COMPARATIVE STUDY OF TOTAL PHENOLIC CONTENT, ANTI-OXIDANT ACTIVITY AND FREE RADICAL SCAVENGING POTENTIAL OF TWO DIFFERENT SOLVENT EXTRACTS OF *MOMORDICA DIOICA* ROXB. EX WILD. & *MOMORDICA CHARANTIA* L. (CUCURBITACEAE)

ABSTRACT

The fruits of *Momordica dioica* Roxb. ex Wild. & *Momordica charantia* L. (Cucurbitaceae) are used as vegetable in India. The fruits are used in the management of diabetes and other diseases. Since these effects may be correlated with the presence of anti-oxidant compounds, methanol and 70% acetone extracts of these plants were evaluated and compared for their total phenolic content, anti-oxidant activity and radical scavenging activity. Total phenolic content of methanol extract of both fruits was found to be relatively higher than acetone extract. The anti-oxidant potential of the extracts were assessed by employing different *in vitro* assays such as reducing power assay, DPPH, hydrogen peroxide scavenging assay, OH radical scavenging capacities, peroxidation inhibiting activity through linoleic acid emulsion system and anti-hemolytic assay. Though all the extracts exhibited dose dependent reducing power activity, acetone extracts of the samples were found to have more hydrogen donating ability.

Keywords: *Momordica dioica*, *Momordica charantia*, Cucurbitaceae, Anti-oxidant activity, Polyphenols, Free radicals, Bitter melon.

INTRODUCTION

Momordica charantia L. (Cucurbitaceae) is a perennial, monoecious climber found throughout India. This plant is often cultivated for its fruits which are used as vegetable[1]. It is traditionally known for its medicinal properties such as anti-diabetic, anti-cancer, anti-inflammatory, anti-virus, cholesterol lowering effect, hepatoprotective, anti-fertililty, anti-bacterial, anti-hypertensive and anti-oxidant[2-6]. Among the secondary metabolites of *M. charantia*, the cucurbitane type triterpenoids are one of the major bioactive constituents[7]. Phytochemical investigation of fruits of *M. charantia* L. have revealed the presence of ascorbic acid, alkaloids, triterpenoids, saponins, tannins, flavonoids and fixed oil[1]. *Momordica dioica* Roxb. Ex Wild (Cucurbitaceae) is a perennial, dioecious climber found throughout India. It is often cultivated for its fruits which are also used as vegetable[1]. *M. dioica* Roxb. Ex Wild is claimed to be anti-inflammatory, hepatoprotective, anti-microbial, anti-fungal, anti-diabetic[8,9]. Phytochemical investigation of fruits have revealed the presence of the traces of alkaloids, lectin, B-sitosterol, saponin glycosides, triterpenoids, long chain aliphatic hydrocarbons, tannins and fixed oil [10-12].

Oxidative stress is an important contributor to the pathophysiology of a variety of pathological conditions such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases[13]. Human body has multiple mechanisms especially enzymatic and non enzymatic antioxidant systems which protect the cellular molecules against reactive oxygen species (ROS) induced damage[14]. However the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous anti-oxidants are constantly required to maintain an adequate level of anti-oxidants in order to balance the ROS in human body. The objective of present work was to verify & compare the anti-oxidant activity and free radical scavenging potential of two different solvent extracts of *M. dioica* Roxb. ex Wild and *M. charantia* L. by employing various *in vitro* models.

MATERIALS AND METHODS

Chemicals

Potassium ferricyanide, ferric chloride, 2, 2-diphenyl-1-picryl-hydrazyl, potassium persulfate,

linoleic acid, ferrous chloride, ammonium thiocyanate, hydrogen peroxide, ferrous ammonium sulfate, ethylenediamine tetracetic acid (EDTA) disodium salt, trichloroacetic acid (TCA), ammonium acetate, glacial acetic acid, acetyl acetone, gallic acid, butylated hydroxyl toluene (BHT) and ascorbic acid were obtained from Merck or Sigma India. All other reagents used were of analytical grade.

Plant Material

The fresh green fruits of *M. charantia* and *M. dioica* were collected during the month of August 2010 from local market of Mumbai, Maharashatra. Freshly collected fruits were cut, dried in tray dryer at 55° for 24 h. The dried samples were powdered and used for solvent extraction.

Solvent Extraction

The air dried powdered plant samples were defatted by petroleum ether. After defatting, dried powder was extracted by Soxhlet apparatus successively with methanol followed by 70% acetone. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°. The extracts were concentrated by rotary vacuum evaporator and then dried. The dry extracts obtained with each solvent were weighed. The percentage yield was expressed in terms of air dried weight of plant material. The extracts thus obtained were used directly for the estimation of total phenolic content and also for the assessment of anti-oxidant potential and free radical scavenging potential through various *in vitro* methods.

Determination of Total Phenolic Content

The total phenolic content was determined according to the reported method[15]. 100 mcL (2 mg/2 mL) of extract solution was mixed with 5.8 mL distilled water and 500 mcL of Folin–Ciocalteu reagent, followed by addition of 1500 mcL of Na_2CO_3 solution (20%) after 1 min. Subsequently, the mixture was incubated at 40° for 30 min and its absorbance was measured at 760 nm. Gallic acid was used as a standard for calibration curve. The total phenolic content was expressed as gallic acid equivalents using the linear equation method.

Determination of Anti-oxidant Activities

- **Reducing Power**

To determine the reducing power of the methanolic and 70% acetone extracts of fruits of *M. charantia* and *M. dioica*, 25-100 mcg/mL of extracts in 1 mL of phosphate buffer with 5 mL of 0.2 M phosphate buffer (pH 6.6) and 5 mL of 1% potassium ferricyanide solution were incubated at 50° for 20 min. After the incubation, 5 mL of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 mL) was mixed with 5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Then the absorbance of reaction mixture was noted spectroscopically at 700 nm[16].

- **Free Radical Scavenging Activity on DPPH**

The anti-oxidant activity of the methanolic and 70% acetone extracts of fruits of *M. charantia* & *M. dioica* was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH[17]. A methanolic and 70% acetone extracts of sample at various concentrations (10–60 mcg/mL) were added to 5 mL of a 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27°. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using following formula[17].

% DPPH radical scavenging activity = (control OD - sample OD/control OD) x 100

- **Anti-oxidant Activity in Linoleic Acid Emulsion System**

The anti-oxidant activity of methanolic and 70% acetone extracts of fruits of *M. charantia* and *M. dioica* was determined using the thiocyanide method[18]. Each sample (500 mcg) in 0.5 mL of absolute ethanol was mixed with 0.5 mL of 2.51% linoleic acid in absolute ethanol, 1 mL of 0.05 M phosphate buffer (pH 7.0), and 0.5 mL of distilled water and placed in a screw capped tube. The reaction mixture was incubated in dark at 40° in an oven. Aliquots of 0.1 mL were taken every 12 h during incubation and the degree

of oxidation was measured by sequentially adding ethanol (9.7 mL, 75%), ammonium thiocyanate (0.1 mL, 30%) and ferrous chloride (0.1 mL, 0.02 M in 3.5% HCl). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. The anti-oxidant activity was calculated as percentage of inhibition relative to the control.

AA = 100 - (sample absorbance at 48 h - sample absorbance at 0 h / control absorbance at 48 h - control absorbance at 0 h) x 100

- **Hydrogen Peroxide Scavenging Activity**

To determine the hydrogen peroxide scavenging activity of the methanolic and 70% acetone extracts of fruits of *M. charantia* and *M. dioica*, 1.4 mL of each extract at various concentrations (100 - 700 mcg/mL) in distilled water was added to 0.6 mL of the hydrogen peroxide solution (40 mM in phosphate buffer pH 7.4). The absorbance of mixture was noted at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide solution. Percentage of hydrogen peroxide scavenging by the extracts and ascorbic acid (positive control) was calculated by following formula[19].

% scavenged of hydrogen peroxide = $(A_o - A_1)/A_o \times 100$

where A_o is the absorbance of the control and A_1 the absorbance of the mixture containing either the extract or standard.

Anti-hemolytic Activity

Preparation of Erythrocyte Suspension

The erythrocytes from cow's blood were separated by centrifugation at 3000 rpm for 20 min. The final erythrocyte suspension (4%) was produced by adding the sufficient volume of saline phosphate buffer (pH 7.4) after washing the cells thrice with saline phosphate buffer.

Hemolysis Assay

In pretreatment stage, 1 mL (10 mg/mL) of each extract was added to 500 mcL of erythrocyte suspension and incubated at 37° for 40 min. A positive control for this experiment was prepared by pre-treating the erythrocyte suspension with 1 mL of 10 mg/mL solution of ascorbic acid (positive control) in saline phosphate buffer. The non-pretreated erythrocyte suspension was used as the negative control. The volume of all pretreated and non-pretreated erythrocyte suspensions was adjusted to 9 mL by adding saline phosphate buffer. Oxidative stress was then induced by adding 1 mL of 10 mM hydrogen peroxide and incubated at 37° for 150 min. After incubation, the released hemoglobin into the supernatant of the mixtures was estimated by noting the absorbance at 540 nm. Erythrocyte hemolysis in pure water was considered as 100%, while that of the pretreated and non-pretreated erythrocytes was expressed as a percentage of this value[20].

- **Hydroxyl Radical Scavenging Activity**

Various concentrations (100, 150, 200, 250, 300 & 400 mcg) of each extracts were added to 1.0 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90° for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0 mL of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated by the following formula[21].

% HRSA = 1 - (difference in absorbance of sample - difference in absorbance of blank) x 100

RESULTS AND DISCUSSION

Total Phenolic Content of Extracts

The percentage yield and total phenolic content of extracts obtained from fruits of *M. dioica* & *M. charantia* using methanol and 70% acetone solvents are shown in Table I. The maximum percentage yield and extractable total phenolic content were found in methanolic extract of both plants. Since the 70% acetone was a subsequent solvent to methanol, percentage yield of acetone extract of all the samples were much lower. Total phenolic content in *M. charantia* might be contributed by the presence of gallic acid[22].

Reducing Power Assay

The results of anti-oxidant potential of the methanolic and 70% acetone extracts of fruits of *M. charantia* and *M. dioica*, estimated using potassium ferric cyanide reduction method are shown in Fig. 1. The yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each extract. The presence of anti-oxidants in the herbal extracts causes the reduction of Fe^{3+}/ferric cyanide complex to ferrous form. Therefore the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[23]. Methanolic and 70% acetone extracts of *M. dioica* showed the higher reducing power and the values were comparable to that of tannic acid (positive control). In *M. charantia*, both methanolic and 70% acetone extracts exhibited lower reducing power activity as compared to tannic acid (positive control). Both methanolic and 70% acetone extract of *M. dioica* showed higher reducing power at all the concentrations than that of *M. charantia*. Reducing power of all extracts increased with increase in concentration. Hence the reducing power of these plant extracts was dose dependent. Phenolic contents of all the extracts appears to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products.

DPPH Radical Scavenging Activity

The results of free radical scavenging activity of the methanolic and 70% acetone extracts of fruits of *M. dioica* and *M. charantia* are shown in Fig. 2. The decrease in absorbance of the DPPH radical was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a colour change from purple to yellow. A lower value of IC_{50} indicates a higher anti-oxidant activity. DPPH radical scavenging activity of each extracts is directly proportional to the concentration of total phenolics including tannins of respective extracts. Similar to reducing power, percentage DPPH radical scavenging activities of all the extracts are dose dependent. In *M. dioica*, the higher DPPH scavenging activity is shown by acetone extract (IC_{50} value is 40 mcg/mL). IC_{50} values for MEMD, MEMC, AEMD, AEMC are 55 mcg/mL, 55 mcg/mL, 40 mcg/mL and 60 mcg/mL respectively. In the present study, the order of DPPH radical scavenging activity of sample extracts is AEMD > MEMD = MEMC > AEMC. This radical scavenging activity of extracts could be related to the nature of phenolics.

Anti-oxidant Activity in Linoleic Acid Emulsion System

Peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good oxidising agents having more than 1000 mV of standard reduction potential[24]. They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation[25]. As lipid oxidation of cell membranes increases, the polarity of lipid phase surface charge and formation of protein oligomers increase; and molecular mobility of lipids, number of SH groups, and resistance to thermal denaturation decreases. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural

Table I: Percentage yield and total phenolic content of methanolic and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Sample	% yield of extract	Total phenolic content (GAE/g)
MEMC	33.5	4.42 ± 0.11[a]
AEMC	22.1	3.36 ± 0.24
MEMD	35.1	4.12 ± 0.14
AEMD	15.3	3.22 ± 0.23

[a]*Values are mean ± standard deviation (n = 3)*

MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia, MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica

Table II: IC_{50} value of hydrogen peroxide scavenging assay for methanolic and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Sample	IC_{50} value in mcg/mL
MEMD	300
AEMD	500
MEMC	600
AEMC	700
Ascorbic acid (Positive control)	23

MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia. Ascorbic acid (positive control)

Table III: Percentages of hydroxyl radical scavenged for methanolic and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Sample	100 mcg	200 mcg	300 mcg	400 mcg
	Hydroxyl radical scavenging activity (%)			
MEMD	14.72 ± 0.77[a]	37.50 ± 0.67[a]	43.41 ± 0.60[a]	64.56 ± 0.44[a]
AEMD	11.54 ± 2.11	30.63 ± 0.62	36.00 ± 0.99	44.77 ± 0.66
MEMC	26.29 ± 1.01	35.09 ± 1.71	47.42 ± 0.42	60.08 ± 0.96
AEMC	10.54 ± 0.75	26.88 ± 1.00	39.60 ± 1.12	50.82 ± 1.07

[a]*Values are means of triplicate determinations (n = 3) ± standard deviation*

MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia

modification, which induce dysfunction of immune systems. The anti-oxidant effect of each extracts and BHT (positive control) on the peroxidation of linoleic acid was investigated and the results are shown in Fig. 3. At a concentration 250 mcg/mL in the final reaction mixture, both the extracts of *M. dioica* and *M. charantia* inhibited 62.05–71.54% peroxidation of linoleic acid after incubation for 48 h. However, those values were significantly lower than those of the BHT (positive controls) (97%). In summary, the result shows that the inhibitory potential decreases in the following order: BHT (positive controls) > AEMD > AEMC > MEMD > MEMC.

Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activity of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanins,

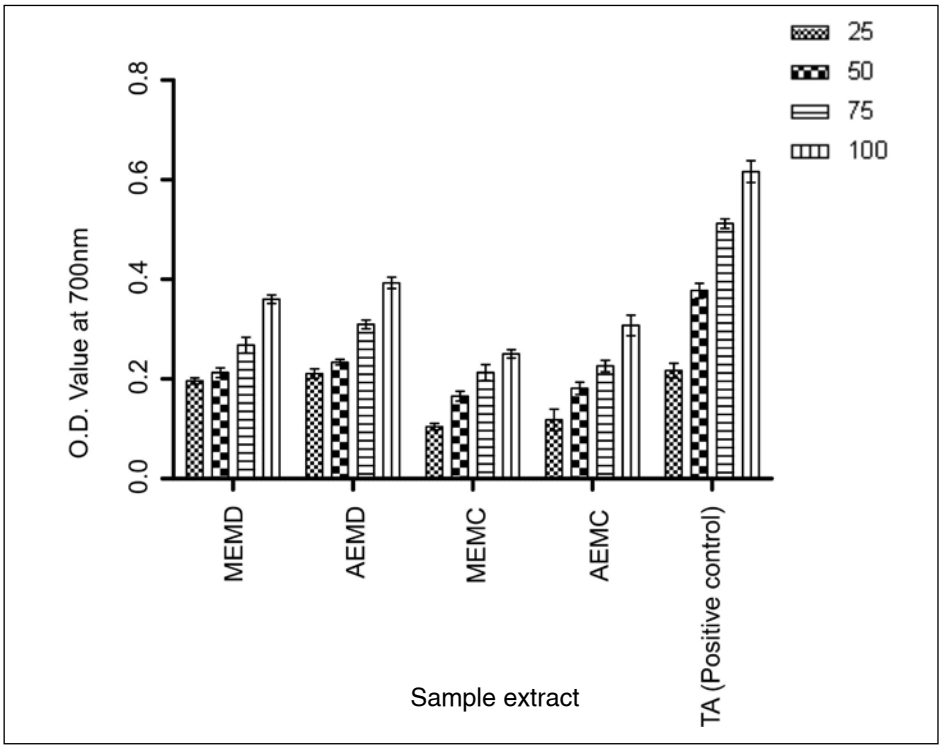

Fig.1: Reducing power of methanol and 70% acetone extracts of fruits of *M. charantia* & *M. dioica*

Values are means of triplicate determinations (n = 3) ± standard deviation, MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia, TA (positive control) - tannic acid

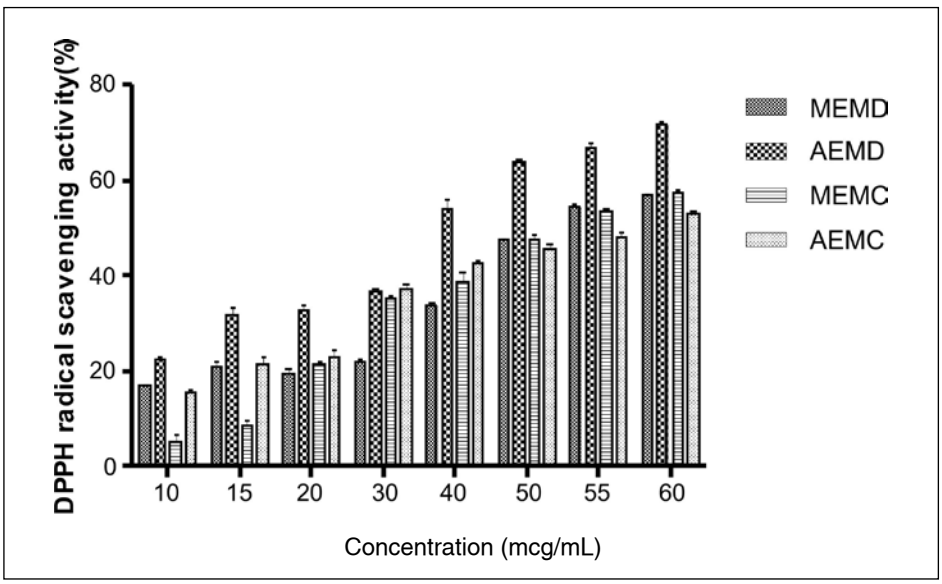

Fig. 2: DPPH radical scavenging activity of methanol and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Values are means of triplicate determinations (n = 3) ± standard deviation, MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia

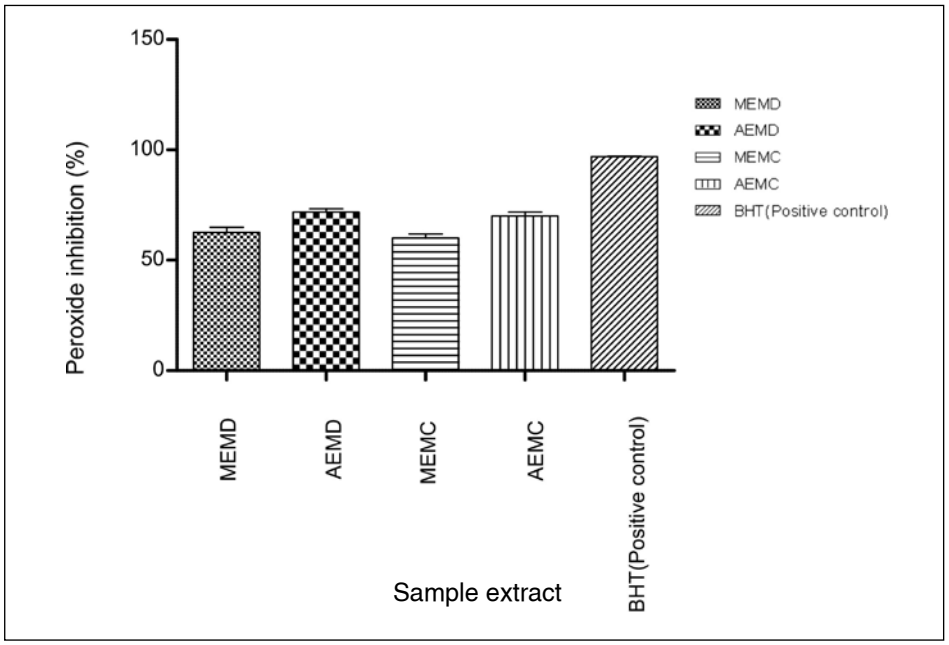

Fig. 3: Anti-oxidant activity through linoleic acid emulsion system for methanol and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Values are means of triplicate determinations (n = 3) ± standard deviation, MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia, BHT (positive control)-butylated hydroxyl toluene

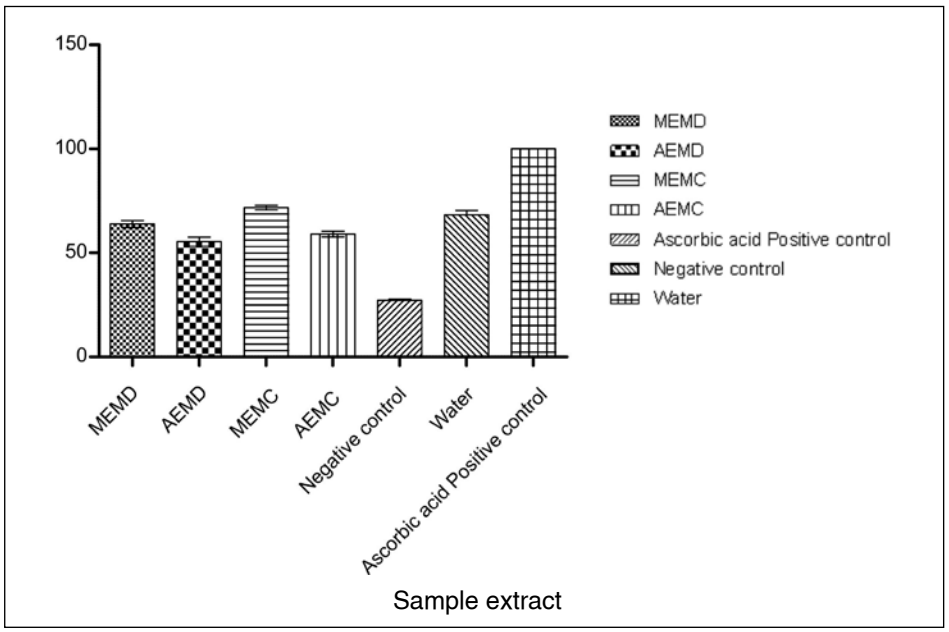

Fig. 4: Hemolytic assay of methanol and 70% acetone extracts of fruits of *M. dioica* & *M. charantia*

Values are means of triplicate determinations (n = 3) ± standard deviation, MEMD - methanol extract of M. dioica, AEMD - 70% acetone extract of M. dioica, MEMC - methanol extract of M. charantia, AEMC - 70% acetone extract of M. charantia. Ascorbic acid (positive control)

tannins and flavonoids which can donate electrons to hydrogen peroxide, thus neutralizing it to water[26]. The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner but showed weaker activity than ascorbic acid (positive control). IC_{50} values for all extracts and control are given in Table II. In present study, hydrogen peroxide scavenging activity of extracts follows the order: Ascorbic acid (positive control) > MEMD > AEMD > MEMC > AEMC.

Anti-hemolytic Assay

The maximum erythrocyte hemolysis (100%) was achieved using pure water and other hemolytic values from free radical damage were expressed as the percentage based on the former. The results of anti-hemolytic assay for methanolic and 70% acetone extracts of fruits of *M. charantia* & *M. dioica* are shown in Fig. 4. A mean of 27.22% hemolysis of erythrocytes was obtained with ascorbic acid (positive control). Percentage of hemolysis in samples pre-treated with MEMD, AEMD, MEMC & AEMC were found to be 64.83%, 55.97%, 70.59%, 57.79% respectively. There was no significant reduction in percent hemolysis by MEMD & MEMC than positive which can be attributed to saponin content of these plants[27]. Saponins are secondary metabolites present in plants and are known to possess hemolytic activity. Since the plant materials were extracted successively, the saponins were preferentially present in the methanolic extract. However, AEMD and AEMC significantly reduced the percentage of hemolysis when compared to the positive control.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[28]. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. The values of hydroxyl radical scavenging (%) for methanol and 70% acetone extracts of fruits of *M. dioica* and *M. charantia* are shown in Table III. In the present investigation, all the extracts exhibited between 11.54% and 64.56% hydroxyl radical scavenging activity up to 400 mcg/mL concentration in the reaction mixture. The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of chain reaction.

The present investigation suggests that the phenolic constituents from fruits of *M. dioica* and *M. charantia* possess potential anti-oxidant activity. Further isolation and preparation of bioactive compounds from the above mentioned samples and their impact on various health improvements/control of free radical mediated diseases through *in vivo* studies are needed.

ACKNOWLEDGEMENT

University Grant Commission, New Delhi, for financial support.

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(Received 23 May 2012) (Accepted 12 July 2012)