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MORUS INDICA: ANTIOXIDANT ACTIVITY IN FOOD AND BIOLOGICAL SYSTEMS

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ABSTRACT

Morus Indica-MI (Mulberry) varieties M5, V1 and S36 leaves were analyzed for their phytochemical composition and antioxidant potency of 100% methanol (M), 80% M (M& water), dechlorophyllised, aq. cold and hot extracts in food and biological system. The radical scavenging activity (RSA), reducing power assay, FRAP and inhibition of oxidation in oil and microsomes was evaluated at different concentrations. Among three varieties, S36 was rich in phytochemicals. All the extracts of MI varieties have performed in a dose dependent manner. The dechlophyllised extract of all the varieties have shown significantly ($P < 0.05$) high RSA, reducing power and inhibition of oxidation in oil and microsomes. Also the IC50 of S36 in RSA and activity in oil and microsomes was significantly ($P<0.05$) high. Above results indicate that the MI varieties can be used as nutraceutical in preventing or reducing the oxidative stress at the biological level and also as functional ingredient in health food formulations.

KEYWORDS : Morus indica, Phytochemicals, antioxidant activity, food and biological systems.

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INTRODUCTION

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects such as phytochemicals and antioxidants[1,2]. The term Phytochemicals is generally used to refer compounds found in plants that are not normally involved in primarily metabolic process such as photosynthesis and cell respiration of plants. The biological activities elicited by a plant are primarily due to the presence of these chemical constituents in the plant. Currently much research is focused on the beneficial effects of bioactive phytochemicals present in micro level in our daily diet[3]. Several epidemiological studies suggest that plants rich in antioxidants play a protective role in health and diseased condition and their consumption lower risk of cancer, heart disease, hypertension and stroke. The major groups of phytochemicals that may contribute to the total antioxidant capacity of plant include polyphenols and vitamins (C and E)[4]. With the focus being shifted towards finding alternatives for synthetic food ingredients, natural substances having antioxidant properties need to be further explored[5]. Many plant species have been investigated in search of novel antioxidants and there is still demand to find more information concerning the antioxidant potential of plant species as they are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards the identification of plant materials, rich in antioxidants[6]. However, besides the role played in human and animal nutrition, knowledge of proximate, phytochemical and micronutrient composition is fundamental for understanding modes of action of medicinal plants in general. It is the diverse composition of these components in plants that places them at advantage position over and above chemotherapy in management of complex diseases such as diabetes mellitus[7]. Each medicinal plant species has its own nutrient composition besides having pharmacologically important photochemicals. These nutrients are essential for the physiological functions of human body [8,9,10]. *Morus indica* (Mulberry) of the family Moraceae has been widely cultivated in countries all over the world including temperate to tropical areas. Different parts of the plant are used as herbal medicine for blood serum glucose reduction, cholesterol and lipids levels reduction, antiphlogistic, diuretic and expectorant effects. It occupies an important position in the holistic system of Indian medicine 'Ayurveda' which has its roots in antiquity and has been practiced for centuries. The leaves of mulberry are nutritious, palatable, nontoxic and also enriched with different active principles[11]. The antihyperglycemic properties of mulberry leaves was investigated in streptozotocin induced diabetic rats[12,13]. There are few reports on the antioxidant activity of *Morus indica*[14,] but the complete information on phytochemical composition and antioxidant activity of the plant and its varieties is scarcely reported. In the present study, three different varieties of *Morus indica* (L), M5, V1 and S36 were studied for their phytochemical composition and antioxidant activity in food and biological systems.

MATERIALS AND METHODS

Chemicals

Tocopherol, β carotene, 2,2-diphenyl-1-picrylhydrazyl. (DPPH), ,5-Dithio (bis) nitro benzoic acid (DTNB), were purchased from Sigma Aldrich, Bangalore, India. All other chemicals and reagents used in the study were of analytical grade.

Plant material

The leaves of three varieties of Morus *indica* (M5, V1 and S36) were collected from Central Sericulture Research and Training Institute (CSRTI), Mysore. The samples were identified by Dr. Shivamurthy, Department of Studies in Botany, University of Mysore and voucher specimen was retained in the laboratory for future reference. The leaves were washed, dried in the oven overnight at 50°C, powdered, passed through 60 mesh and stored at 4°C till further use.

Phytochemical composition

In dehydrated sample, different phytochemicals were estimated by using standard methods. Total phenols were extracted from a weighed portion (50-500 mg) of dried sample with 50ml 50% aqueous methanol of pH 2 and 70% aq. acetone for 2hrs and analyzed by Folin-Ciocalteu micro method. Results are expressed as mg Gallic acid equivalent g^{-1} dry weight[15]. Flavonoid content was determined by a pharmacopoeia method using Rutin as a reference compound[16], alkaloids by the method based on the reaction with bromocresol green (BCG) using Atropine as a standard[17], saponins by using vanillin/sulfuric acid reagents and Diosgenin as standard[18], tannins by the method of Makkar et al[19] were estimated. α-Tocopherol was extracted by direct saponification of dried sample and estimated based on formation of a red complex from reaction of α,α' –bipyridyl with ferrous ion due to reduction of ferric ion by tocopherol[20]. β- Carotene was quantified by column chromatography, followed by measuring the absorbance of elute at 450nm against standard β-carotene[21]. Reduced glutathione was determined based on the development of a yellow compound due to reaction of 5,5-Dithio (bis) nitro benzoic acid (DTNB) with compounds containing sulphydryll groups[22].

Preparation of solvent extracts

A 15g dehyadrated sample of all the three varieties individually was extracted with 100 ml solvent [100% methanol, methanol and water (80:20) and 100% water (cold)] for 6h in a mechanical shaker. The extracts were filtered and filtrates were evaporated at 40°C under reduced pressure to dryness in a rotary evaporator (Superfit, India). The residue of each extract was stored in airtight container at 4°C until further use. In case of hot water extract, the sample was added to boiling water and extracted for 15 min and filtered. The filtrate of both cold and hot water extracts was freeze dried and stored at 4°C until further use.

Dechlorophyllised extract

Since the samples were rich in chlorophyll, an extract was prepared by separating the chlorophyll. Briefly, hexane was added to the 80% methanol extract and shaken for 30 minutes, as chlorophyll is readily soluble in hexane, a chlorophyll rich hexane layer is formed on the top of the extract leaving the other polar components especially phenolic compounds in the water and methanol layer and this extract was further dried and stored in air-tight container at 0°C until used. The Total polyphenol content of all the extracts was estimated[23.]

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl. (DPPH) Radical scavenging activity

The ability of extracts to scavenge DPPH radicals was determined according to the method of Blois (1958) [24].

Reducing power assay

The ability of extracts to reduce iron (III) to iron (II) was determined as per Yildirim et al (2003) method [25].

Ferric Reducing Antioxidant Power (FRAP)

Measurement of ferric reducing antioxidant power of the herbal extract was carried out based on Benzie and Strain (1999) procedure [26].

Inhibition of lipid oxidation in oil emulsion

The antioxidant activity of the above extracts was determined in an edible oil emulsion and liver microsomes by modified method of TBARS -thiobarbituric acid reactive substances. Five grams of oil (sunflower) was weighed and an emulsion was prepared in phosphate buffer of 0.01M, 7pH. To the known concentration of the extracts, 300µl of emulsion, 450µl of Fenton's reagent was added and the volume was made up to 2ml with phosphate buffer and incubated at 50°C for 2hrs. A control was run without extract. After incubation, 1ml of 0.67% TBA was added and heated in boiling water bath for 30min. and cooled immediately. The inhibition of lipid peroxidation in oil was determined by TBA, in which the secondary oxidation products (TBARS, expressed as MDA equivalents)

formed by oxidation of oil was determined by measuring the absorbance at 535 nm [27].

Inhibition of lipid oxidation in microsomes

A healthy male adult rat was sacrificed to get fresh liver. The procedure described here is partially modified from that reported by Shapiro & Rodwell (1971)[28]. Briefly, the liver was immediately removed from the rat and placed in cold triethanolamine HCl buffer (0-4°C) at pH 7.4. The liver was thoroughly chilled and homogenized. The homogenate was centrifuged in ultra centrifuge, at 60,000 g for 60 min. The 60,000g microsomal pellet was then rinsed with buffer and frozen in a freezer (-20°C). The resuspended microsomes to be used for the assay were diluted with buffer to give a protein concentration of 5-10mg/ml. To an aliquot of liver microsomes (1mg protein concentration), Fenton's reagent and different extracts were added and incubated at 50°C for 2 hrs. After incubation, 1ml of TCA (10%) and 1ml of TBA were added and heated in boiling water bath (15min) and cooled in an ice bath immediately. After cooling, 2ml of butanol was added and the pink color developed was read at 535nm. A control was run without plant extract [29].

$$AOA\% = \frac{\text{Absorbance of control- Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Statistical analysis

All the experiments were run in triplicates. Results were expressed as the mean ± standard deviation (SD). Data was subjected to one way ANOVA and Tukey's multiple comparison tests using SPSS software (version 11) ($P < 0.05$)

RESULTS

The phytochemical composition of MI varieties is given in table 1. Among three varieties S36 was found to be rich in phytochemicals (glutathione- 788mMoles, β carotene-87.30mg/100g powder, flavonoids-1.15mg/g extract, alkaloids- 100.66mg/g and tannins-1546.67mg). M5 was rich in α tocopherol-34 mg, saponins-135.3 mg and steroidal saponins-39.63 mg and V1 was rich in polyphenols - 1.406g. The polyphenol content of the extracts was in the order of dechlorophylised>methanol> 80% methanol > Aq. cold and hot.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical scavenging activity *(RSA)*

RSA is a rapid, simple and inexpensive method to measure antioxidant activity and DPPH radical serves as the oxidizing substrate, which can be reduced by an antioxidant compound to its hydrazine derivative via hydrogen donation, and as the reaction indicator molecule. Here in this assay, different solvent extracts of MI varieties were assessed for their ability to scavenge the DPPH by donating the hydrogen ion. All the extracts of MI varieties have performed in a dosed dependent manner. The figure 1 shows IC_{50} values of the samples extracts and dechlorophyllised extract of all the three varieties had significantly high radical scavenging activity followed by 80% methanol >Aq. cold > Aq. hot > methanol extracts and among the three MI varieties S36 showed high activity.

Reducing power activity

The reducing power of a compound is related to its electron transfer ability and may therefore serve as a significant indicator of its potential antioxidant activity[30]. The electron donating capacity was measured at 200 and 300µg (fig 2). The reducing power of the 80% methanol and dechlorophyllised extract was high in all the MI varieties and S36 showed high reducing capacity than M5 and V1.

Ferric reducing antioxidant power (FRAP)

The change in absorbance at 593nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) formed by the action of electron donating antioxidants, this represents a

one-electron exchange reaction. The FRAP of the MI varieties was measured at 200 and 300µg and figure 3 shows 80% methanol extract of all the three MI varieties showed high FRAP. Among the samples S36 exhibited high activity.

Inhibition of lipid peroxidation

In oil emulsion

The IC 50 of different extracts of the samples in inhibition of oxidation in oil emulsion and microsomes is given in figure 4. Among all the samples, methanol and 80% methanol extracts were significantly ($P<0.05$) more effective in inhibiting the oxidation followed by dechlorophyllised, aq. cold and hot extracts.

In microsomes

The IC 50 value of the MI varieties extracts is given in figure 5. The dechlorophyllised extracts of all the MI varieties had exhibited significantly ($P<0.05$) high activity followed by aq cold, aq. hot, 80% methanol and methanol extracts. No significant difference was observed between the aq. cold and hot extracts. All the three varieties of MI were more potent in inhibiting oxidation in biological system than food system.

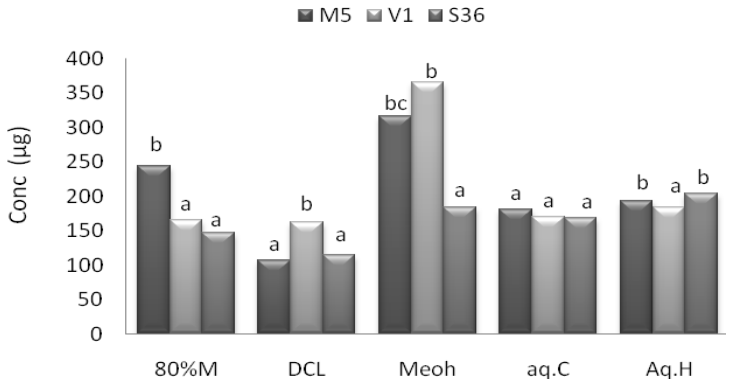
Table 1

Phytochemical composition in different varieties of Morus indica

Phytochemical	M5	V1	S36
	/100g (dry basis)		
α-Tocopherol (mg)	34±3.05[a]	24±2[b]	28±2[b]
Glutathione (m Moles)	520± 0.00[a]	550 ± 4.642[a]	788 ±15.17[b]
β carotene (mg)	69.35 ±1.64[a]	73.50±4.77[a]	87.30±3.12[b]
Polyphenols (mg)	0.505±0.02a	1.406±0.155b	0.622±0.023[c]
Flavonoid (mg/g extract)	0.783±0.01[a]	1.05±0.07[b]	1.15±0.01[b]
Alkaloids(mg/g extract)	46.83±0.288[a]	72.5±6.06[b]	100.66±9.51[c]
Saponin (mg/g extract)	387±12.5.[a]	543±40.41.[b]	550±25[b]
Steroidal saponins (mg/g extract)	1.35.±0.29[a]	1.28±0.25[b]	1.48±0.76[a]
Tannins (g)	0.873±0.081[a]	1.00±0.033[b]	1.54±0.007[b]
Polyphenol content of the extracts (%)			
Meoh	1.16[a]	2.33[b]	1.68[a]
80% Meoh	5.3[b]	2.8[b]	5.3[c]
Decl.Meoh	5.6[b]	3.7[c]	5.0[c]
Aq.Cold	1.0[a]	1.50[a]	1.8[a]
Aq.Hot	1.5[a]	1.91[ab]	2.16[b]

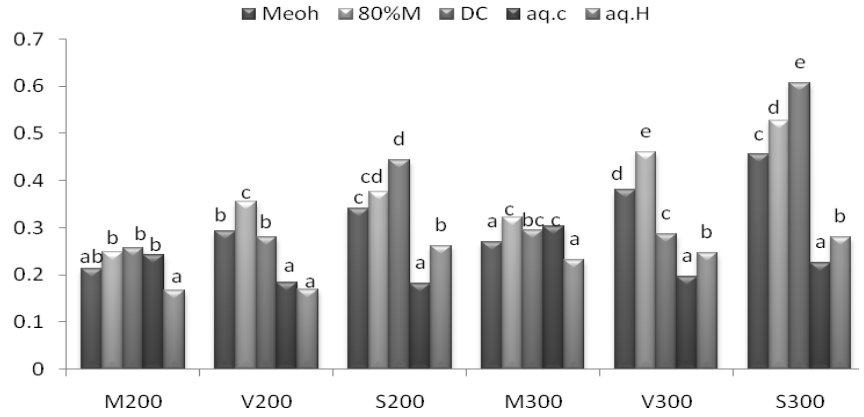
meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h- aqueous hot; values baring different alphabets differ significantly (P<0.05)

Figure 1
IC 50 of the Radical Scavenging Activity of Morus indica extracts



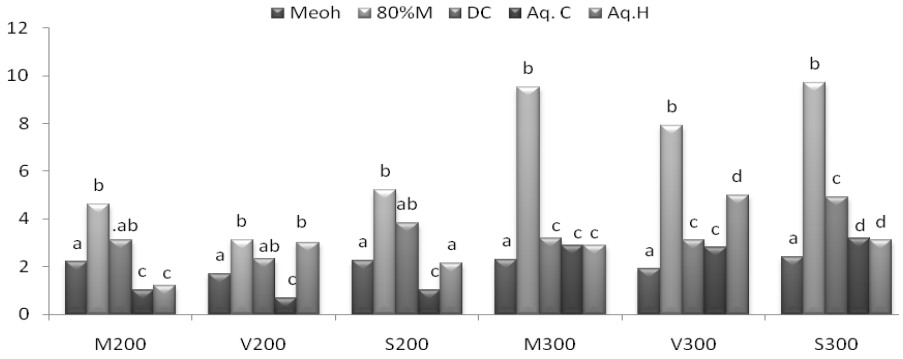
meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h- aqueous hot; Bars with different alphabets differ significantly (P>0.05)

Figure 2
Reducing power assay of Morus indica extracts



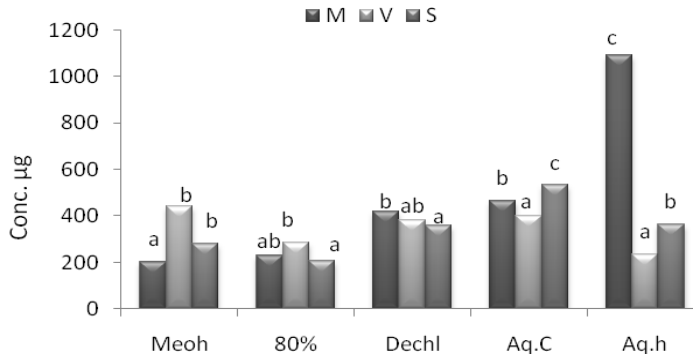
meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h-aqueous hot; Bars with different alphabets differ significantly (P>0.05)

Figure 3
Ferric Reducing Antioxidant Power of Morus indica extracts



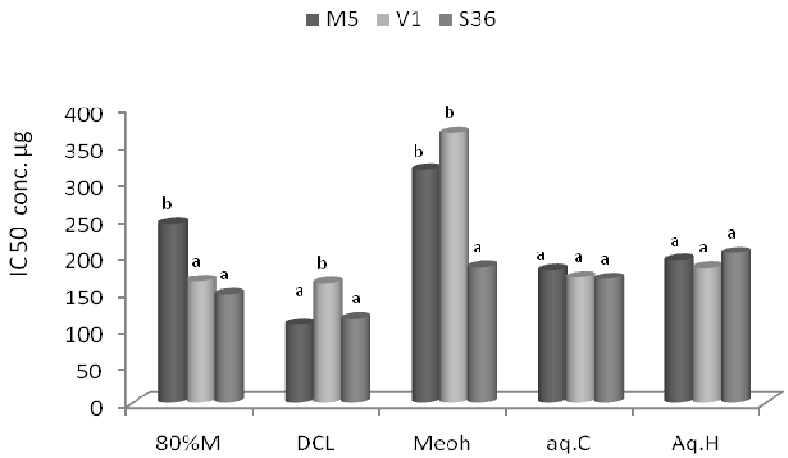
meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h- aqueous hot; ; Bars with different alphabets differ significantly (P>0.05)

Figure 4
IC 50 of the Morus indica extracts in inhibition of lipid peroxidation in oil emulsion



meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h- aqueous hot; ; Bars with different alphabets differ significantly (P>0.05)

Figure 5
IC 50 of the Morus indica extracts in inhibition of lipid peroxidation in microsomes



meoh- methanol; 80%- methanol-8: water-2; dechl. - dechlorophyllised; aq.c- aqueous cold; aq.h- aqueous hot; ; Bars with different alphabets differ significantly (P>0.05)

DISCUSSION

Many plant-derived substances, collectively termed "phytonutrients," or "phytochemicals," are becoming increasingly known for their antioxidant activity[31]. In the present study all the extracts of MI varieties (M5,V1 and S36) have shown some quantity of phenolics which are well known for their antioxidant activity. Phenolic compounds are one of the largest groups of metabolites and there are a great interest in their antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic activity[32]. Several reports indicate the antioxidant potential of medicinal plants may be related to the concentration of their phenolic compounds which include phenolic acids, flavonoids, anthocyanins and tannins [33] Results presented here clearly indicate that the extracts from MI varieties with some percentage of polyphenols

exhibited antioxidant activity in different *in vitro* models.In the process of oxidation, free radicals are considered to play a cardinal role in numerous chronic pathologies. In the present study the MI varieties have shown excellent percent of radical scavenging in a dose dependent manner. The RSA of the MI varieties werecomparable with some medicinal plants viz, *Raphanus sativus*[34], *Aegle marmelos, Psidium guajava*[5] and *Moringa oleifera* [35] explored from our laboratory. In the present study the dechlorophyllised extract with high polyphenol content showed significantly high radical scavenging activity, similar correlation was reported between polyphenols and RSA in case of *Moringa oleifera*[35], *Aegle marmelos and Psidium guajava*[5]. Binding of metal ions, such as iron, *in vivo* is an antioxidant action of itself, preventing metal ion catalyzed generation of reactive species[36]. In reducing assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe2+concentration.The ability of MI varieties to reduce iron (III) to iron (II) is inferior than the standard (ascorbic acid- 0.973) and superior to *Raphanus sativus*[34], *Aegle marmelo*[37] and *Moringa oleifera*[35]. The reducing power of a sample might be due to their hydrogen-donating ability[38]. Accordingly, MI varieties might contain higher amounts of reductone, which could react with free radicals to stabilize and block radical chain reactions. Phytochemicals such as Flavonoids, vitamin C, glutathione directly interacts with radicals like O_2^{-}, $OH^{\cdot}$ and scavenge many free radicals like O^{-}_2, $OH^{\cdot}$ and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing pollutants like ozone, NO_2 and free radicals in cigarette smoke in respiratory tract. Lipid peroxidation is a process in which PUFA undergo oxidative damage resulting in lipid derived radicals such as alkoxy and peroxyl radicals. In biological systems, antioxidants are capable of stabilizing or deactivating free radicals before they attack cells[39]. Vitamin E scavenges peroxyl radicals intermediates in lipid peroxidation and is responsible to protect PUFA present in cell membrane and LDL, against lipid peroxidation [39]. The polarity of phytochemicals play a key role in exhibiting antioxidant role at lipid phase especially at unsaturation site, which influence the chain breaking reaction **.** Similarly in dechlorophyllised extracts of the MI varieties the antioxidants with high partition coefficient may be distributed hydrophobic compartments for the protection of lipids. In the present study, use of two lipid systems was helpful in studying the inhibition potency of different extracts in food and biological level.

CONCLUSION

Results of the present study reveal that the *Morus indica* varieties , M5, V1 and S36 are potential source of phytochemicals which are responsible for the antioxidant activity. The different extracts of MI varieties with strong antioxidant activity indicates its scope for utilization in food and biological systems. In addition to being consumed as healthy antioxidants, the compounds present in MI that are responsible for antioxidant activity could be isolated and then used as food additives to delay the oxidative deterioration of foods and also as nutraceutical or functional ingredient in food formulations against degenerative diseases.

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