Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(12)60041-0

Assessment of effect of hydroalcoholic and decoction methods on extraction of antioxidants from selected Indian medicinal plants

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ARTICLE INFO

Article history:
Received 5 August 2011
Received in revised form 7 September 2011
Accepted 2 October 2011
Available online 28 March 2012

Keywords:
Antioxidant
Hydroalcoholic extraction
Total phenol content
DPPH
Superoxide
Manilkara zapota

ABSTRACT

Objective: To assess the effects of extraction methods on antioxidant activities of selected Indian medicinal flora. **Methods:** Different parts of plants were extracted by hydroalcoholic and decoction methods using water and various concentrations of methanol (ME) *viz.* 75%, 50% and 25% ME. The antioxidant activity of all the different extracts was evaluated using two different antioxidant assays *viz.* 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay and superoxide anion radical scavenging assay. Total phenol and flavonoid content was also estimated. **Results:** The results showed that the extracting solvent significantly altered the antioxidant property estimations of screened plants. High correlations between phenolic compositions and antioxidant activities of extracts were observed. High levels of antioxidant activities were detected in *Manilkara zapota* (*M. zapota*) as compared with other screened plants. **Conclusions:** The results obtained appear to confirm the effect of different methods on extraction of antioxidants and antioxidant property of *M. zapota*.

1. Introduction

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of ROS and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders[1] such as cancer[2], cardiovascular disease[3], neural disorders[4], Alzheimer's disease[5], mild congnitive impairment[6], Parkinson's disease[7], alcohol induced liver disease[8], ulcerative colitis[9], ageing[10], and atherosclerosis[11].

Oxygen derived free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are cytotoxic and give rise to tissue injuries[12]. Excessive amount of ROS is harmful because they initiate bimolecular oxidation which leads to cell death and creates oxidative stress. In addition, oxidative stress causes inadvertent enzyme activation and oxidative damage to cellular system[13]. Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radicals generated from the oxygen is called ROS which can cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O_2^-) and hydroxyl radicals (OH^-), as well as non-free radicals (H_2O_2) and singlet oxygen[14]. In the body, free radicals are derived from two sources: endogenous sources, *e.g.* nutrient metabolism, ageing process, *etc* and exogenous sources *e.g.* tobacco smoke, ionizing radiation, air pollution, organic solvents, pesticides, *etc*[15]. The mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage.

Many medicinal plants contain large amount of antioxidants such as polyphenols, which can play an important role in absorbing and neutralizing free radicals,

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Foundation Project: This work was financially supported by University Grants Commission, New Delhi, India [grant No. F.4-3/2006(BSR)/5-121/2007(BSR)].

quenching singlet and triplet oxygen or decomposing peroxides[16]. Phenolic compounds in plants provide an array of natural source of antioxidants for use in foods and nutraceutical[17]. Polyphenols are antioxidants with redox properties which allow them to act as reducing agents, hydrogen donor and singlet oxygen quenchers[18]. The interest in polyphenol antioxidant has increased remarkably over the last decade because of their protective effects against different diseases including cardiovascular, inflammatory disease as well as cancers[19]. Natural antioxidants tend to be safer and also possess anti-viral, anti-inflammatory, anti-cancer, antimutagenic, anti-tumour, and hepatoprotective properties. The source of natural antioxidants may be all or any part of plants such as fruits, vegetables, nuts, seeds, leaves, roots, barks, peels, plant, *etc*[20-23].

A great number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. There is no single, widely acceptable assay method for evaluating antioxidant capacity applicable to different compounds and different plant extracts, but the most commonly used methods for measuring antioxidant activity are those that involve the generation of free radical species which are then neutralized by antioxidant compounds[24]. However, it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals[25,26]. Considering the above, in the present study, six plants which are traditionally used in treating various diseases and disorders are selected to evaluate their antioxidant potential.

2. Materials and methods

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide reduced (NADH), gallic acid, ascorbic acid, quercetin, Folin-Ciocalteu reagent, aluminium chloride, potassium acetate, Tris-HCl, were obtained from Hi-Media, Mumbai, India; petroleum ether, methanol, *etc* were obtained from Merck, India.

2.2. Plant materials

The dry powder of all the plant parts was purchased locally, in the month of August, 2010, Gujarat, India.

2.3. Plant description

Six plants belonging to different families were used in the present study. The description of the plants with their therapeutic uses[27] was given below.

2.3.1. *Azadirachta indica* (*A. indica*) A. juss.

A. indica belongs to the family of Meliaceae with a vernacular name called "Limbdo". Its bark is often used for uses such as tonic, antiperiodic, refrigerant, anthelmintic, naturant, pectoral astringent. It is often used in vomiting, burning sensation near the heart, fatigue, fever, thirst, bad taste in the mouth, cough, cures alters, inflammations, earache, rheumatism, symphilitic sores, boils, and blood impurities.

2.3.2. *Hemidesmus indicus* (*H. indicus*) (L.) R. Br.

H. indicus belongs to the family of Asclepiadaceae with vernacular names called "Anantmul, Upalsari". Its roots are often used for uses such as bitter, sweet, cooling, antipyretic, astringent, aromatic, refrigerant, emollient, depurative, aphrodisiac, carminative, appetizer, anthelmantic, diuretic, tonic. Its stem is used for bitter, diaphorestic, diuretic, laxative. Its root is used in leprosy, leucoderma, itching, skin diseases, fevers, foul odour from the body, loss of appetite, asthma, bronchitis, diseases of blood, leucoderma, dysentery and diarrhea, thirst, burning sensation, piles, rat bite poisoning, eye troubles, epileptic, fits, in children, wasting diseases, useful in heminarnia, pain in joints, syphilis, leucoderma, sarsaparilla, in anorexia, fever, skin diseases, as remedy for heat or inflammations of the urinary passages, applied to swelling, vitiated condition of pitta, burning sensation, leucoderma, leprosy, pruritus, asthma, bronchitis, helminthiasis, diarrhea, dysentery, haemorrhoids, strangury, leacorrhoea, syphilis, abscess, arthralgia, fever and general dedility.

2.3.3. *Manilkara zapota* (*M. zapota*) L.

M. zapota belongs to the family of Sapotaceae with a vernacular name called "Chiku". The part of leaves are often used. The seeds are aperients, diuretic, tonic and febrifuge. Bark is antibiotic, astringent and febrifuge. Fruits are edible, sweet with rich fine flavour. Chicle from bark is used in dental surgery. Bark is used as tonic and the decoction is given in diarrhea and peludism.

2.3.4. *Psorelea corylifolia* (*P. corylifolia*) L.

P. corylifolia belongs to the family of Fabaceae with vernacular names called "Babchi, Bavachi". Its seeds are often used. The seeds are bitter, acrid, anthalmintic, laxative, stomachic, stimulant, aphrodisiac, diuretic, rubefacient. They are useful in leucoderma, ulcers, scabies, leprosy and vitiated conditions of 'pitta' mucomembranous disorders and dermatitis. It is a good hair tonic.

2.3.5. *Rubia cordifolia* (*R. cordifolia*) L.

R. cordifolia belongs to the family of Rubiaceae with a vernacular name called "Majith". Its root is often used. Roots are sweet, bitter, astringent, thermogenic, anti-inflammatory, anodyne, anti-setic, digestive, carminative, constipating, anti-dysenteric, anthelmintic, depurative, vulnerary, emmenagogue, diuretic, galactapurifier, alterant, ophthalmic, febrifuge, rejuvenating and tonic. They are useful in vitiated conditions of kapha pitta, rheumatoid arthritis, neuralgia, cephalgia, dyspepsia, flatulence, colic, diarrhea, dysentery, helminthiasis, leprosy, skin diseases, leucoderma, pruritus, wounds, ulcers, amenorhoea,

dysmenorrhoea, strangury, ophthalamopathy, intermittent, fever, pharyngitis, cough, diabetes, discolouration of the skin and the mucous tissues, otopathy, urethrorrhea, haemorrhoids, jaundice, hepatopathy, splenopathy, arthralgia, leucorrhoea, pectotoral diseases and general debility.

2.3.6. *Tinospora cordifolia* (*T. cordifolia*) (Willd.) Miers ex Hook. F. Thoms.

T. cordifolia belongs to the family of Menispermaceae with a vernacular name called "Gulvel". Its stem is often used. The stem is bitter, astringent, sweet, thermogenicanodyne, anthelmintic, alterant, antiperiodic, antispasmodic, anti-inflammatory, antipyretic, antiemetic, digestive, carminative, appetister, stomachic, constipating, cardiotonic, depurative, haematinic, expectorant, aphrodisiac, rejuvenating, galactapurifier and tonic. It is useful in vitiated condition of vata, burning sensation, hyperdipsia, helmenthiasis, dyspepsia, flatulence, stomachalgia, intermittent fevers, chronic fevers, inflammations, gout, vomiting, cardiac debility, skin diseases, leprosy, erysipelas, anaemia, cough, asthma, general debility, jaundice, seminal weakness, uropathy and splenopathy.

2.4. Extraction

Extraction was done by two different methods as described below.

2.4.1. Hydroalcoholic method

The dried powder of plant parts was individually extracted by hydroalcoholic cold percolation method[28]. 10 g of dried powder was taken in 100 mL of petroleum ether in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. After 24 h, it was filtered through eight layers of muslin cloth, centrifuged at 5 000 rpm for 15 min and the supernatant was collected and air dried under reduced pressure to obtain the dried residue. Petroleum ether was evaporated from the powder. This dry powder was then taken individually in 100 mL of each solvent *i.e.* methanol (ME), 75% ME, 50% ME, 25% ME and water and was kept on a rotary shaker at 120 rpm for 24 h. Then the procedure followed was same as above, and the residues were weighed to obtain the extractive yield of all the extracts and were stored in air tight bottles at 4 ℃.

2.4.2. Decoction method

For the decoction method[29], 5 g of dried powder was extracted with 100 mL of deionized water at 100 ℃ for 30 min in a water bath. It was filtered with eight layers of muslin cloth and centrifuged at 5 000 rpm for 10 min. The supernatant was collected and the solvent was evaporated to dryness. The residue was weighed to obtain the extractive yield, and it was stored in air tight bottle at 4 ℃.

2.5. Quantitative phytochemical analysis

2.5.1. Determination of total phenol content

The amount of total phenol content, in different solvent extracts, was determined by Folin-Ciocalteu reagent method[30]. 0.5 mL of extract and 0.1 mL (0.5 N) Folin-Ciocalteu reagent were mixed and the mixture was incubated at room temperature for 15 min. Then 2.5 mL saturated sodium carbonate solution was added and further incubated for 30 min at room temperature and the absorbance was measured at 760 nm. Gallic acid was used as a positive control. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of extracted compounds).

2.5.2. Determination of flavonoid content

The amount of flavonoid content, in different solvent extracts, was determination by aluminium chloride colorimetric method[31]. The reaction mixture (3.0 mL) consisting of 1.0 mL sample (1 mg/mL), 1.0 mL methanol, 0.5 mL (1.2%) aluminium chloride and 0.5 mL (120 mM) potassium acetate, was incubated at room temperature for 30 min. The absorbance of all samples was measured at 415 nm. Quercetin was used as positive control. The flavonoid content is expressed in terms of quercetin equivalent (mg/g of extracted compound).

2.6. Antioxidant assays

2.6.1. DPPH free radical scavenging assay

The free radical scavenging activity of different solvent extracts was measured by using DPPH with the modified method of Mc Cune and Johns[32]. The reaction mixture (3.0 mL) consisting of 1.0 mL DPPH (0.3 mM), 1.0 mL extract (different concentrations) and 1.0 mL methanol, was incubated for 10 min, in dark, after which the absorbance was measured at 517 nm. Ascorbic acid was used as positive control. Percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [1 - (A/B)] \times 100$$

Where, B is the absorbance of the blank (DPPH plus methanol) and A is absorbance of the sample (DPPH, methanol, plus sample).

2.6.2. Superoxide anion radical scavenging assay

The superoxide anion radical scavenging activity of different solvent extracts was measured by the method as described by Robak and Gryglewski[33]. Superoxide anion radicals are generated by oxidation of NADH and assayed by the reduction of NBT. The reaction mixture (3.0 mL) consisted of 0.5 mL Tris-HCl buffer (16 mM, pH 8), 0.5 mL NBT (0.3 mM), 0.5 mL NADH (0.936 mM), 0.5 mL PMS (0.12 mM) and 1.0 mL of different concentrations of different solvent extracts. The superoxide radical generating reaction was started by the addition of PMS solution to the mixture. The reaction mixture was incubated at 25 ℃ for 5 min and then the absorbance was measured at 560 nm against a blank sample. Gallic acid was used as a positive control. The percentage inhibition was calculated as described above.

3. Results

3.1. Extractive yield

The dry powder of the six plants was extracted by hydroalcoholic cold percolation method and decoction extraction method. The extractive yield of all the six plants in different solvent was given in Figure 1. In all six plants, the extractive yield was higher by decoction method than hydroalcoholic method. Different plant extracts can be ranked from high to low in the following order: *M. zapota* > *R. cordifolia* > *H. indicus* > *T. cordifolia* > *P. corylifolia* > *A. indica* in different extracts. Amongst all the six plants, the maximum yield was in *M. zapota*.

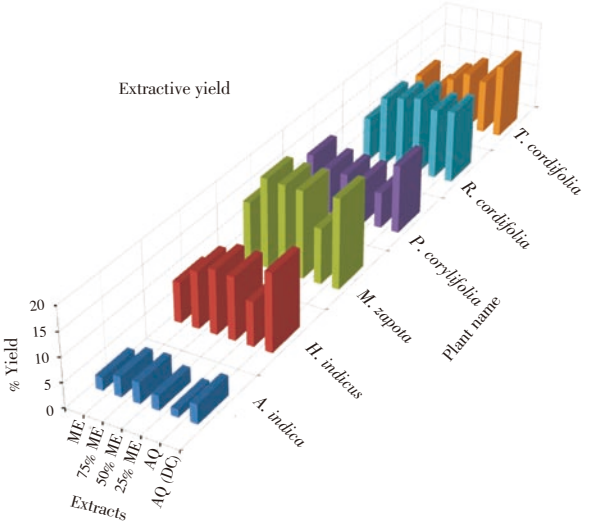

Figure 1. Percentage extractive yield of different extracts of screened plants.
AQ: Aqueous extract; DC: Decoction.

3.2. Total phenol and flavonoid content

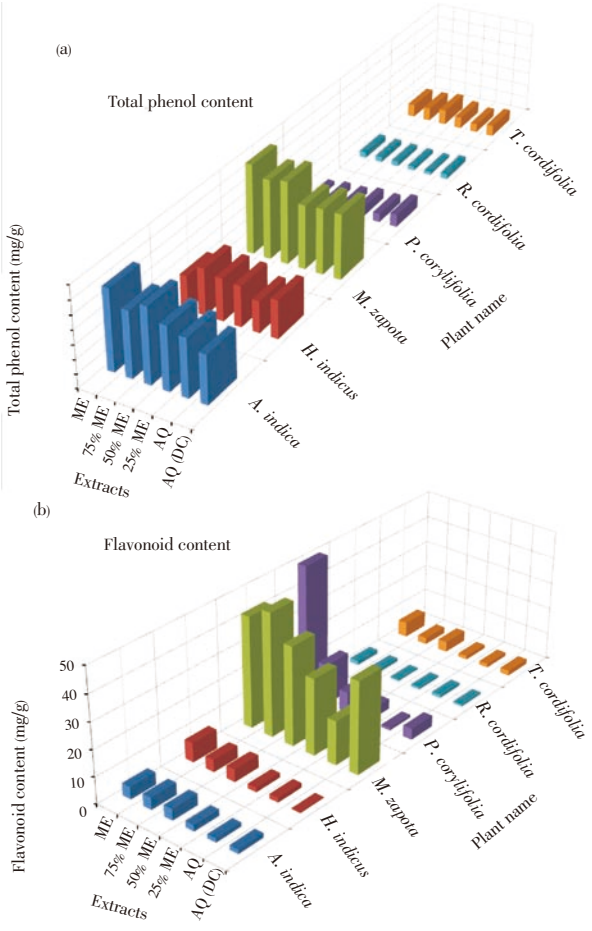

Figure 2. Total phenol and flavonoid content of different extracts of screened plants.
AQ: Aqueous extract; DC: Decoction.

Total phenol content and flavonoid content of screened plants was shown in Figure 2. In all the plants in all the extracts, total phenol content was considerably more than flavonoid content except in *P. corylifolia*. The flavonoid content of pure ME extract was considerably more than total phenol content in *P. corylifolia* (Figure 2). Maximum total

Table 1
IC_{50} values of DPPH free radical (DPPH) scavenging and superoxide anion radical (SO) scavenging activities.

Assay	Plant	IC_{50} values (μ g/mL)					
		Hydroalcoholic					Decoction aqueous extract
		ME	75% ME	50% ME	25% ME	Aqueous extract	
DPPH	*A. indica*	34	33	50	121	214	125
	H. indicus	33	53	79	212	480	328
	M. zapota	34	30	49	67	120	74
	P. corylifolia	375	620	–	–	–	–
	R. cordifolia	–	–	–	–	–	–
	T. cordifolia	315	560	325	–	–	–
	Standard (ascorbic acid)	11.4					
SO	*A. indica*	360	350	275	305	360	225
	H. indicus	840	490	510	690	920	700
	M. zapota	210	225	220	305	360	305
	P. corylifolia	–	–	–	–	–	–
	R. cordifolia	–	–	–	–	–	–
	T. cordifolia	–	–	–	–	–	–
	Standard (gallic acid)	185					

–: > 1000.

phenol content was present in *M. zapota*. Except *A. indica*, in all the other plants, the phenol content was more in aqueous extract by decoction method than cold percolation method. When phenol content of pure solvent extracts of the six plants was compared, generally phenol content was more in ME than both aqueous extracts except in *P. coylifolia*. Therefore, in hydroalcoholic extracts, the phenol content decreased to some extent or remained same. Maximum phenol content was in pure ME extract of *M. zapota* (Figure 2). Hence, it can be stated that ME was able to extract more phenolic compounds as compared with water (aqueous).

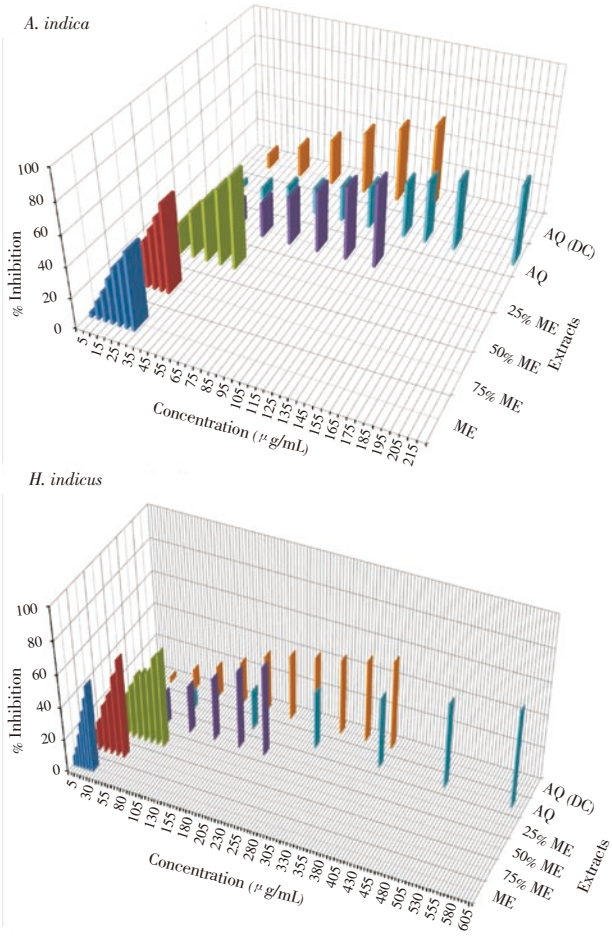

Figure 3. DPPH free radical scavenging activity of different extracts of *A. indica* and *H. indicus*.
AQ: Aqueous extract; DC: Decoction.

3.3. Antioxidant activity

3.3.1. DPPH free radical scavenging activity

The DPPH free radical scavenging activity of screened plants was shown in Figures 3-5. Six different extracts of six plants were evaluated for their DPPH free radical scavenging activity. Out of 36 extracts investigated, 13 extracts showed IC_{50} value of more than 1 000 μg/mL (Table 1) while the remaining 23 showed varied levels of DPPH free radical scavenging activity (Table 1). IC_{50} values ranged from 30 to 620 μg/mL (Table 1). Ascorbic acid was used as a standard and its IC_{50} value was 11.4 μg/mL (Table 1). Amongst all the extracts, the lowest IC_{50} value was of 75% ME extract of *M. zapota* (30 μg/mL) and the highest IC_{50} value was of 75% ME extract of *P. corylifolia* (495 μg/mL). Amongst all extracts of all the plant studied, ME, 75% ME and 50% ME showed better DPPH free radical scavenging activity than 25% ME, and both aqueous extracts. Amongst all the plants *M. zapota* showed the best DPPH free radical scavenging activity.

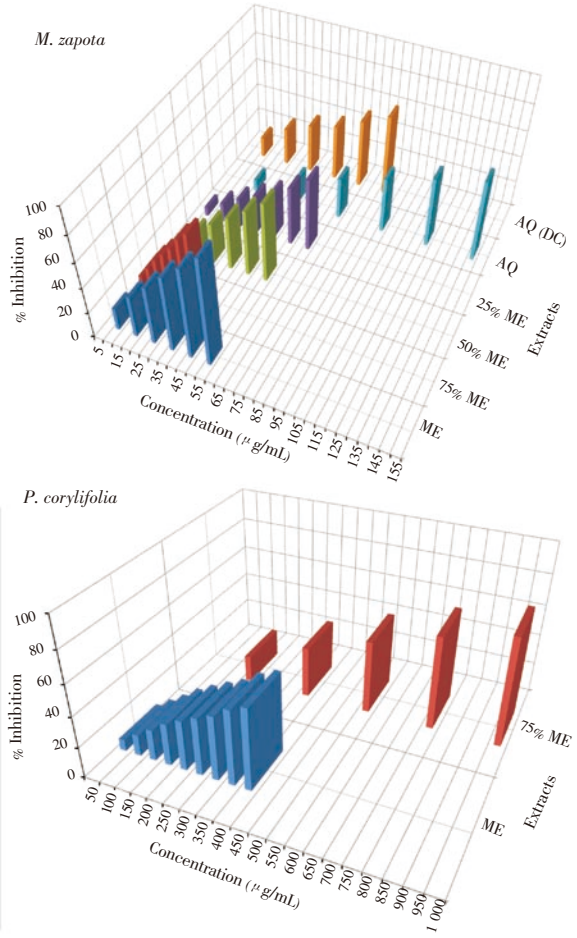

Figure 4. DPPH free radical scavenging activity of different extracts of *M. zapota* and *P. corylifolia*.
AQ: Aqueous extract; DC: Decoction.

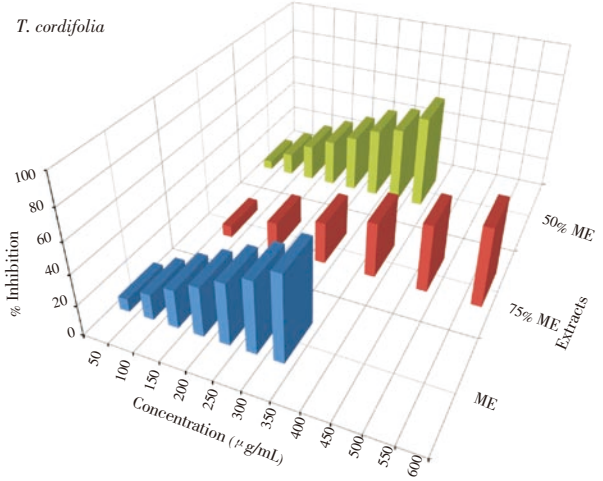

Figure 5. DPPH free radical scavenging activity of different extracts of *T. cordifolia*.

3.3.2. Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of screened plants was shown in Figure 6 and 7. Six different

extracts of six plants were evaluated for their superoxide anion radical scavenging activity. Out of 36 extracts investigated, 18 extracts showed IC_{50} value of more than 1 000 μg/mL (Table 1) while the remaining 18 showed varied levels of superoxide anion radical scavenging activity (Table 1). IC_{50} values ranged from 210 to 920 μg/mL (Table 1). Gallic acid was used as a standard and its IC_{50} value was 185 μg/mL (Table 1). Among all the extracts, the lowest IC_{50} value was of ME extract of *M. zapota* and the highest IC_{50} value was of aqueous extract of *H. indicus* (Table 1). Amongst all different extracts of all the plant studied, ME, 75% ME and 50% ME showed better superoxide anion radical scavenging activity than 25% ME and both aqueous extracts. Amongst all the plant studied, different extracts of *M. zapota* showed the best superoxide anion radical scavenging activity, in some case comparable with standard gallic acid.

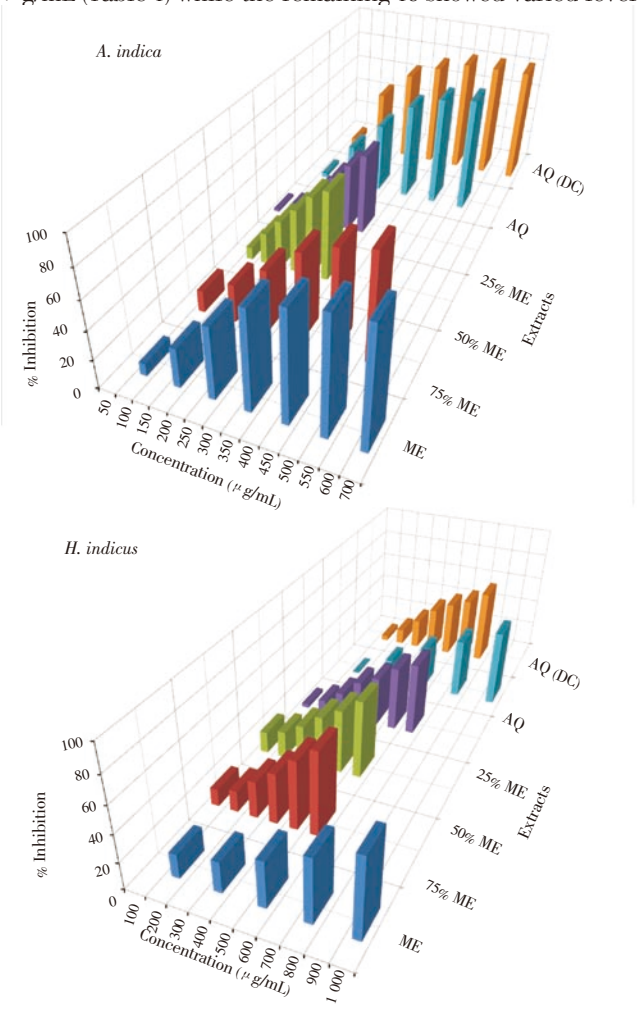

Figure 6. Superoxide anion radical scavenging activity of different extracts of *A. indica* and *H. indicus*.
AQ: Aqueous extract; DC: Decoction.

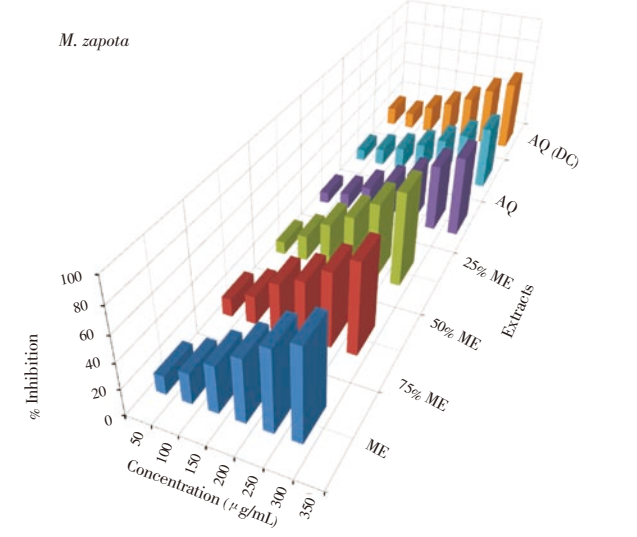

Figure 7. Superoxide anion radical scavenging activity of different extracts of *M. zapota*.
AQ: Aqueous extract; DC: Decoction.

4. Discussion

The extractive yield depends on solvents, time and temperature of extraction as well as the chemical nature of sample. Under the same time and temperature conditions, the solvent used and the chemical property of sample are the most important factors[34]. The traditional healers or practitioners make use of water primarily as a solvent but there are many reports where organic solvents showed better activity as compared with aqueous extracts[35,36]. In the present study, when aqueous extracts of all the six plants were compared, the extractive yield was maximum in aqueous extract by decoction method than by cold percolation method. This may be because the phytoconstituents present in these plants are extracted better on application of heat. Similar results were obtained in some plants of Zingiberaceae as reported by Chandarana *et al*[37]. On the other hand, when aqueous extracts by cold percolation method and pure methanol extracts are compared, a different result was obtained. Three plants had more yield in pure methanol while the other three plants had more yield in aqueous extract, which implies that there is no universal criteria for extraction. It varies from plant to plant again may be because of the nature of secondary metabolites present in them and also their proportion. When hydroalcoholic extracts of the six plants are considered, the yield again either increased or decreased than pure solvents.

Phenolics are secondary plant metabolites that are ubiquitously present in plant products[38]. Plant phenolics are biosynthesized following different routes, the shikimic acid pathway being the most biosynthetic route involved[39]. Plant phenolics have been reported to have several biological activities including antioxidant activity[40]. Many of the phenolics have been shown to contain high levels of antioxidant activities[41]. Among the six plants investigated in the present work, *M. zapota* had considerably greater total phenol content, and methanol was able to extract more phenolic compounds than aqueous, which is also supported by other researchers[42-44]. It is important to examine the correlation between the content of total phenols and the antioxidant potential because some authors have reported that there is no correlation between the content of phenolic compound and the radical scavenging capacity[22,45]. The results obtained in this study do not support these claims.

Because multiple reaction characteristics and mechanisms are likely to be involved, no single assay will accurately reflect all antioxidants in a mixed or complex system. Thus, to fully elucidate a full profile of antioxidant capacity of different extracts, different antioxidant capacity assays were used in this study. DPPH, superoxide anion radical

scavenging activity assays are most commonly accepted ones which have been used in the present investigation[46,47]. DPPH has been used extensively as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging activities of compounds[48]. The use of the DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidant and radical scavengers[49,50]. The DPPH radical scavenging activity of extract has been attributed to the ability of these extracts in pairing with the old electron of DPPH radical[51]. The antioxidants are able to reduce the stable DPPH radical to yellow-colored and the antioxidant power is indicated by the degree of discoloration which could be determined by measuring of a decrease in the absorbance at 517 nm[52]. Because of the ease and convenience of this reaction, it has now widespread use in the free radical scavenging activity assessment[40]. The lowest IC_{50} value was in *M. zapota* irrespective of the solvent used. Incidentally this plant in different methanol concentrations, *i.e.* ME, 75% ME and 50% ME had maximum phenol content thus supporting the general view that phenol content is a good indicator of antioxidant activity.

Scavenging of superoxide anion radical is important for protection against early events in oxidative damage[53]. Superoxide anion is the most common free radical *in vivo* and is generated in a variety of biological system and the concentration of superoxide anion increases, under condition of oxidative stress[54]. In the PMS-NADH-NBT system, superoxide anions were derived from dissolved oxygen by the PMS-NADH coupling reaction, which then reduced to NBT. The decreased absorbance at 500 nm with antioxidants indicates consumption of the superoxide anion in the reaction mixture[33]. The lowest IC_{50} value was in *M. zapota* in ME, 75% ME and 50% ME except in aqueous extract by decoction method in which *A. indica* had the lowest IC_{50} value. Incidentally these three extracts of *M. zapota* had the highest phenolic content which again suggests that there is a direct correlation between phenol content and antioxidant activity. This is in accordance with results of others[23,55,56].

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems[57]. The difference in the antioxidant activity of the different hydroalcoholic extracts may be ascribed to the difference in the total phenolic compositions. As far as the free radical scavenging efficiency was concerned, the antioxidant activity of the different hydroalcoholic extracts analyses was found to have a strong positive correlation with their total phenolic content. Results from this study also provide a better understanding for the selection of an appropriate solvent and extraction method especially for *M. zapota* so that its full potential can be utilized. It could also be useful for further investigations.

This study indicated that different types of extraction method and solvent had great influence on the antioxidant property of obtained extracts. The highest total phenol content and the best antioxidant activity *i.e.* the lowest IC_{50} value in DPPH as well as in superoxide anion radical scavenging activity was shown by *M. zapota*. Therefore, it can be stated that there was a direct correlation observed between total phenolic content and antioxidant activity. Methanolic extracts (ME, 75% ME and 50% ME) showed better antioxidant activity and more phenol content as compared with aqueous extracts. Hence, it can be concluded that *M. zapota* is the best source of natural antioxidants. Organic solvent extraction (either 100% or 75%) is better than aqueous extraction by either method *i.e.* cold percolation or decoction method. The specific components that confer the antioxidant properties on the *M. zapota* extracts are currently unknown. A further study is underway to identify and characterize chemicals contributing to antioxidant properties of *M. zapota* extracts.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors thank Prof. Singh SP, Head, Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India for providing excellent research facilities. One of the author, Mr. Mital Kaneria is also thankful to University Grants Commission, New Delhi, India for providing financial support as Junior Research Fellow.

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