric method described by Govindarajan *et al.*[8] Briefly, sodium nitroprusside (5 mM) in phosphate buffer saline was mixed with various concentrations of (25–100 µg/ml) 2.5 ml of apigenin-7-O-β-D-glucuronide methyl ester dissolved in methanol and incubated at 25°C for 30 min. After 30 min, 1.5 ml of the incubation solution was removed and diluted with 1.5 ml of Griess reagent (1% sulfanilic acid, 2% phosphoric acid, and 0.1% N-1-napthylethylene diamine dihydrochloride). The optical density was measured at 546 nm. The ascorbic acid was taken as standard. The percentage inhibition of NO radical was calculated using the formula:

$$\text{Percentage of inhibition} = \left(\frac{\text{O.D.of control} - \text{O.D.of test}}{\text{O.D.of control}}\right) \times 100$$

Statistical Analysis

The results were expressed as the mean ± standard error of the mean. The statistical difference between the test and control groups were evaluated by one-way analysis of variance (ANOVA) by GraphPad Prism 6.0

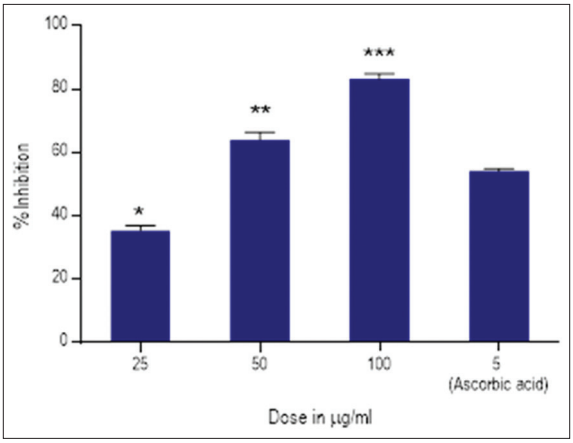

Figure 1: Scavenging activity of Apigenin-7-O-β-D-glucuronide methyl ester by DPPH assay. Values are expressed as mean±S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 represents significant difference compared with control group by student's *t*-test (n=3)

software and followed by Dunnett's *t*-test. *$P \leq 0.05$, ** $P \leq 0.01$, and ***$P \leq 0.001$ represent a significant difference between the control with the test group.

RESULTS AND DISCUSSION

DPPH Scavenging Activity by Apigenin-7-O-β-D-glucuronide Methyl Ester

The antioxidant activity of apigenin-7-O-β-D-glucuronide methyl ester was conducted with different doses, namely 25, 50, and 100 µg/ml. As shown in Table 1 and Figure 1, a dose-dependent DPPH free radical scavenging activity was found to be at 100 µg/ml was 83.26%, whereas the standard ascorbic acid exhibited 54.21% at 10 µg/ml, as shown in Figure 2, half maximal inhibitory concentration (IC_{50}) was determined and found to be 36.38 µg/ml. Ascorbic acid was employed as positive control whose IC_{50} value is 4.49 µg/ml. Mirghani *et al.*[9] reported that the

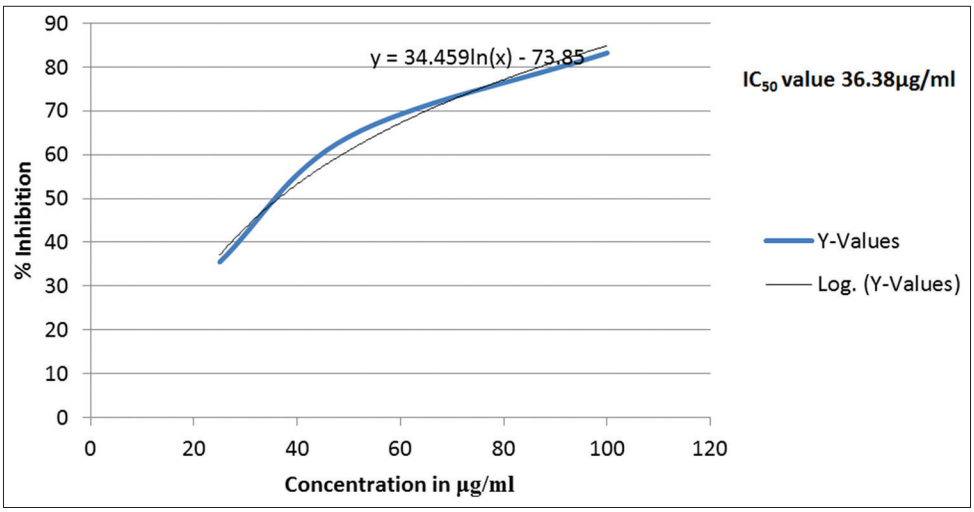

Figure 2: Determination of half maximal inhibitory concentration for 1, 1-diphenyl-2-picrylhydrazyl assay by apigenin-7-O-β-D-glucuronide methyl ester

Table 1: Determination of scavenging activity of apigenin-7-O-β-D-glucuronide methyl ester by DPPH assay

Apigenin-7-O-β-D-glucuronide methyl ester (µg/ml)	Percent inhibition				IC_{50} (µg/ml)
	1	2	3	Mean	
25	34.11	37.21	35.15	35.49±0.91	36.38
50	63.98	66.52	61.83	64.11±1.36	
100	82.15	85.16	82.47	83.26±0.95	
5 µg/ml (ascorbic acid)	55.09	53.86	53.68	54.21±0.44	4.49

DPPH: 1, 1-diphenyl-2-picrylhydrazyl, IC_{50}: Half maximal inhibitory concentration

Table 2: Determination of scavenging activity of apigenin-7-O-β-D-glucuronide methyl ester by NO assay

Dose (µg/ml)	Percent inhibition				IC_{50} (µg/ml)
	1	2	3	Mean	
25	40.08	43.29	40.98	41.45±0.96	29.743
50	73.62	69.37	74.96	72.65±1.69	
100	84.51	89.02	85.04	86.19±1.42	
5 (BHT)	55.91	58.03	56.31	56.75±0.65	3.938

NO: Nitric oxide, IC_{50}: Half maximal inhibitory concentration, BHT: 3,5-di-tert-butyl-4-hydroxytoluene

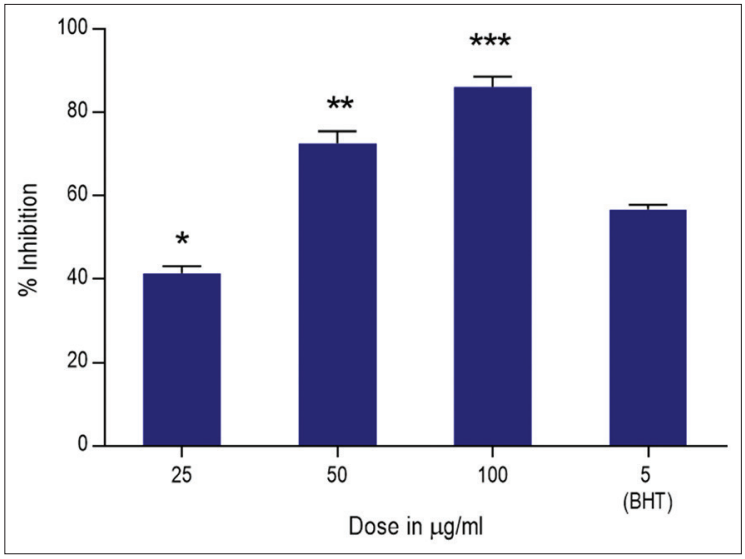

Figure 3: Scavenging activity of Apigenin-7-O-β-D-glucuronide methyl ester by nitric oxide radical assay. Values are expressed as mean±S.E.M.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ represents significant difference compared with control group by student's *t*-test (n=3)

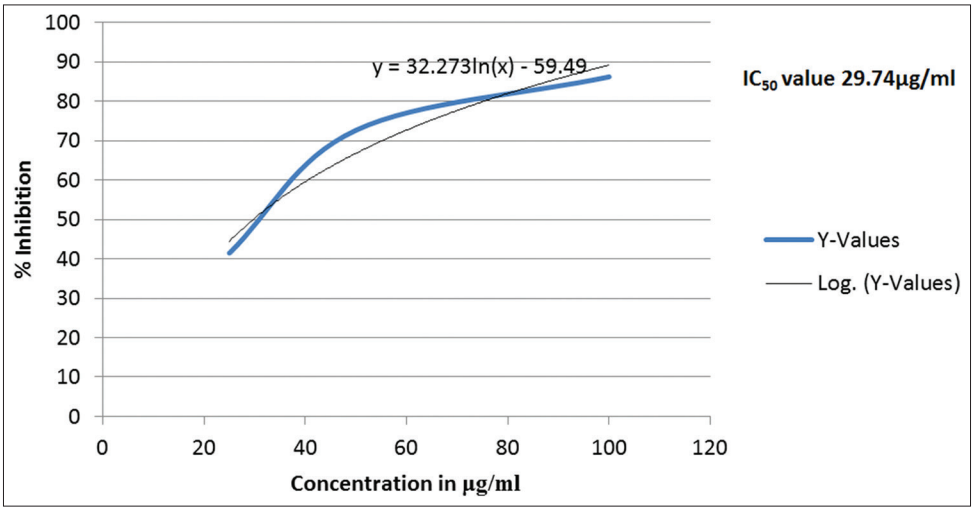

Figure 4: Determination of IC_{50} for nitric oxide assay by apigenin-7-O-β-D-glucuronide methyl ester

flavonoid was isolated from the methanolic extract of *Helianthus annuus* leaves shown 65% DPPH radical scavenging activity.

NO Scavenging Activity by Apigenin-7-O-β-D-glucuronide Methyl Ester

NO radical scavenging activity of apigenin-7-O-β-D-glucuronide methyl ester was determined by the method of Griess Illosvoy reaction developed by Garrat, in 1964.[10] Antioxidant activity of apigenin-7-O-β-D-glucuronide methyl ester was evaluated with different doses, namely 25, 50, and 100 µg/ml. As shown in Table 2 and Figure 3**,** a dose-dependent NO radical scavenging activity was found to be at 100 µg/ml was 86.16%, whereas the standard BHT exhibited 56.75% at 10 µg/ml. Further, the IC_{50} was determined and found to be 29.74 µg/ml. BHT was employed as positive control whose IC_{50} value is 3.93 µg/ml as shown in Figure 4.

CONCLUSION

From the results of *in vitro* antioxidant activities, it can be concluded that the isolated compound apigenin-7-O-β-D-glucuronide methyl ester from ethyl acetate leaf extract of *M. zapota* exhibited a dose-dependent significant DPPH and NO free radical scavenging activity, which may be due to donation of hydrogen from the flavonoids. This study has showed that the importance of isolated bioactive flavonoid apigenin-7-O-β-D-glucuronide methyl ester as source for novel source of antioxidant and leads for drug discovery and development in pharmaceutical field.

ACKNOWLEDGMENTS

We are very happy to convey our sincere thanks to esteemed Professor U.S.N Murthy, Chief Scientist,

Head, Department of Biology, Indian Institute of Chemical Technology, Hyderabad, have permitted us to carry out pharmacological studies in his laboratory.

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Source of support: This work was partially supported by UGC-MRP. F. No. 4 N 2-643/2013 sanctioned to Prof. Duddukuri Govinda Rao, GITAM University, and Visakhapatnam; Conflict of interest: None Declared