IN VITRO ANTIOXIDANT ACTIVITY OF SELECTED HERBAL EXTRACTS OF INDIAN TRADITIONAL MEDICINE

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ABSTRACT

The antioxidant activity of the aqueous extracts of selected plants Phyllanthus amarus, Hydrocotyl asiatica, Terminalia chebula, Tephrosia purpurea , Ricinus communis, Solanum nigrum , Cinchorium intybus, Vitex negundo, Aloe vera, Citrullus colocynthis, Momordica Charantia (fruit) and Momordica Charantia (leaf) that are used widely in several herbal formulations were investigated in comparison with a known antioxidant ascorbic acid in *in vitro* studies. The superoxide radical scavenging activity was determined by riboflavin photoreduction method, hydroxyl radical scavenging activity by deoxyribose method, and lipid peroxidation inhibiting activity by thiobarbituric acid method. The extracts showed good antioxidant activities when compared to standard ascorbic acid. The presence of polyphenolic constituents might be responsible for the antioxidant activity and the claimed hepatoprotective and antidiabetic activities. The above plants used in Indian traditional health care system (Ayurveda) have shown good antioxidant activity to indicate their beneficial role in disorders associated with free radical damage.

Key words: Antioxidant activity, aqueous extracts, free radicals, ascorbic acid

1. INTRODUCTION

The role of free radicals has been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, aging, arthritis, diabetes etc. and the compounds that can scavenge free radicals have great potential in ameliorating these disease processes. Antioxidants play an important role to protect the human body against damage by reactive oxygen species. Human body has endogenous mechanisms such as superoxide dismutase, glutathione peroxide, catalase and vitamin E (α-tocopherol) to reduce free radical induced injury. Sometimes these protective mechanisms are found to be insufficient compared to the insult produced to the body and exogenous antioxidant like vitamin C is administered and hence the search for exogenous antioxidants is continued. Much attention has been focused on antioxidant compounds present in edible plants, because of safety concerns associated with synthetic antioxidants. In Asia a number of plants are used in the treatment of a number of diseases like liver cirrhosis, diabetes, arthritis and cancer etc even though there is lack of sufficient scientific proof. Many plant extracts and plant products have been known to have significant antioxidant activity.

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2. METHODOLOGY

Materials

The selected aqueous extracts of *Phyllanthus amarus* (PA), *Hydrocotyl asiatica* (HA), *Terminalia chebula* (TC), *Tephrosia purpurea* (TP), *Ricinus communis* (RC), *Solanum nigrum* (SN), *Cinchorium intybus* (CI), *Vitex negundo* (VN), *Aloe vera* (AV), *Citrullus colocynthis* (CC), *Momordica charantia* (fruit) (MCF) and *Momordica charantia* (leaf) (MCL) were supplied by M/S Laila Impex (a leading herbal drug export company), Vijayawada, A.P, India. Nitroblue tetrazolium (NBT) was purchased from SISCO Research laboratories Pvt Ltd. Mumbai, 2-deoxy-D- ribose was purchased from Sigma Chemical Company, USA. All other chemicals and reagents used were of analytical quality.

Methods

Determination of superoxide scavenging activity

Riboflavin photoreduction method: Superoxide scavenging activity of the extract was determined by McCord and Fridovich method (McCord and Fridovich 1969) which depends on light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The assay mixture contained 0.3 ml of different concentrations of the extract and 0.2 ml ethylene diamine

tetra acetic acid (6µM containing 3 µg NaCN), 0.1ml NBT (50 µM), 0.05 ml riboflavin (2µM) and 2.35 ml phosphate buffer (58 mM, pH 7.8) to give a total volume of 3 ml. The tubes were uniformly illuminated with an incandescent light for 15 minutes and the optical density was measured at 560 nm. The percentage inhibition by the extract of superoxide production was evaluated by comparing the absorbance values of control and experimental tubes.

Determination of hydroxyl radical scavenging activity

Deoxyribose method: Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radical generated from the Fe^{3+}/ascorbate/ EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose and eventually results in formation of thiobarbituric acid reacting substances (TBARS) (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), H_2O_2 (1mM), ascorbate (0.1 mM), phosphate buffer (20 mM, P^H 7.4) and 0.2 ml different concentrations of the extract in a final volume of 1 ml was incubated for I hour at 37^{0}C. Deoxyribose degradation was measured as TBARS by the method of Ohkawa (Ohkawa et. al 1974) and percentage of inhibition was calculated from the control where no test compound was added.

Determination of lipid peroxidation inhibiting activity

Induction of lipid peroxidation by Fe2+/ Ascorbate system: Inhibition of lipid peroxidation was determined by the thiobarbituric acid method. 0.1 ml of different concentrations of the extract was incubated at 37 ^{O}C with 25% (W/V) rat liver tissue homogenate (0.1 ml) containing Tris-HCl buffer (40 mM, pH 7.0), 0.1 ml KCl (30 mM), 0.1 ml ascorbic acid (0.06 mM) and 0.1 ml ferrous iron (0.16 mM) in a total volume of 0.5 ml for 1 hour. At the end of the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml sodium dodecyl sulphate (8.1%), 1.5 ml thiobarbituric acid (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was then made up to 4 ml by adding distilled water and kept in oil bath at 95 ^{o}C for 1 hour. After the mixture had been cooled, 1 ml distilled water and 5 ml of butanol- pyridine mixture (15: 1 V/V) were added. Following vigorous shaking, the tubes were centrifuged and absorbance of the upper layer containing the chromophore was read at 532 nm. The percentage inhibition of lipid peroxidation by the extract was determined by comparing the absorbance values of the control and experimental tubes (Ohkawa et. al 1974)

Data and Statistical analysis: Data were expressed as mean ± standard error of mean (SEM). The significance was determined by applying student's paired 't' test.

3. RESULTS

Superoxide scavenging activity

The herbal extracts PA, HA, TC, TP, RC, SN, CI, VN, AV, CC, MCF, MCL and AA were found to scavange the superoxides generated by photoreduction of riboflavin. The extracts and ascorbic acid at various concentrations (12.5 – 400 µg) scavenged the superoxide radicals in a dose dependent manner (Table 1). The amount of aqueous extracts of PA, HA, TC, TP, RC, SN, CI, VN, AV, CC, MCF, MCL and AA needed for 50% scavenging of superoxide radicals was found to be 82.50 µg , 299.50 µg, 61.50 µg, 255.10 µg, 50.10 µg, 326.05 µg, 120.45 µg, 61.50 µg, 70.10 µg , 276.15 µg, 374.50 µg, 325.10 µg and 79.95 µg respectively (Fig 1). The superoxide scavenging activity of the extracts/AA is in the following order. RC > TC & VN > AV > AA > PA > CI > CC > TP > HA >MCL > SN > MCF (Table 4).

Hydroxyl radical scavenging activity

The herbal extracts PA, HA, TC, TP, RC, SN, CI, VN, AV, CC, MCF, MCL and AA at different concentrations (25 - 500µg) scavenged the hydroxyl radicals in a dose dependent manner (Table 2). The quantity of herbal extracts PA, HA, TC, TP, RC, SN, CI, VN, AV, CC, MCF, MCL and AA needed for 50% inhibition of hydroxyl radicals was found to be 208.98 µg, 351.57µg, 203.19 µg, 226.72 µg, 201.53 µg, 349.07 µg, 172.29 µg, 153.79 µg, 195.35 µg, 401.45 µg, 288.08 µg, 362.56 µg and 184.96µg respectively (Fig 2). The hydroxyl radical scavenging activity of the extracts/AA is in the following order. VN > CI >AA >AV > RC> TC >PA > TP > MCF > SN > HA >MCL > CC (Table 4).

Inhibition of lipid peroxidation

The extracts PA, HA, TC, TP, RC, SN, CI, VN, AV, CC, MCF, MCL and AA at different concentrations (12.5 – 500 µg) inhibited the lipid

peroxidation in a dose dependent manner (Table 3). The amount needed for 50% inhibition of lipid peroxide was found to be 359.18 µg, 158.72 µg, 361.93 µg, 216.58 µg, 329.67 µg, 267.22 µg, 422.80 µg, 410.10 µg, 267.22 µg, 268.00 µg, 224.97µg and 191.63 µg respectively. (Fig 3). The lipid peroxidation inhibition activity of the extracts/AA is in the following order. HA >AA > TP >MCL >SN & CC > MCF > RC>PA > TC > VN>AV > CI (Table 4).

4. DISCUSSION & CONCLUSIONS

Several phytochemicals possessing polyphenolic structures are advocated as neutraceuticals to supplement food for better health care during recent years. Most of them are claimed to possess antioxidant activity. Ayurveda and naturopathy the medical systems indigenous to India advocate the use of plant extracts / mixtures of extracts for treating various disorders apart from others from times immemorial in humans without preclinical evidence, which is required to make the systems popular and scientific. The claimed usefulness of herbs in several disorders might be due to their antioxidant activity. To support the use of the selected plant extracts in herbal mixture and in Ayurveda and naturopathy, the antioxidant potential of the aqueous extracts of *Phyllanthus amars* (PA), *Hydrocotyl asiatica* (HA), *Terminalia chebula* (TC), *Tephrosia purpurea* (TP), *Ricinus communis* (RC), *Solanum nigrum* (SN), *Cinchorium intybus* (CI), *Vitex negundo* (VN), *Aloe vera* (AV), *Citrullus colocynthis* (CC), Momordica Charantia (fruit) (MCF) and Momordica Charantia (leaf) (MCL) of Indian origin were investigated in comparison with the known antioxidant ascorbic acid (AA) following *in vitro* studies. The antioxidant activity of ascorbic acid was well established (Kusuma Devi V and Rehman F, 2002). The quantities of the extracts needed for the *in vitro* inhibition of the oxygen radicals such as superoxide, and hydroxyl radicals, the extracts of PA, TC, RC, CI, VN and AV were relatively similar to the known antioxidant ascorbic acid. The quantities of the extracts needed for the *in vitro* inhibition of the lipid peroxidation, the extracts of HA, TP, SN, CC, MCF and MCL were relatively similar to the known antioxidant ascorbic acid. Herbal drugs containing antiradical constituents are gaining importance in prevention and treatment of stress related disorders. The free radical scavengers like polyphenolics are well known for their therapeutic activity in disorders such as cancer, diabetes and skin diseases (Jose JK and Kuttan R 1995).

Earlier reports on the above plants indicated the presence of compounds representing polyphenols, flavonoids, terpenoids, tannins, alkaloids etc (Rasstogi *et al* 1991, Masateru *et al* 2004 and Monika B *et al*, 2005). The terpenoids and flavonoids having glycosidic linkage were likely to be extracted into aqueous extracts. Earlier studies in our laboratory on aqueous extracts of umbelliferous fruits showed potent antioxidant activity (Satyanarayana S et al., 2004). The terpenoids were reported to protect lipids, blood and body fluids against the attack of free radicals, some types of reactive oxygen, hydroxylic groups, peroxides and superoxide radicals (Kawamori T et al., 1996). In experimental studies, terpenoids have prevented the occurrence of cancer in many tissues including lung, breast, colon, stomach, prostate, pancreas, liver and skin (So FV et al., 1996; Reddy BS et al., 1997). The presence of these structures might be responsible for the comparable antioxidant activity with that of known antioxidant ascorbic acid. It was found that potential of the extracts to scavenge oxygen radicals depends on the type of radicals encountered (Sabu MC et al., 2002). The presence of the above compounds in various extracts might be responsible for the observed antioxidant activity since polyphenolic compounds like gallic acid, (+) catechin, (-) epicatechin are known to increase antioxidant activity (Takuro K et al., 1999). Since reactive oxygen species are involved in stress and stress related disorders, the extracts of these plants may be beneficial in preventing the initiation or progression of such disorders. Liver disorders like cirrhosis, jaundice etc being stress related disorders, the presence of above mentioned chemical constituents might prevent initiation and progression of such disorders. From the literature it is evident that the above plants are used / claimed to be used for the treatment of stress related disorders like liver cirrhosis, diabetes, cancer, arthritis etc (Astrid B., 2002, Jun HW *et al*., 2006, Ben WC *et al.,* 2007) in humans. Hence, the claimed pharmacological activities of the above plants might be partly due to their antioxidant activity.

Table-1: Percentage inhibition of superoxide radical by Extracts / Ascorbic acid in *in vitro* studies (N=5)

Extract / AA	Quantity(µg) (Mean ± SEM)					
	12.5	25	50	100	200	400
P.amaras	6.12±0.82***	12.12±1.45**	26.70±1.17***	55.74±2.19	73.68±2.30	78.02±4.14
H.asiatica	3.18±0.81***	5.96±0.63***	9.76±1.36***	21.32±2.45***	40.92±2.57***	2.88±2.18**
T.chebula	11.26±0.66*	21.54±1.52	45.64±2.00	74.52±2.41	83.48±3.54	-
T.purpurea	5.48±0.81***	8.60±0.29***	11.92±0.78***	33.52±3.33***	52.24±3.66**	3.88±3.59**
R.communis	11.24±0.82*	22.96±1.82	50.54±2.32	66.50±2.47	71.68±3.20	5.30±1.62*
S.nigrum	3.02±0.17***	5.60±0.58***	10.46±1.21***	26.90±1.48***	50.56±3.17**	1.40±1.07**
C.intybus	5.82±2.25***	19.26±1.46	36.74±1.58*	43.06±2.21	68.50±1.26	74.38±2.11*
V.negundo	11.20±0.76*	22.34±2.90	47.86±2.77	62.72±2.93	74.32±2.42	81.88±2.78
A.vera	11.68±0.91*	20.96±2.16	44.40±2.85	59.14±3.57	79.22±3.74	82.10±3.46
C.colocynthis	7.02±0.74**	19.82±1.67	22.32±2.51***	38.90±2.11***	50.18±2.37**	56.62±1.34***
M.charantia(F)	4.54±0.69***	11.84±1.48***	17.82±1.81***	22.04±2.74***	33.42±2.27***	50.86±3.65***
M.charantia(L)	3.28±0.48***	8.98±0.76***	17.92±1.08***	30.98±1.18***	37.50±2.46***	57.98±1.54***
Ascorbic acid	15.80±1.85	21.10±1.15	42.36±1.99	56.34±1.32	69.36±1.40	79.36±1.40

*** Significant at P< 0.001; ** Significant at P< 0.01; * Significant at P< 0.05 compared to ascorbic acid control

Table-2: Percentage inhibition of hydroxyl radical by Extracts / Ascorbic acid in *in vitro* studies (N=5)

Extract / AA	Quantity(µg) (Mean ± SEM)						
	25	50	100	200	300	400	500
P.amaras	11.30±1.59*	25.12±3.88**	34.82±2.53***	50.52±2.90*	57.52±1.93	65.70±2.39	-
H.asiatica	10.16±1.30**	25.30±2.45**	37.82±3.77**	44.32±2.41***	47.48±1.58***	51.78±1.58***	55.58±1.89
T.chebula	7.82±0.36***	17.62±2.26***	29.58±2.44***	51.60±2.56*	62.52±2.12	68.46±2.86	-
T.purpurea	12.16±1.28*	26.50±3.38**	29.90±1.49***	39.74±2.09***	54.34±2.31**	66.32±2.81	-
R.communis	12.26±1.18	25.84±1.80**	33.12±2.27***	48.48±1.43**	58.38±2.19	61.60±1.54**	-
S.nigrum	10.62±1.34**	24.18±2.07**	31.44±2.66***	37.56±1.37***	50.26±2.71***	52.64±2.08***	56.48±1.39
C.intybus	11.82±1.86*	25.04±1.88**	45.22±1.46	58.58±1.33	62.00±2.98	68.16±1.96	-
V.negundo	14.48±0.88	32.54±2.59	51.38±2.35	53.38±2.89	64.21±2.71	69.24±1.96	-
A.vera	9.38+1.64**	16.92+1.23***	23.68+1.48***	51.16+2.00*	57.06+1.11*	62.08+1.23*	-
C.colocynthis	1.88±0.98***	3.96±0.769***	14.34±1.02***	21.94±1.68***	36.72±2.75***	51.60±1.16***	55.21±2.76
M.charantia(F)	5.66±2.34***	12.62±1.56***	26.96±2.32***	44.64±0.72***	51.12±0.98**	57.28±1.71*	-
M.charantia(L)	3.88±0.81***	6.58±1.57***	15.44±2.18***	22.64±2.29***	48.36±1.01***	55.22±2.66***	57.92±2.26
Ascorbic acid	13.00±1.04	33.48±1.25	44.86±2.24	53.08±1.25	58.66±1.32	64.64±1.59	-

*** Significant at P< 0.001; ** Significant at P< 0.01; * Significant at P< 0.05 compared to ascorbic acid control

Table-3: Percentage inhibition of lipid peroxidation by Extracts /Ascorbic acid in *in vitro* studies (N=5)

Extract/AA	Quantity(µg) (Mean ± SEM)						
	12.5	25	50	100	200	400	500
P.amaras	5.24±0.52***	9.08±1.46***	17.50±1.24***	26.64±1.20***	35.06±2.82***	51.62±2.84***	-
H.asiatica	9.501±1.64**	23.24±1.03	35.20±2.41	40.94±2.46	57.20±2.44	64.08±3.11	-
T.chebula	4.60±0.28***	9.80±1.90***	31.72±2.97	35.58±1.69*	45.52±2.06**	51.10±2.94***	58.24±2.48
T.purpurea	7.08±1.94***	22.80±1.68	29.14±1.29	44.32±2.00	53.60±2.51	66.88±1.72	-
R.communis	9.22±1.63**	22.26±1.49	28.52±1.08	38.76±2.39	42.80±2.23***	53.86±2.37**	-
S.nigrum	7.08±0.56***	26.26±1.62	37.00±2.90	43.78±3.32	56.36±2.48	66.64±1.24	-
C.intybus	-	3.82±0.89***	6.52±0.19***	9.24±2.71***	30.74±1.40***	50.56±1.68***	54.70±2.05
V.negundo	4.86±0.17***	8.80±1.48***	17.58±1.62***	21.50±1.78***	36.54±1.80***	50.76±2.15***	54.16±2.98
A.vera	3.86±0.82***	7.18±1.87***	11.32±2.96***	13.04±2.94***	32.70±2.21***	50.88±1.29***	54.72±2.21
C.colocynthis	9.74±1.41**	14.24±1.53**	23.56±2.79*	29.40±1.94***	50.18±1.50	62.80±2.24	-
M.charantia(F)	6.64±1.72***	12.92±1.72***	19.16±1.39***	31.42±2.17**	48.86±1.04**	61.88±1.76	66.38±1.52
M.charantia(L)	7.72±2.51***	18.90±1.57	24.34±1.65	38.58±1.34	55.18±3.17	70.38±1.68	-
Ascorbic acid	12.16±1.52	18.82±1.88	25.84±2.24	36.88±1.52	51.20±2.82	58.64±2.61	-

*** Significant at P< 0.001; ** Significant at P< 0.01; * Significant at P< 0.05 compared to ascorbic acid control

Table 4: IC 50 Values of selected aqueous herbal extracts/ Ascorbic acid

Extract/AA	IC 50 Quantity(µg)		
	Superoxide radical	Hydroxyl radical	Lipid peroxidation
P. amaras	82.50	208.98	359.18
H. asiatica	299.50	351.57	158.72
T. chebula	61.50	203.19	361.93
T. purpurea	255.10	226.72	216.58
R .communis	50.10	201.53	329.67
S. nigrum	326.05	349.07	267.22
C .intybus	120.45	172.29	422.80
V. negundo	61.50	153.79	410.10
A. vera	70.10	195.35	417.85
C. colocynthis	276.15	401.45	267.22
M. charantia(F)	374.50	288.08	268.00
M. charantia(L)	325.10	362.56	224.97
Ascorbic acid	79.95	184.96	191.63

IC= Inhibitory concentration

Fig.1: Quantity of the extract / Ascorbic acid required for 50 % inhibition of superoxide radicals in *in vitro* studies.

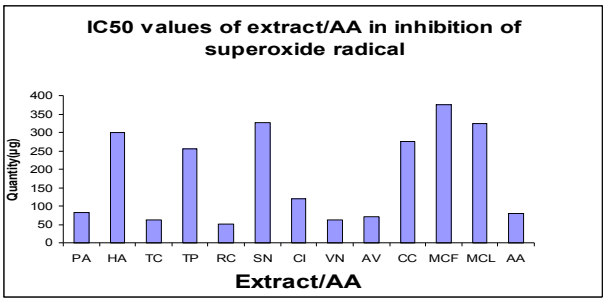


Fig.2: Quantity of the extract / Ascorbic acid required for 50 % inhibition of hydroxyl radicals in *in vitro* studies.

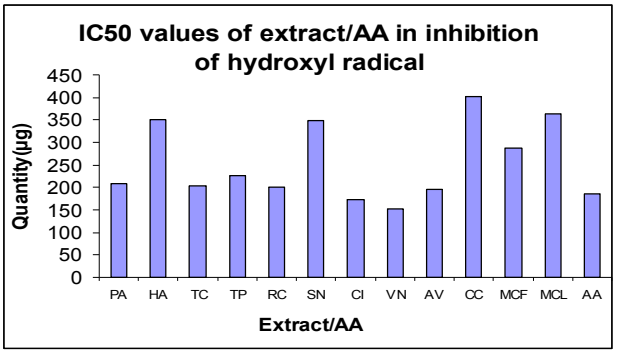
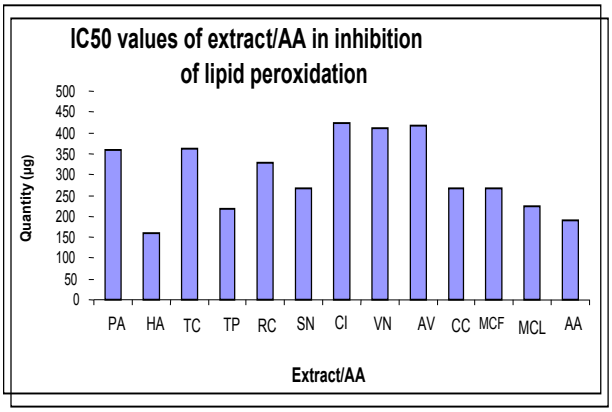


Fig.3: Quantity of the extract / Ascorbic acid required for 50 % inhibition of lipid peroxidation in *in vitro* studies.



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