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Der Pharmacia Lettre, 2015, 7 (12):406-413
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ISSN 0975-5071
USA CODEN: DPLEB4

Effects of solvents on microwave assisted extraction of phytoconstituent from the fruits of *Pithecellobium dulce*

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ABSTRACT

Various traditional systems of medicine enlightened the importance of Pithecellobium dulce. There are reports on the medicinal values of the leaves and seeds of this plant. The present study was discusses the microwave assisted soxhlet extraction of Pithecellobium dulcefruit powder and its screening on pharmacognostical and preliminary phytochemical analysis followed by total phenolic, flavonoid and carbohydrate estimation. The crude extracts were obtained by microwave assisted soxhlet extraction with solvents; 20 % ethanol and Methanol which was further partitioned based on polarity. The fruit extracts were studies for its in vitro antioxidant activity by DPPH, Phospho molybdenum and Ferric ion reducing method. The total phenolic and flavonoid content was found to be higher in methanolic extract which was obtained bypartitioning with chloroform (MEC) 46.8 ± 2.83 mg/g of Gallic acid equivalent and 1.27 ± 0.03 mg/g of Quercetin equivalent respectively. The total carbohydrate content was found to be higher in 20% ethanol; 113.4 ± 0.04 mg/g of dextrose equivalent. The in vitro antioxidant assay revealed free radical scavenging potential of Pithecellobium dulce fruit, compared with standard antioxidant. This antioxidant activity is endorsed for its high phenolic content. Thus our findings provide evidence that P.dulce fruit is a potential source of natural antioxidant and a good source of carbohydrates.

Key words: Microwave assisted extraction, phenolic, flavonoid, carbohydrate, *P. dulce*

INTRODUCTION

Since pre historic times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. About 80 % of individuals from developed countries use traditional medicines, which has compounds derived from medicinal plants. Therefore, such plant should be investigated for better understanding of their properties, safety and efficiency. These medicines initially took the form of crude drugs such as tincture, tea, powder and other herbal formulations[1].

*P.dulce*which is chosen for the present study is locally called as manila tamarind belongs to the family *Mimosaceae.* It is a small to medium sized evergreen spiny tree height which grows up to 18-20m, native of tropical America and cultivated through the plains of India and Andaman[2]. The seed oil was used as an edible and also for manufacturing soap [3]. The bark was reported to have astringent in dysentery, febrifuge and it contains upto 37% of tannins. Polyphenols from the bark extract possess antivenomous activity [4]. The seed are found to contain steroids, saponins, glycosides, lipids and polysaccharides which are reported to have antidiabetic and antioxidant activity [5]. Afzelin(Kaemperol-3-O-α-L-rhamopyranoside) was isolated from the leaves of *P.dulce* which have antimycobacterial property. The leaves also reporte to have anti-inflammatory, analgesic, anti-oxidant and anti-

diabetic activity[6-9]. The *P.dulce*fruits have been studied for anti-inflammatory activity due to saponin content, free radical scavenging, and gastro protective, antidiabetic and hepatoprotective effects were reported [10-13]. The aqueous extract of flowers reported to have cardio protective activity [14].

Microwave assisted soxhlet extraction (MASE) has been widely used for extracting phyto constituents from different plant materials for shorter treatment and enhanced efficiency. It is a new extraction technique which combines microwave and traditional soxhlet solvent extraction method [15].Further it requires less solvent, shorter extraction time, better product with lower cost[16].Microwave energy acts as nonionizing radiation that cause rotation of the dipole and highly localized temperature can cause selective migration of target compounds from the material to the surroundings at more rapid rate[17].Hence MASE is an interesting alternative to conventional extraction methods, especially in the case of secondary plant metabolites like flavonoids ,phenolics, and sapononinsextraction[18].

The purpose of the study is to develop a novel MASE method for extracting different constituents of the plants,by optimising the solvent with selective extraction capability from the plant *P.dulce* .The respective yields are calculated to optimise the solvent and qualitative and quantitative estimation of individual constituents were also used to confirm the choice of the solvent.

MATERIALS AND METHODS

2.1 Collection of plant**:**
The fresh ripen fruits of *P.dulce*were collected from the local flora in Vellore district during the month of March to May 2014. The plant was authenticated by Professor Jayaraman, National Anatomy research centre, Chennai. The ripened fruits were dried at room temperature under shade and powdered using mechanical grinder. The powder is stored in a refrigerator for further use.

2.2 Experimental procedure:
2.2.1 Physico chemical properties of crude drug:
In order to determine the quality and purity of the crude drug the physico chemical properties of the fruit powder was subjected to quantitative estimation of different ash values such as total ash, water soluble ash and acid insoluble ash content as per Indian Pharmacopoeia.

2.2.2 Microwave assisted soxhlet extraction:[MASE]
The extraction was performed using Catalyst Systems CATA R scientific microwave oven. Two different methods of microwave extractions were performed by using two different solvents which are described below.

2.2.2.1 Hydroalcoholic extract (HAE): The powdered material was extracted with 20% ethanol with microwave temperature of 50% (350 Watt), for 5 hours(which was previous optimized in our laboratory for this plant). The extract was concentrated under reduced pressure. To this excess ethanol was added and stored in refrigerator at 4 ˚C for 48h to precipitate polysaccharide.

2.2.2.2 Methanolic extract (ME): The powdered material was extracted with methanol with microwave power of 35 % (210 Watt) for 6hours. The extract was concentrated on rotary evaporator. The concentrated methanolic extract was suspended in waster and successfully partitioned with petroleum ether (MEP), chloroform (MEC) and ethyl acetate (MEE). The fractions were concentrated under reduced pressure. The remaining aqueous layer was concentrated to furnish an aqueous layer [19].

2.2.3Preliminary phytochemical screening:
Crude extracts of HAE and ME was dissolved in respective solvents and subjected to the preliminary phytochemical test as per methods described by Kokate 1999.

2.2.4 Determination of Total phenolic content
The total phenolic content of *P.dulce*extract was determined by using folinciocalteau reagent followed by slight modification[21]. Gallic acid was used a standard for estimation of phenolic content. Plant extract (1 mg/ml) was mixed with 2 mL of folin-ciocalteau reagent and were neutralized with 4 mL of sodium carbonate solution (7.5 %

w/v). The absorbance wasmeasured at 765 nm using UV-Visible spectrophotometer (Jasco V-670). The content of total phenolic compounds was expressed as mg/g Gallic acid equivalent (GAE) of dry extract.

2.2.5 Determination of total flavonoid content**:**
The total flavonoid content was determined by calorimetric aluminium chloride method with some modification[21]. Quercetin was used as a standard for the estimation of flavonoid. The absorbance was measured at 415 nm using UV-Visible spectrophotometer (Jasco V-670). The total flavonoid content was expressed in terms of quercetin equivalence.

2.2.6 Determination of total carbohydrate content**:**
The total carbohydrate content was determined by phenol sulphuric acid method with slight modification[23]. Dextrose is used as a reference standard. The absorbance was measured at 490nm. It is expressed as mg/g dextrose equivalent of dry extract.

2.3 *Invitro*Antioxidant activity:
Three assays namely DPPH free radical scavenging activity,Phospho-molybdenum and ferric ion reducing antioxidant power assay were used to evaluate the antioxidant activity of the extracts.

2.3.1 DPPH radical scavenging activity:
The antioxidant activity of the *P. dulce* extracts was carried out using DPPH. Methanolic solution of the extracts at various concentrations (10-200µg/ml) was added to 2ml of 0.2mM DPPH solution allowed to stand for 30min under room temperature. The absorbance of the samples was measured at 517nm. Radical scavenging activity was expressed as percentage inhibition and was calculated using the formula

% inhibition = [Control (Abs)-Sample (Abs)/Control (Abs)]X100

2.3.2 Phospho-molybdenum assay:
The extracts of *P. dulce* in different concentration ranging from 10µl to 50µl were added to each test tube individually containing 3ml of distilled water and 1ml of molybdate reagent. The absorbance of the reactive mixture was measured at 695nm. Ascorbic acid was used as a positive reference standard [22].

2.3.3 Ferric ion reducing antioxidant power assay:
The crude extracts of *P. dulce* in various concentrations (10-50µl) were mixed with 0.2M Phosphate buffer and 1% potassium ferricyanide. The absorbance was measured at 700nm. Ascorbic acid was used as a reference standard.

2.4 Data analysis:
Statistical analysis was determined by variance (ANOVA) followed by students t test.

RESULTS

3.1 Physicochemical parameters of*P.dulce*fruits**:**
The physicochemical drug evaluation is an important parameter in detecting adulteration and improper handling of drugs. Moisture content of drug should be at minimal level to avoid microbial growth during storage. Ash values are used to determine quality and purity of crude drug. Higher amount of acid insoluble content indicates the presence of silicate and earthy materials. Similarly the presence of inorganic element in the drug will be indicated from water soluble ash content. Thus physicochemical parameter can serves as valuable source of information and provides appropriate standard to establish the quality of the plant material in future studies.

Table 1 summarisesthe results obtained with respect to physicochemical parameters of *P. dulce* powder. The loss on drying is found to be 8.08% whereas the total ash content was found to be 78.94% and the water and acid insoluble fractions are 89.28% and 34.06% respectively. This reveals that the crude drug possess more percentage of organometallic soluble content and also presence of inorganic minerals indicating the mineral rich components in the plant.

Table 1: Physicochemical parameters of *P. dulce* fruit powder

Test	Percentage
Loss on drying	8.08
Total ash content	78.94%
Water soluble ash content	89.28%
Acid insoluble ash content	34.06%

Fluorescence is the phenomenon exhibited by the various chemical constituents present in the plant material. Crude drug are often assessed qualitatively in this way and it is an important parameter of pharmacognostic evaluation. The results of fluorescence analysis are given in the table 2 which indicates the presence of possible chromophores in the drug.

Table 2: Fluorescent analysis of *P. dulce*fruit powder

S. No	*P.dulce* powder + solvents	Short UV	Long UV	Visible light
1	Powder	Brown	Dark green	Dark brown
2	Methanol	Dark brown	Dark green	Brown
3	Acetic acid	Brown	Fluorescent green	Brown
4	Water	Brown	Fluorescent green	Brown
5	Ethanol	Brown	Dark green	Brown
6	Petroleum ether	Brown	Green	Brown
7	Chloroform	Dark brown	Dark green	Brown
8	Ethyl acetate	Brown	Green	Brown
9	Dichloro methane	Brown	Dark green	Brown
10	1 N HCl	Greenish grey	Green	Dark brown
11	1 N Sodium hydroxide	Green	Green	Brown
12	Ferric chloride	Black	Dark green	Dark brown
13	50 % Sulphuric acid	Black	Dark green	Dark brown
14	5 % Potassium hydroxide	Green	Green	Brown
15	50 % Nitric acid	Fluorescent bluish grey	Green	Dark brown

3.2 Microwave assisted soxhlet extraction: [MASE]

3.2.1 Hydroalcoholicextract (HAE):

20 % alcohol was used as extracting solvent to extract polysaccharides .The percentage yield was found to be 72.8% which is dark brown and sweet in taste which is a characteristic of carbohydrates. The extract is found to be crystalline in nature.

3.2.2 Methanolic extract (ME):

Methanol was chosen as the extracting solvent to separate phenolics and flavonoids. Methanol extract on partitioning with different solvents yielded three types of extraction. The separation is based on solubility in the respective solvents and their yields are given in table 3.

Table 3: Physical characteristic and percentage yield of *P. dulce*fruit extracts

S. No	Solvent used	Colour	Odour	Yield (%)
1	Petroleum ether	Dark yellow	Odourless	8.3
2	Chloroform	Reddish brown	Sweet	4.6
3	Ethyl acetate	Brown	Caramel	5.25

3.2.3 Preliminary phytochemical screening:

The results of qualitative phytochemical analysis are summarized in the table 4. The crude MEP fraction reveals the presence of phenols, flavonoids and trace amount of carbohydrate. MEC fraction shows the presence of phenols, flavonoid, carbohydrate and saponins. MEE fraction was positive for phenols, flavonoids, carbohydrate and saponins. HAE crude extracts reveals the presence of phenols, carbohydrates and trace amount of saponins.

Table 4: Phytochemical analysis of *P. dulce* fruit extracts

S. No	Tests	MEP	MEC	MEE	HAE
1	Alkaloids	-	-	-	-
2	Phenols	+++	+++	+++	++
3	Flavanoids	+	+	+	-
4	Carbohydrates	+	+	+	++
5	Steroids	-	-	-	-
6	Saponins	-	+	+	+
7	Fats and oils	-	-	-	-
8	Terpenoids	-	-	-	-

3.2.4 Total phenolic, Flavonoid and carbohydrate content:

Phenolics,flavonoids and carbohydrates as important phytochemicals ,are present in vegetables, fruits and cereal grains. These secondary metabolites are natural antioxidants that have multiple biological effects and play an important role in the defence against various diseases. Hence we have estimated the total phenolic, Flavonoid and carbohydrate contents of the fruit extract *P.dulce*. The total phenolic content were found to be 6.9±0.005 (MEP), 46.8±0.001(MEC), 38.08±0.001(MEE) and 6.5±0.005 (HAE)mg gallic acid equivalents respectively. The total flavonoid content was found to be 0.21±0.0 (MEP), 1.27±0.03 (MEC), 0.969±0.03 (MEE) and 0.1008±0.05 (HAE) mg/g quercetin equivalentsrespectively. The total carbohydrate content were found to be 5.4±0.03 (MEP), 36.04±0.64 (MEC), 60.9±0.07 (MEE) and 113.4±0.04 (HAE) mg dextrose equivalents(figure 1).

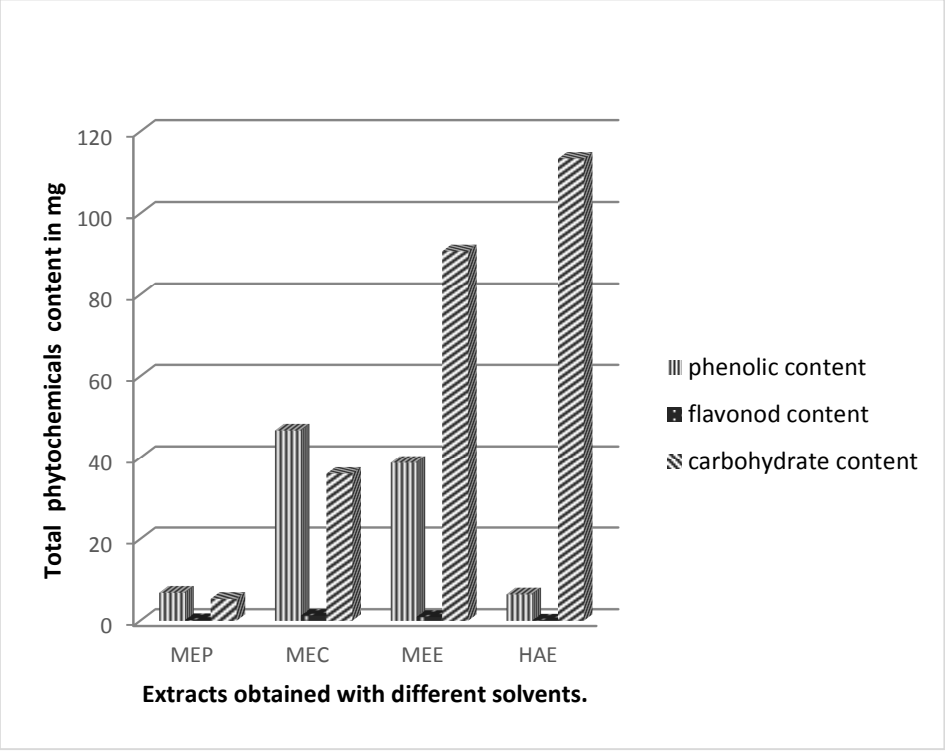

Figure 1: comparison of total phenolic, flavonoid and carbohydrate content in the *P. dulce*fruit extracts

3.3 Antioxidant activity:

3.3.1 DPPH Assay:

The scavenging effect of MEP, MEC, MEE, and HAE at concentration of 200 µg/ml was found to be 67.1% (MEP), 37.5 % (MEC), 85.8 % (MEE), and29.6 % (HAE) respectively whereas at the same concentration ascorbic acid is showed 93.5 %. (Figure 2) The present investigation shows that all the extracts exhibits DPPH radical scavenging activity in the concentration dependent manner and the results are compared with the standard compound.

3.3.2 Phospho-molybdenum assay:
Phospho molybdenum assay is based on the reduction of phosphate molybdenum (IV) to phosphate molybdenum (V) and subsequent formation of a bluish coloured complex at acidic pH. The total antioxidant activity of MEP, MEC, MEE, and HAE at concentration of 50 µg/ml was found to be 69.9 % (MEP), 74.1 % (MEC), 72.4 % (MEE), and 40.9 % (HAE) respectively (Figure 3) while the standard showed 94.6%.This reveals that the ethyl acetate extract contains effective antioxidants than the other extracts, which is due to the higher amount of phenolic in this extract (38.08mg/g).

3.3.3Ferric ion reducing antioxidant power assay (FRAP):
FRAP measures the reducing potency of the extract and the standard antioxidant. Higher the inhibition higher is the reducing power. The reducing effect of MEP, MEC, MEE, and HAE at concentration of 50 µg/ml was found to be 24.32 % (MEP), 27.37 % (MEC), 61.77 % (MEE), and 52.8 % (HAE) respectively (Figure 4).

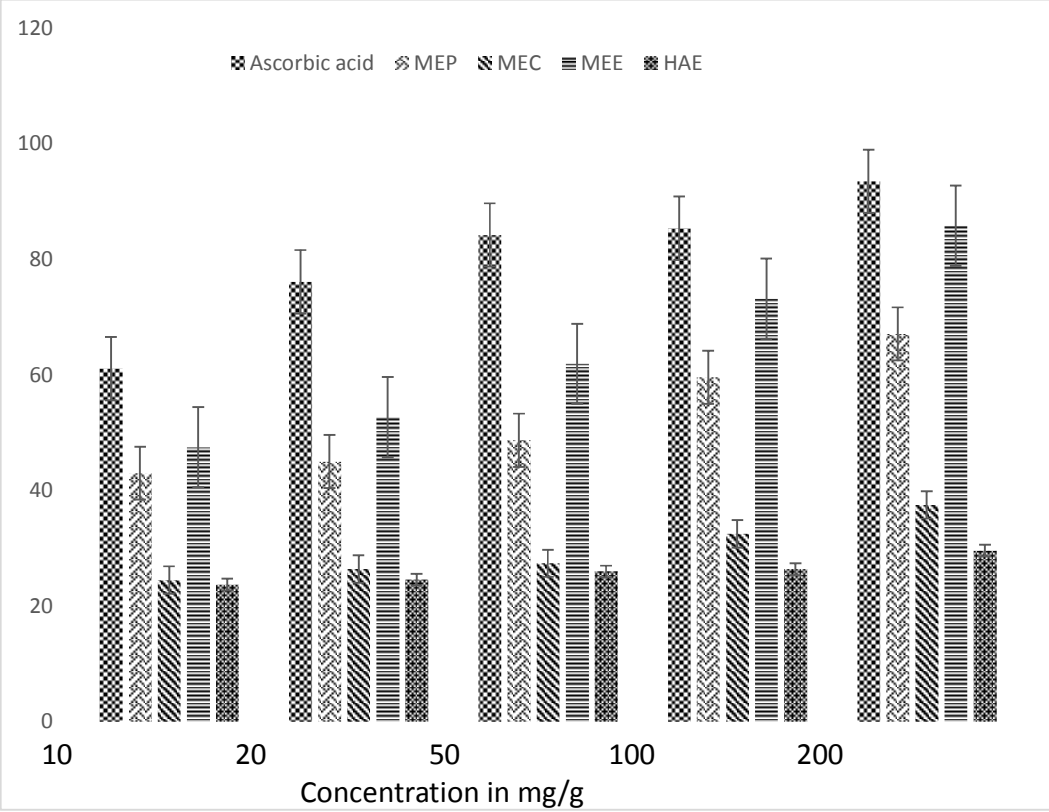

Figure 2: Antioxidant activity for different fruit extracts of *P. dulce*by DPPH Method

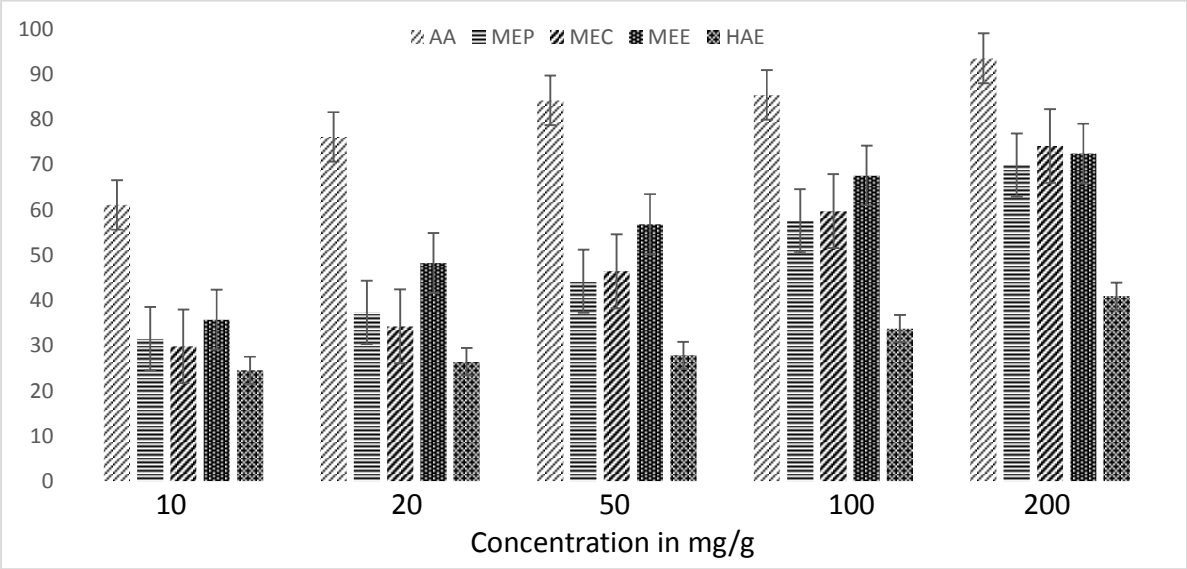

Figure 3: Antioxidant activity for different fruit extracts of *P. dulce*by Phospho molybdenum assay

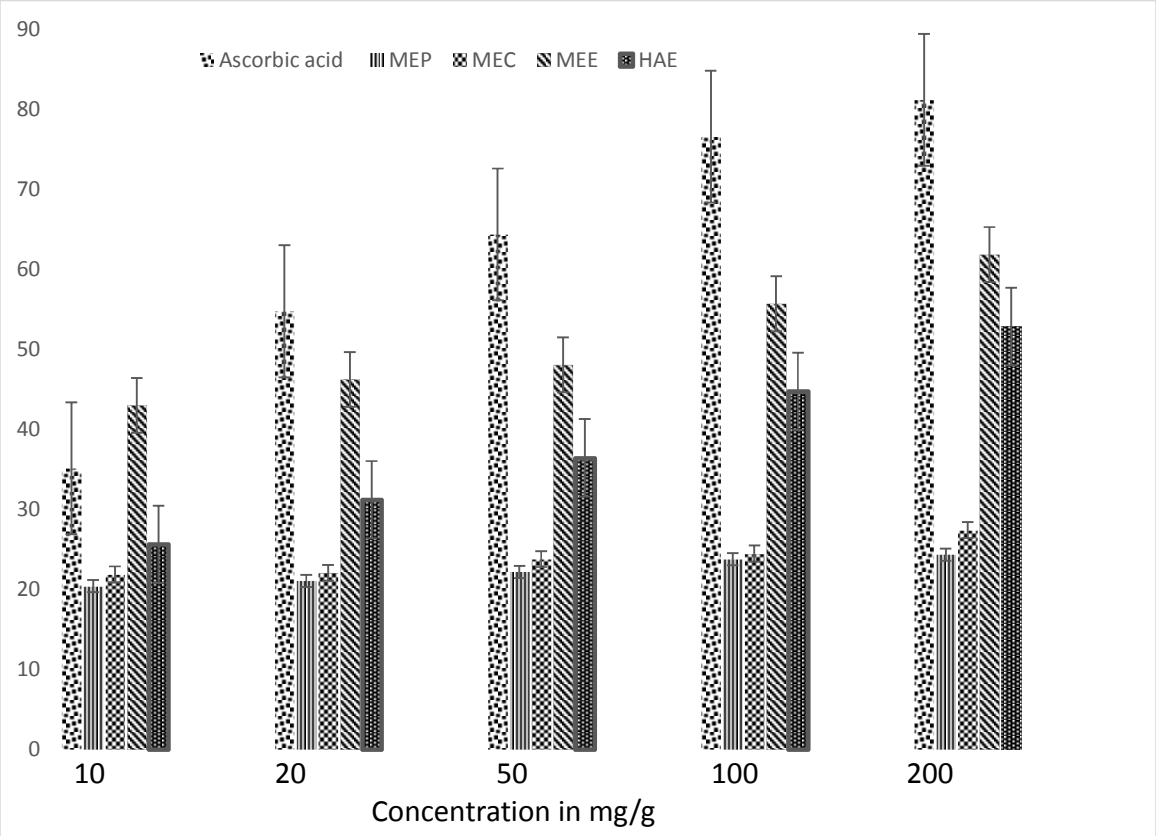

Figure 4: Antioxidant activity for different fruit extracts of *P. dulce*by ferric ion reducing antioxidant assay

DISCUSSION

The present study made us to conclude that the solvents used in microwave assisted extraction play a significant role in extracting the different constituents present in *P. dulce*. The first method of extraction made us to conclude that it is the best solvent to extract carbohydrate and the yield was found to be 72.8%. The second method reveals that methanol as the best solvent to extract phenolic and flavonoids. However, small percentage of carbohydrates is also found to be present in this extract. This extract on further separation with petroleum ether, chloroform and ethyl acetate yields the phenolic, flavonoids in higher amount which could be concentrated further to separate the individual compounds. Higher yield and fast extraction performance ability with less solvent consumption and protection offered to thermo labile constituents are some of the advantage of this promising microwave assisted extraction techniques. The total phenolic content was found to be higher in chloroform extract 46.8± 0.0001 mg/g of Gallic acid equivalent. The total flavonoid content was found to be higher in chloroform extract 1.27 ± 0.03 mg/g of Quercetin equivalent. The total carbohydrate content was found to be higher in 20% ethanol 113± 0.04. The present study reveals that the order of phenolic and flavonoid contents in the extracts as MEC>MEE>MPE>HAE. The total carbohydrate content is in the order of HAE>MEE>MEC>MEP. The antioxidant activity studies reveals that the plant is richer in phenolic and hence their activities also was highly remarkable.

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