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ISOLATION OF GREEN BIOREDUCTANTS FROM TWO PLANT SOURCES AND EVALUATION OF REDUCING ABILITY BY FREE RADICAL SCAVENGING ASSAYS

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ABSTRACT

Green chemistry approaches are popular in synthesising metal oxide nanomaterials due to benignness in terms of human and environmental toxicity. Phytocompounds potentially reduce metal ions in to their respective nanoparticles. In this study, we isolated two bioreductants, punicalagin and gallic acid from Punica granatum peel and Emblica officinalis fruit respectively using previously designed methods. Isolated phytocompounds were analysed using High Performance Liquid Chromatography to qualify isolates. To assay their reducing ability 2, 2 diphenyl 1 picrylhydrazyl assay and hydrogen peroxide reduction assay were performed. Results of HPLC analysis showed that punicalagin had similar retention time as that of standard (17.5 min) with lesser intensity HPLC peak. Gallic acid from E officinalis had retention time (2.5 min) and intensity similar to its standard. Gallic acid showed a higher antioxidant activity against both free radicals compared to punicalagin isolate. Gallic acid showed an activity of 76% and 64% against DPPH and H_2O_2 respectively at highest tested concentration of 0.2 mg/mL. Punicalagin showed 61% and 64% inhibition against DPPH and H_2O_2. From this study, authors conclude that isolated phytocompounds are good candidates as bioreductants in synthesis of nanomaterials and if their shelf life is extended they will be good alternatives to chemical reductants.

Keywords: ***Punicalagin, Gallic acid, HPLC, DPPH, H_2O_2.***

I. INTRODUCTION

Metal and metal oxide nanomaterials are important class of materials which have found application in all fields of life. Most of their synthesis methods involve reduction mechanism from their respective salts and also commonly with hydrothermal/solvothermal and co-precipitation methods [1]. Most commonly used chemical

methods involves the use of inorganic and organic reducing agents, many of which are toxic and hazardous to environment [2]. Hence, use of biological molecules from different plant and animal sources have become a popular green chemistry approach. Biomolecules present in plant extracts act as potent reducing agents, which reduce metal ions into metallic nanoparticles in a single step green synthesis process. The experiments can be conducted at room temperature and pressure and can be readily scaled up [3]. Plant extracts in different organic solvents give away various biomolecules which have varying degrees of reducing ability. In most of the cases, phenolics, flavonoids and tanninoid principles of plant extracts are held responsible for their reduction mechanism [4]. Many a times, plant parts are freshly extracted in respective solvents and then used up in nanomaterial synthesis. At the same time, tedious solvent separation techniques are used to elute different active principles and identify them followed by drying and resuspending them before using them in nanomaterial synthesis. Reducing ability of most plant extracts are confirmed by different antioxidant assays in comparison with standard antioxidants [5]. Therefore, in the current study as an alternative to use of crude extracts in nanomaterials processing, we have made an attempt to isolate 2 important bioactive principles having potent antioxidant (reducing ability) activity which can be used directly in bio-reduction of metal ions into nanometals. The first isolate considered here is punicalagin. Punicalagin is an ellagitannin, a type of phenolic compound which is a potent antioxidant whose bioactivity can be explained by its ability to hydrolyse into Ellagic acid (EA), a secondary metabolite *in vivo* and across mitochondrial membrane *in vitro* [6]. It is found richly in the fresh juice, arials and peels of pomegranate fruit [7, 8]. Gallic acid is one of the active principle of *E officinalis* fruit. The fruit is commonly called as Indian gooseberry or Amla. It is a potent antioxidant of natural source and can be isolated in large quantity from fresh aqueous solution of *E officinalis.* In this study, gallic acid was isolated using enzymatic separation method using cellulase enzyme [9]. To assay their reducing ability, DPPH free radical scavenging assay and H_2O_2 reduction assay were performed. These are the standard antioxidant assays which are used to assay reducing ability of various antioxidants used to scavenge free radicals like hydroxyl free radical $OH^{\cdot}$ and superoxide radical [10]. DPPH scavenging activity is a measure of antioxidant (reducing) ability of materials. DPPH is a free radical with characteristic absorption at 517 nm. Materials with reducing properties, donate protons which in turn decrease absorption, which is taken as measure of radical scavenging [11]. H_2O_2 gets decomposed rapidly into oxygen and water and produces hydroxyl radicals ($OH^{\bullet}$) that can initiate lipid peroxidation and cause DNA damage in the body. The H_2O_2 reducing ability assay mainly focuses on the ability of plant extracts to scavenge H_2O_2 [12]. Considering the importance of radical scavenging methods in assessment of removal of oxygen functionalities, we used these two standard methods to assess reducing ability of both the isolates from selected plant sources.

II. MATERIALS AND METHODS

2.1 Materials

The plant materials used in the current study was procured from local market and identified at DOS in Botany, University of Mysore. All chemicals and reagents used for RF-UFLC and HPLC were obtained from Merck Chemicals, Germany. Ultrapure water used in the experiments was obtained from Pure labs (ELGA) with

resistivity of 18.2 MΩ. Cellulase, ascorbic acid, BHT standard antioxidants were obtained from Sigma-Aldrich. DPPH used in the antioxidant assay was obtained from Nice Chem Pvt Ltd.

2.2 Methods

The *P granatum* peel was separated from fruit and dried in shade for 8-10 days. The *E officinalis* fruit was deseeded and the fruits were dried in shade for 4-5 days. The plant materials were pulverized using electrical blender and stored in air tight container until further use.

2.2.1 Isolation of Punicalagin from *P granatum* peel

20 g of pomegranate peel powder was extracted in 200 ml methanol using Soxhalet apparatus at 60^{o} C for 15 cycles until a dark brown coloured crude extract was obtained. Excess methanol from liquid was evaporated using rotary evaporator and further dried in hot air oven at 50^{o} C. The resultant crude methanolic extract powder was subjected to gel chromatography using Sephadex LH-20 resin column using the method of *Hanu et al* with modification [13]. Resultant pale yellow coloured punicalagin was stored at 4 ^{o} C until further use. Fig 1 shows the schematics of punicalagin isolation from methanolic extract of *P granatum.*

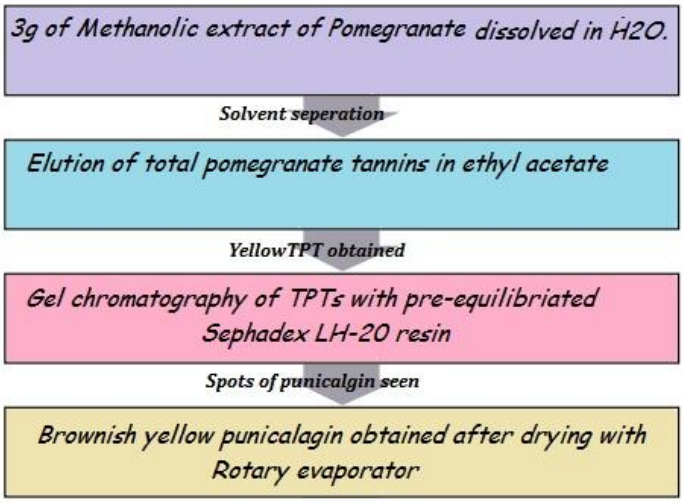

Figure 1-Shematics of isolation of punicalagin

2.2.2 Isolation of Gallic acid from *E officinalis* fruit

Isolation of Gallic acid from *E. officinalis* fruit powder was carried out using enzymatic reduction method using cellulase. 100g of dried *E officinalis* powder was added to enzyme solution of cellulose (2.5 mg/mL) and dispersed well. *pH* of the mixture was adjusted to 4.5 (as this gives the maximum yield of gallic acid by this method) using 0.1 M HCl. The mixture was sealed in an air-tight reagent bottle and wrapped with aluminium foil. This was incubated at a temperature of 50^{o} C for 8 hr with continuous shaking. After 8 hrs the mixture was filtered using Whatmann's filter paper # 1 and vacuum evaporated at 55^{o} C and the resultant powder which was pale yellow colored was stored at 4^{o} C until further use.

2.3 Analytical methods

The HPLC, UFLC with UV spectroscopy was carried out at JSS college of Pharmacy, Mysore. HPLC was carried out using Shimazdu HPLC instrument with diode array detector.

2.3.1 Qualification of Punicalagin from *P granatum*

0.5 g of punicalagin standard with 98% purity was obtained from Sigma-Aldrich and the analysis was carried out by our previous protocol [14]. Using HPLC with diode array detector. 100 mg punicalagin sample obtained from Sephadex LH-20 column was dissolved in 10 ml of 1:1 deionized water and methanol mixture. Buffer solutions were prepared using deionized water and 1% acetic acid (solvent A) and methanol and 1% acetic acid (solvent B). The injection volume was kept at 20 µl for each run. Gradient mode was used to obtain chromatogram with flow rate maintained at 0.5 µl/min.

2.3.2 Qualification of Gallic acid

Standard gallic acid was obtained from JSS college of pharmacy, Mysuru as generous gift sample. 25 mg of this sample was dissolved in 50 ml methanol in a 50-ml capacity standard flask. About 250 mg sample presuming to contain gallic acid was dissolved in 50 ml volumetric flask and the volume was made up to 50 ml. Mobile phase consisted of ultrapure water with 2% acetic acid (solution A) and acetonitrile (Solution B) which were mixed in 90:10 ratio (A: B). 5 ml of sample was diluted with 50 ml of mobile phase. Chromatographic separation was done using Phenomenex C Column (250 X 4.6 mm, 5µ ID). The flow rate was adjusted to 1.2 ml/min and run time was adjusted to 10 min. Gallic acid was detected at a wavelength of 272 nm using a PDA detector with retention time 2.5 min. 10µl/min of injection volume was used for injection [15].

2.4 Antioxidant assays

2.4.1. DPPH free radical scavenging activity

Concentration dependent DPPH radical scavenging activity of punicalagin isolated from *P granatum*peel and gallic acid from *E officinalis* fruit was determined by DPPH scavenging assay described by Blois [16]. Ascorbic acid was used as standard oxidant. Different concentrations of test substances were prepared in ultrapure water, which ranged from 0.025 mg/ml to 0.2 mg/ml. The test samples were added to equal volumes of 0.1 mM DPPH solution. The reaction mixture was incubated in an opaque box for 30 min with continuous shaking at ambient temperature. Further the absorbance was recorded at 517 nm. Percentage radical scavenging was calculated using the formula;

$$\% \text{ DPPH scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

Where A_c is absorbance of Control and A_s is absorbance of sample.

2.4.2- H_2O_2 reducing assay

H_2O_2 reducing ability of test samples was determined using the method of Glucin*et al* [17]. H_2O_2 solution was prepared in 1 X phosphate buffer saline which was maintained at neutral *pH*.0.6 ml of this was added to 1 ml of punicalagin or gallic acid isolates at different concentrations. The solutions were then incubated for 10 min and read at 230 nm. The absorbance of the positive control of 0.0.1 mg/ml Butylated hydroxy toluene (BHT) were

measured. The percentage H_2O_2 scavenging activity of punicalagin and gallic acid was calculated using the formula

$$\% H_2O_2 \text{ inhibition} = (Ac - As/Ac) \times 100$$

Where, Ac is control absorbance and As is sample absorbance.

III. RESULTS AND DISCUSSION

3.1.1. Qualification of Punicalagin from *P granatum*

Using gradient mode of HPLC with diode array detector (Shimzdu), punicalagin sample isolated from pomegranate peel was analysed using standard punicalagin (Sigma-Aldrich). The retention time of standard punicalagin was recorded as 17.5 min in our study and same retention time was noted with the isolate though with lesser intensity which shows that there was a high degree of similarity between standard punicalagin and the isolate. Fig 2A and 2B shows HPLC chromatogram of punicalagin standard and isolate.

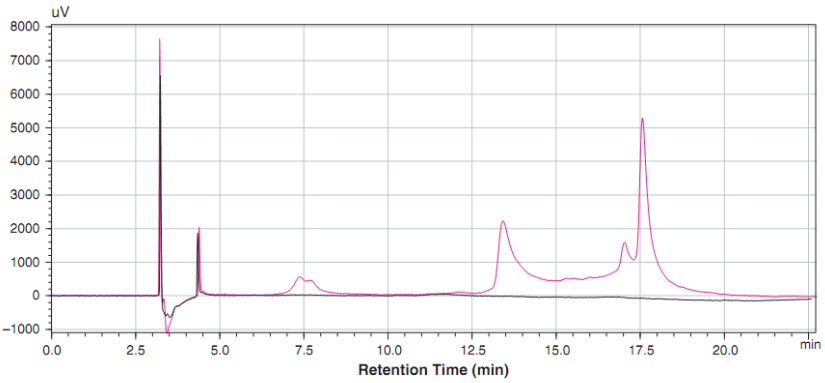

Figure 2A- Punicalagin Std-Blank comparison

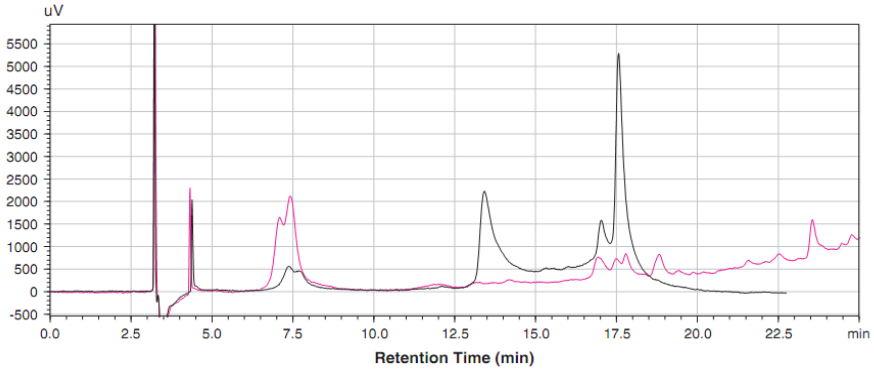

Figure 2B-Punicalagin sample-standard comparison

P granatum is rich in hydrolysable tannins like punicalagin, ellagic acid and gallic acid, all of which are potent antioxidants [18]. The peel of *P granatum* is rich in punicalagin which is primarily an ellagitannin. It is known to have antioxidant, antibacterial, antifungal, anti-inflammatory and anti-atherosclerotic property [19-21]. Considering its potent reducing ability, punicalagin was isolated from methanolic extract of *P granatum* peel using Sephadex LH-20 chromatography by the method of Hanu et al. Punicalagin is a high molecular weight

ellagitannin (M. W. 1084.71g/mol) which readily elutes in methanol [22]. Sephadex LH-20 resin is basically used for gel chromatography techniques for the isolation of biological molecules by size exclusion principle. Due to the unique physicochemical properties, Sephadex LH-20 is one of purification materials which are useful for both analytical and industrial scales. Sephadex LH-20 has been widely used to isolate natural products such as individual catechins [23]. Considering this fact, a column chromatography was performed using LH-20 resin and further using HPLC with diode-array detector, the *P granatum* isolated punicalagin was compared withstandard punicalagin (Sigma-Aldrich), which showed a detectable peak of punicalagin with lesser intensity. Since the climatic conditions, cultivars and species affect the quantity of phytocompounds present inany plant part, thelesser intensity peak of punicalagin could have been observed from our data [24]. This is reflected in its antioxidant activity also. (as discussed in subsection **3.2).**

3.1.2 Qualification of gallic acid from *E officinalis*

Using RF-UFLC method presence of gallic acid in *E officinalis* extract prepared using cellulase solution was determined and compared with standard gallic acid. standard gallic acid as well as the *E officinalis* extract showed a retention time of 2.5 min, identifying gallic acid as the single major component in extract as there were no other major peaks in the chromatogram. Fig 3A and 3B shows RF-UFLC chromatogram of gallic acid standard and the isolate.

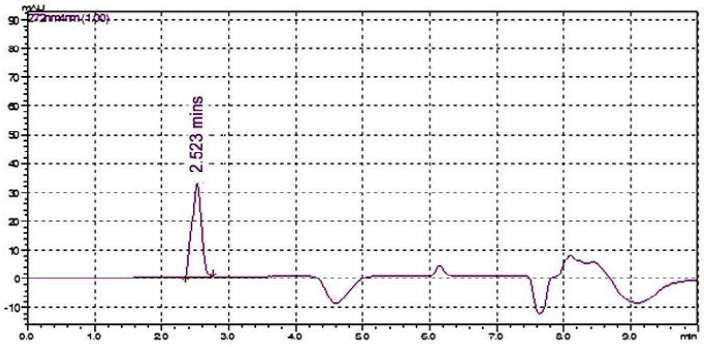

Figure 3A-RF-UFLC Chromatogram of gallic acid standard showing retention time of 2.5 min

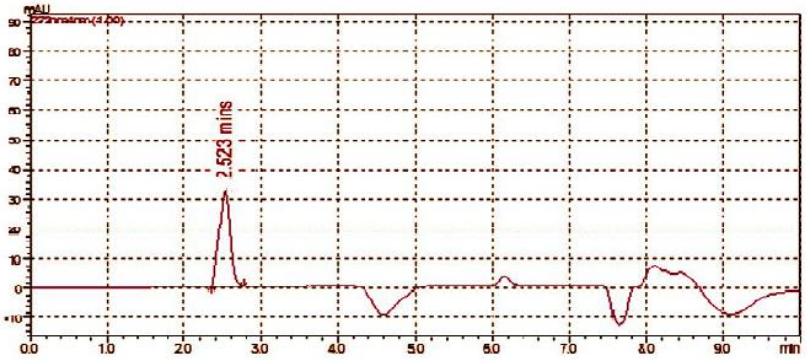

Figure 3B-RF-UFLC Chromatogram of gallic acid isolate from *E. officinalis* with same retention time

Gallic acid is one of the major phenolic antioxidant present in *E officinalis* fruit along with vitamin C and tannic acid [25]. It is also known to have potent antibacterial properties and sequesters reactive oxygen species [26]. Gallic acid can be easily extracted from aqueous extract of *E officinalis* fruit, even in large quantity[27]. It has a

molecular weight of 188.4 g/mol. Use of hydrolytic enzymes in extraction of gallic acid typically involves enzymes like cellulase, beta-glucosidase and pectinase, which interact with the cell wall of the fruit, breakdown its structural integrity which facilitates increase in release of Gallic acid notably [28-29]. Hence using the method of Yadav *et al* gallic acid was extracted from *E officinalis* fruit which was further subjected to RF-UFLC chromatography. Results of the experiment showed that gallic acid emerged as single major component whose retention time and peak intensity was similar to that of gallic acid standard used in the study.

3.2 Radical scavenging assays

3.2.1 DPPH free radical scavenging activity

In DPPH assay, the radical scavenging activity is measured at ambient temperature. DPPH is a stabilized free radical. It has an intense purple color and gives maximum absorption at 517 nm. Substances having antioxidant activity, when they react with radical form of DPPH, gets converted to non-radical form of DPPH (DPPH-H) which is pale in colour which can be read using UV-Vis spectroscope at 517 nm, wherein the reduction in absorption can be measured [30]. It has been shown in earlier studies that the extraction process and preservation technique of phenolics from pomegranate juice plays a major role in quantity of phenolics and industrial processes gives a better yield of phenolic compounds than the laboratory methods, which in turn effects the antioxidant activity of phenolics [31]. Upon observing the results of HPLC, the retention peak of punicalagin appeared less intense, which reflected in DPPH radical scavenging activity of the substance. Fig 4A shows the radical scavenging activity of gallic acid isolate in comparison with standard ascorbic acid and 4B shows activity of punicalagin.

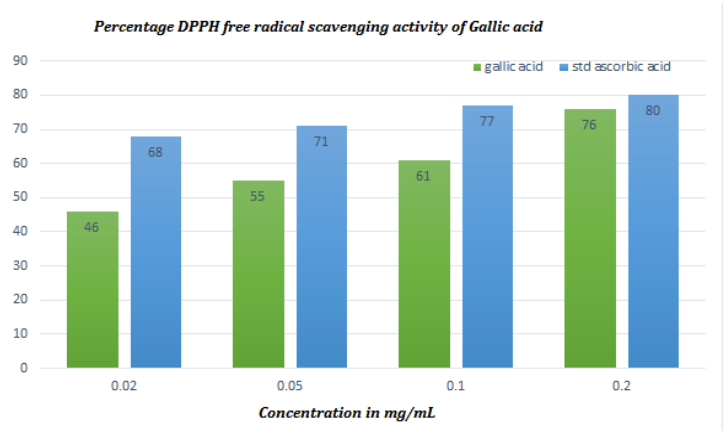

Figure 4A-DPPH free radical scavenging activity of Gallic acid isolate

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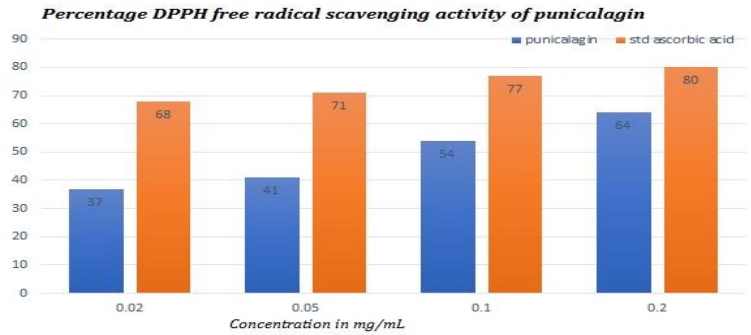

Figure 4 B-DPPH free radical scavenging activity of punicalagin

As observed from the graph of punicalagin's antioxidant activity against DPPH radical, there was a concentration dependent increase in antioxidant activity, with the highest concentration (0.2 mg/ml) showing a 64% activity which was much lesser than the standard antioxidant chosen in the study, the ascorbic acid, which showed 80% activity at the same concentration. The gallic acid isolated from *E officinalis* showed more promising results with a good scavenging ability (more than 50%) up to 2 concentrations less than the highest. There was a 76% antioxidant activity shown by highest concentration of gallic isolate of *E officinalis*, matching ~80% activity shown by standard antioxidant. From HPLC peak of isolate it was evident that there was the maximum yield of compound by enzyme method followed in our study and the intensity of gallic acid peak was matching nearly to that of the standard. Earlier studies have shown that minimum processing protocols are essential for maximum yield of antioxidants from *E officinalis*, and presence of other active components shows improved antioxidant activity of *E officinalis* extract than gallic acid alone [32]. This explains the slight reduction in antioxidant activity of gallic acid compared to the standard antioxidant used in the study.

3.2.2.H_2O_2 scavenging activity

H_2O_2 scavenging activity of punicalagin and gallic acid isolated from respective plant material was measured at 320 nm using BHT as positive control. Figure 5A and 5B shows comparative antioxidant activity of punicalagin and gallic acid with standard positive control BHT. The percentage radical scavenging activity of punicalagin against H_2O_2 was 24%, 37%, 50% and 61% for 0.025, 0.05, 0.1 and 0.2 mg/ml of punicalagin respectively, whereas gallic acid caused a 29%, 34%, 53%, and 64% scavenging respectively.

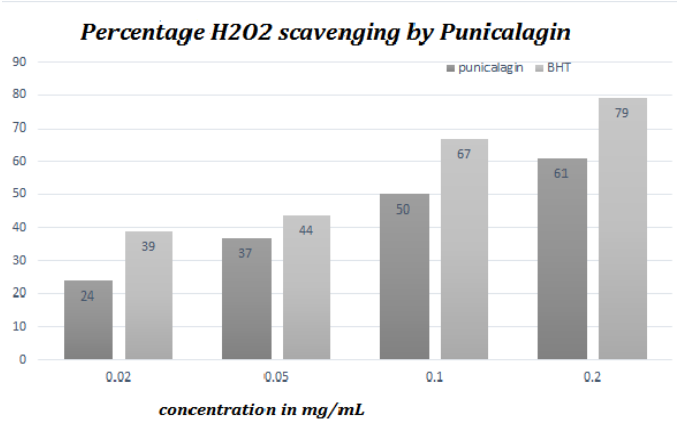

Figure 5 A-H_2O_2radical scavenging activity of punicalagin

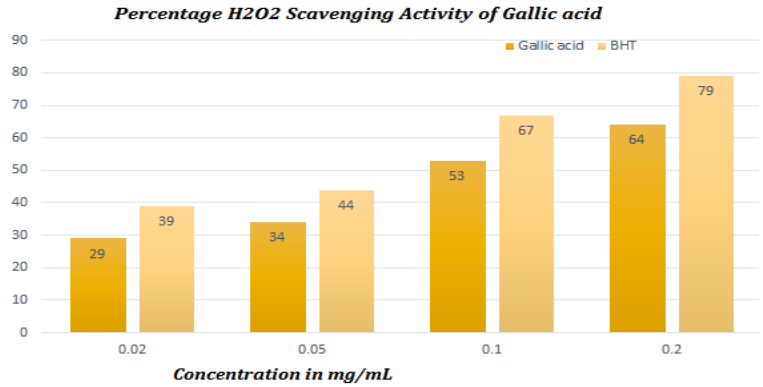

Figure 5 B-H_2O_2radical scavenging activity of Gallic acid

In either case the scavenging activity increased with increase in concentration of test substances.Hydrogen peroxide is a non-free radical species of oxygen. Within biological systems, the H_2O_2 has a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction [33].Plant polyphenols are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers [34]. Both punicalagin and gallic acid are rich in phenolic groups. Therefore, it gives them the ability to donate electron to convert H_2O_2 to H_2O by reduction process [35]. Earlier evaluation on antioxidant activity of individual phenolic acids has shown that among all phenolic acids, the gallic acid shows the highest antioxidant activity [36].This has reflected in our study also. Gallic acid showed a good quenching of free radical at all tested concentrations when compared to the standard and caused more than 50% scavenging at the 2 higher concentrations. Punicalagin did show some good antioxidant activity with slightly lesser activity compared to gallic acid. This might be probably because of separation technique and quality of the plant material used in the study.

IV. CONCLUSIONS

From the current study, the authors conclude that two potent phenolic compounds namely punicalagin and gallic acid were isolated from peel of *P granatum*and fruit of *E officinalis* respectively. Reducing ability of both isolates was assayed using radical scavenging activity against free radicals of DPPH and H_2O_2, wherein both the isolates showed fairly good antioxidant activity with gallic acid showing a higher activity in comparison with punicalagin. The enzymatic isolation technique followed here gave a yield of good quality gallic acid which showed potent reducing ability. Since the methodology followed for gallic acid isolation is economic, it can be scaled for a large scaled production of bioreductant for use in metal oxide nanomaterial synthesis.

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