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An *in vitro* study of concentration specific antioxidant activity of commonly using herbs.

Jyoti Agrawal and Anand Kar

School of Life Sciences, Devi Ahilya University, Takhshila campus, Indore- 452017 (M.P.), India
* Corresponding autho: **Jyoti Agrawal**, E-mail: *jyotiagrawal111@rediffmail.com*

Abstract

We aimed to analyze the concentration dependent *in vitro* antioxidative or pro-oxidative activity of three commonly using herbal extracts. For this, extracts of AV *(Aloe vera),* AS (*Annona aquamosa*) and PM (*Pterocarpus marsupium*) were prepared. I*n-vitro* free radicals scavenging assays such as 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Nitric oxide (NO), reducing power assay and ferrous sulphate ($FeSO_4$) induced lipid peroxidation (LPO) in rat tissue homogenates and in isolated erythrocytes were performed. Ascorbic acid was used as standard antioxidant for all chemical *in vitro* assays. Total polyphenols and flavonoid contents were also estimated. Concentration dependent protective effects were observed, these were also tissue specific for all tested herbs. In case of all assay systems very low and higher concentrations were found to be ineffective and less effective. Whereas, moderate drug concentrations used were observed to be significantly protective. However, pro-oxidative effects were also seen with PM and AV in LPO assay. All three test drugs were found to be antiperoxidative. However, higher concentration showed decreased efficacy indicated their harmful or less protective effects at higher doses. Significant amount of polyphenols and flavonoids present in the test drugs supported their antioxidative property. Of course more studies are required for confirmations.

Keywords: Antioxidants, DPPH, harmful effects, reducing power assay, lipid peroxidation.

Introduction

Diabetes mellitus (DM) has been known to be linked with increased oxidative injury (Yang et al., 2009; Szentmihalyi et al., 2010). Conversely, the excessively generated free radicals results numerous pathological complications including DM (Ganga et al., 2015). As, routinely using herbs are rich source of polyphenols, flavonoids, saponins and other health boosting secondary metabolites, so specially in India they are used as home remedy as normal diet. Special reference to diseased person these are taken without prescription of physician (Szentmihalyi et al., 2010). Although, scientifically >800 herbs have been known to possess antioxidative as well as antidiabetic activities (Buyukbalci and Nehir, 2008) and are thought to devoid of side effects. But, recently few reports on side effects of non-prescribed random eating of herbs have been come to the vision, furthermore in different clinical and pre-clinical investigations hundreds of herbs have been known for their hepatotoxic, renotoxic and cardio-toxic effects (Panda and Kar, 2000; Posadzki et al., 2013).

Though, protective efficacy of many herbs were studied but to the best of our knowledge, health effects of accidental or intentional higher drug doses were not known till now (Mohammad, 2012; Posadzki et al., 2013). Hence, there was a strong need to established scientific evidences regarding safety concern of herbs

which are casually taken by people (Yama et al., 2012). Here, we aimed to investigate the possible negative health effects of commonly using antidiabetic herbs. In preliminary study an *in vitro* study was designed, since this provide a simplified tissue system for study (Alam et al., 2013) with clear cut tissue and free radical specific effects. Thus, the same can be considered as a first step of investigation before going for *in vivo* studies (Ganga et al., 2015).

Therefore, in this research work, the concentration dependent effects of AV *(Aloe vera),* AS (*Annona aquamosa*) and PM (*Pterocarpus marsupium*) were measured. Although, every assay system has some limitations so the differential antioxidative effects was studied various antioxidant assay methods (Niki, 2010).

Materials and Methods

Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA; while phosphoric acid and thio-barbituric acid (TBA), Bovine serum albumin (BSA), Folin-Ciocalteu reagent and all other chemicals were supplied by Hi Media Laboratories Ltd., Mumbai, India or from E-Merck (India) Ltd., Mumbai, India.

Animals

Healthy in-bred Wistar rats (150-200 gm) were taken for tissue samples which were reared in institutes' animal house under standard conditions. Standard ethical guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India were followed (Reg. No.-779/PO/Ere/S/03/CPCSEA).

Preparation of herbal drug extracts

Good quality dried powdered test herbs (100 g of each) were purchased from registered shop of ayurvedic herbs named "Akhand Aoushdhi Bhandar" local market, Indore (India) and identified by the departmental taxonomist. The extracts of herbs were prepared using 20 volume alcoholic or aqueous solutions. The extracts were vacuum dried to obtain fine powder (Tahiliani P & Kar A 2003; Sharma et al., 2013; Agrawal et al., 2013; 2014). These powders were dissolved for oral administration to the experimental animals.

Determination of total polyphenols and flavonoids

Total polyphenols of the test extract was measured by the protocol of Leontowicz et al (2003) using Folin-Ciocalteu. The results were expressed in mg gallic acid equivalent / 100 g dry weight of the extract. Similarly, total flavonoids were determined following the method of Leontowicz et al (2003) with some modification as followed in our laboratory earlier (Dixit and Kar, 2009; Parmar and Kar, 2008; 2009).

The 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay

Methanolic stock solution of DPPH and different concentrations of test drugs were prepared. One ml of extract was mixed with 0.5 ml of 0.15 mM DPPH and incubated for 30 min in dark at 20˚C. Control tube was prepared by adding methanol in place of extract. Optical density (OD) was taken at 517 nm. Ascorbic acid was used as standard and percent (%) scavenging activity was determined (Leontowicz et al., 2003). The scavenging activity of any drug was expressed in % inhibition [% scavenging = (control OD – sample OD/ control OD) X 100], as done earlier (Dixit and Kar, 2009; Parmar and Kar, 2008; 2009).

Nitric Oxide (NO) radical scavenging assay

This assay was performed following the protocol of Wang et al (2012). Sodium nitropruside was used as a NO free radical donor *in vitro*. In assay mixture 0.5 ml sodium nitropruside (10 mM in 0.2 M PBS, pH 7.4) was added with 0.5 ml of different concentrations of drugs and incubated for 150 min at 20˚C in dark. Then 1 ml Griess reagent was added to read OD at 542 nm against blank. Results were expressed as percentage nitrite produced with respect to the control, which was devoid of drug.

Reducing power assay

The ferric ion reducing power of the extracts was determined by the method of Lim et al (2007). In brief 0.75 ml extract of individual drug (0.3-5000 µg/ml diluted in Phosphate buffer saline (PBS) of 0.2 M, pH 6.6) were mixed with 0.75 ml PBS and 0.75 ml of potassium ferricyanide (1% w/v in DW), followed by incubation at 50˚C for 20 min. The reaction was stopped by adding 0.75 ml 10% TCA and centrifuged at 3000 rpm for 10 min. Then 1.5 ml supernatant was mixed with 1.5 ml DW followed by 1.5 ml of freshly prepared $FeCl_3$ (0.1% w/v) solution. After 10 min, OD was read at 700 nm. Higher OD of reaction mixture indicates greater reducing power. Ascorbic acid was used as standard.

Lipid peroxidation (LPO) assay

To detect *in vitro* free radical scavenging activity, LPO assay is widely used (Panda and Kar, 2008). LPO was measured in liver and kidney, as these organs are directly involve in drug metabolism. As higher content of phospholipids in erythrocyte's membrane make it more vulnerable for oxidative stress, RBCs were also considered in the study. $FeSO_4$ (10 mM) was used as oxidizing agent, antiperoxidative effects of drugs were evaluated following the protocol of Ohkawa et al. (1979).

Results

Results indicated marked inhibitory effects against free radical generation in studied assays, where concentration dependent effects were seen in all the test extracts and in all assay systems. In all assays drugs exhibiting more than 50% free radical scavenging potential were considered to be safe and effective.

In DPPH assay, AV and PM showed maximum inhibition ($P<0.001$ for both) at 125 mg/ml while AS showed maximum inhibition ($P<0.001$) at 15.25 mg/ml concentration, which is comparable to the standard. Higher and lower concentrations for each were observed to be less effective for all three herbs. In the same PM showed the highest inhibition (Table 1). For NO scavenging assay, most protective concentration of AS and PM were 5 and 2.5 mg/ml respectively, these were again equivalent to the standard. However, AV exhibited maximum inhibition at 1.25 mg.ml concentration. In case of all three tested drugs lower drug concentration were also observed to be less effective or ineffective. Here also, higher drug concentrations were found to be significantly less effective ($P>0.001$) than their lower drug concentrations (Table 2).

Table 1: Scavenging of DPPH free radicals (in %) by different test drugs. Drug concentrations are in mg/ml of DW.

Drugs	250	125	62.5	31.5	15.25
AV	80.01±0.004	89.57±0.005[c]	87.56±0.003	84.88±0.001	79.55±0.001
AS	68.098±0.01	73.61±0.006	80.16±0.015	84.94±0.004	87.62±0.004[c]
PM	82.87±0.01	91.02±0.006[c]	86.41±0.014	77.60±0.008	68.40±0.020
ASC a	97.53±0.09[b]	96.7±0.05	96.98±0.05	95.55±0.12	87.56±0.05

Data are expressed in % inhibition (mean ±SE of n=3) in comparison to that of the corresponding next higher drug concentration, [a] $P<0.05$; [b] $P<0.01$ and [c] $P<0.001$. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Table 2: Scavenging of Nitric oxide (NO) free radicals (in %) by different test drugs, drug's concentrations are in mg/ml of DW.

Drugs	10	5	2.5	1.25	0.62
AV	9.63±0.052	39.69±0.01	73.01±0.029	76.74±0.033[c]	38.73±0.005
AS	93.73±0.002	96.14±0.002[c]	80.72±0.017	75.17±0.03	75.48±0.022
PM	78.13±0.031	90.18±0.007	96.50±0.015[c]	80.11±0.016	47.46±0.029
A acid	93.73±0.39	93.91±0.39[a]	91.74±0.31	86.92±0.94	85.78±0.52

Data are expressed in % inhibition (mean ±SE of n=3) in comparison to that of the corresponding next higher drug concentration, [a] $P<0.05$; [b] $P<0.01$ and [c] $P<0.001$. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

In reducing power assay, AS showed comparatively the highest inhibition of free radicals at 0.15 mg/ml concentration. Both AV and PM exhibited maximum protection at 0.31 and 0.62 mg/ml concentration respectively. All test herbs e.g. AV, AS and PM showed decreased degree of hydroxyl ion inhibition at higher and lower drug concentrations as showed in table 3.

Table 3: Reducing power of different test drugs (in % reduction of OH free radicals). Drug's concentrations are in mg/ml of DW.

Drug	1.25	0.62	0.31	0.15	0.07
AV	29.7±.001	55.85±0.003	82.9±0.001[c]	73.05±0.33	32.35±0.116
AS	35.05±0.014	46.45±0.26	58.39±0.015	87.96±0.28[c]	39.41±0.055
PM	32.28±0.044	70.05±0.046[c]	23.39±0.114	21.3±0.050	27.66±0.135
ASC a	100±0.00	100±0.00	100±0.00	98.23±0.4	56.57±0.92

Data are expressed in % inhibition (mean ±SE of n=3) in comparison to that of the corresponding next higher drug concentration, [c] $P<0.001$. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Similar pattern was seen in in vitro LPO analysis where liver and kidney tissue homogenate and erythrocyte exhibited differential effects of drug extracts in concentration dependent manner.

In liver tissue homogenate, $FeSO_4$ treatment was observed to cause significant increase in tissue LPO ($P<0.001$) which was observed to be decrease in tubes treated with different concentrations of drugs. Except AS, both drugs showed significant inhibition of LPO at 0.1 mg/ml dose. For AV 1 mg/ml and for AS and PM 10 mg/ml concentrations were observed to be comparatively more effective and safe. While, higher concentrations of drugs (20 and 30 mg/ml) were observed to be considerably less effective (Table 4). All three drugs showed more or less similar pattern in kidney tissue homogenate and in isolated erythrocytes where highest drug doses of AS were ineffective against LPO. Somewhere higher concentration showed increased values of LPO in erythrocytes than only $FeSO_4$ treated cells (Table 5 and 6). Presence of polyphenolic and flavonoids in the test drugs are given in table 7.

Table 4: Inhibition of LPO by different test drugs (mg/ml of DW) induced by $FeSO_4$ (10 mM in DW) in liver tissue homogenate.

Cont.	1.93±0.106
FeSO4	7.016±0.017[@]

Drugs	30	20	10	1	0.1
AV	7.370.57	5.70±0.208b	3.41±0.13c	2.24±0.018c	2.03±0.53c
AS	3.81±0.32b	3.05±0.11c	2.82±0.17c	4.78±0.201c	6.61±0.21
PM	6.190.049b	3.670.34c	2.40±0.133c	4.30±0.11c	5.67±0.18a

Data are expressed in mean ±SE (% increase) of three measurements (each differ significantly $P<0.05$) and presented as nM MDA formed /h/mg protein. [@] $P<0.0001$ increased than control tube. [a] $P<0.01$; [b] $P<0.001$ and [c] $P<0.0001$ significant decrease and [#] $P<0.05$ increase in LPO as compared to the only FeSO4 treated tissue homogenate. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Table 5: Inhibition of LPO by different test drugs (mg/ml of DW) induced by $FeSO_4$ (10 mM in DW) in kidney tissue homogenate.

Cont.	1.74±0.06
FeSO4	4.62±0.15[@]

Drugs	40	30	20	10	1
AV	4.28±0.15	3.74±0.20[a]	3.05±0.291[b]	2.48±0.253[c]	1.92±0.24[c]
AS	3.55±0.17[b]	2.94±01.08[c]	2.24±0.141[c]	2.54±0.239[c]	3.49±0.29[c]
PM	3.83±0.171[a]	2.02±0.122[c]	2.40±0.133[c]	2.29±0.097[c]	3.27±0.94[c]

Data are expressed in mean ±SE (% increase) of three measurements (each differ significantly $P<0.05$) and presented as nM MDA formed /h/mg protein. [@] $P<0.0001$ increased than control tube. [a] $P<0.01$; [b] $P<0.001$ and [c] $P<0.0001$ significant decrease and [#] $P<0.05$ increase in LPO as compared to the only FeSO4 treated tissue homogenate. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Table 6: Inhibition of LPO by different test drugs (mg/ml of DW) induced by FeSO4 in isolated erythrocytes of rats.

Cont.	0.701±0.022				
FeSO4	1.55±0.058[@]				
Drugs	**30**	**20**	**10**	**5**	**1**
AV	1.52±0.040	1.34±0.045	1.05±0.035[b]	1.50±0.031	1.74±0.037
AS	1.61±0.028	1.47±0.023	0.96±0.025[b]	0.99±0.027[b]	1.50±0.035
PM	1.64±0.041	0.92±0.043[b]	0.80±0.01[c]	0.56±0.011[c]	0.84±0.013[c]

Data are expressed in mean ±SE (% increase) of three measurements (each differ significantly P<0.05) and presented as nM MDA formed /h/mg protein. [@] *P*<0.0001 increased than control tube. [a] *P*<0.01; [b] *P*<0.001 and [c] *P*<0.0001 significant decrease and [#] *P*<0.05 increase in LPO as compared to the only FeSO4 treated tissue homogenate. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Table 7: Flavonoids and total Polyphenol of different test drugs.

Drug	Flavonoids	Polyphenols
AV	69.21 ± 6.32	67.37 ± 3.68
AS	79.11 ± 7.81	43.66 ± 4.11
PM	88.67 ± 8.84	63.45 ± 5.25

Data are expressed in mean ± SE of three measurements. Amount of total flavonoids are presented as mg quercetin equivalent / 100 g dry weight of drug and total polyphenols are presented as mg gallic acid equivalent /100 gm dry weight of drug. Where, AV *(Aloe vera)*, AS (*Annona aquamosa*), PM (*Pterocarpus marsupium*).

Discussion

The obtained results revealed that three test herbs exhibited different degrees of antioxidative/ antiperoxidative potential depending on their concentrations, assay system and also on the tissue system used, as observed with other drugs (Sharma et al., 2014, 2015; Dixit and Kar, 2009; Parmar and Kar, 2009; Alam et al., 2013). Though, protective efficacy of the tested herbs was already established but the scientific studies of higher concentrations was extremely meager (Ernst, 2008). Above all, this report appears to be the first one which demonstrated the comparative aspect of antioxidative effects of test drugs. Additionally, the present findings mainly emphases on the lesser antioxidative / protective effects of the test drugs at higher drug concentrations than their corresponding lower concentrations. As also found earlier with some other herbs and their combinations (Dixit and Kar, 2009).

In this study, DPPH served as a stable free radical, which is one of the mostly used free radical used for antioxidant assays and also believed to be a suitable model compound for the analysis of antioxidative potential of drugs (Alam et al., 2013). In present investigation all the test extracts were observed to exhibit significant DPPH scavenging potency, along with these findings the highest drug concentrations used were observed to be less protective than their corresponding lower drug concentrations and also with the standard.

In other assay system the NO scavenging potential of the same test herbs were measured. NO free radicals are continuously generated in biological system. So, drugs having NO radical scavenging activity are supposed to be helpful against oxidative damage (Parmar and Kar, 2008). In this examination all the test extracts possess noteworthy NO scavenging potency at nearly all concentraions. However, higher concentrations of AV were ineffective for the same which might reveal their negative impact in assay system. of course, more studies are needed for confirmation but the present findings may provide supplementary data for further research and can be used to choose more appropriate drug concentration (Ernst, 2008; Dixit and Kar, 2009; Parmar and Kar, 2009).

Similarly hydroxyl radicals are also required for normal functioning of macrophages and microglial cell. But here also, their surplus production may cause DNA damage, cytotoxicity or mutations (…..). Here, reducing power assay was applied to estimate hydroxyl free radical scavenging activity of the test drugs. However, all three herbs showed significant protection in all above mentioned assay systems but the results varied with concentration of drugs and also with the types of free radicals this indicated that different herb might serve via different pathway.

With the reference of LPO, excessively formed free radicals may steal electrons from the lipids in the plasma membrane, resulting cell damage. The products of LPO are more damaging and may cause cancer, mutation or other harms. As used in this study, $FeSO_4$ is known as potent oxidizing agent which forms ferryl-perferryl complex and by Fenton reaction. It leads to cause severe oxidative stress. We also found a higher TBARS level in $FeSO_4$ treated tissue homogenates and in isolated erythrocytes which was consistent with previous finding (Dixit and Kar, 2009; Parmar and Kar, 2009; Panda and Kar, 2009, Sharma et al., 2014). The obtained results revealed significant protective effects of test drugs at relatively lower concentrations. This analysis also pointed out that all three drugs possess considerable amount of total polyphenolics and flavonoids.

In conclusion, obtained results clearly demonstrated that test drugs exhibited concentration dependent protective effects. Significant inhibition of DPPH oxidation, NO formation and decrease in Induced LPO in biological system by test indicating their possible use as natural and safe antioxidants. However, in all assay systems, reduced protective efficacy at higher drug concentrations may point out towards their regulated and systematic use. Besides, studies by *in vitro* methods may not be considered as conclusive without further *in vivo* studies. Obviously, more investigations are needed with different parameters to confirm to the results.

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