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Concentration dependent effects of some commonly used plant extracts on three different tissue systems: An in vitro study

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Abstract: ***The present study was designed to find out the concentration dependent comparative in vitro antioxidative /pro-oxidative activity of three commonly used plant extracts of AI (Azadirachta indica), OS (Ocimum sanctum) and IR (Inula recemose). For this, in-vitro free radicals scavenging assays such as 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Nitric oxide (NO), reducing power assay and ferrous sulphate (FeSO4) induced lipid peroxidation (LPO) assay in liver and kidney homogenates and in isolated erythrocytes were performed. Ascorbic acid was used as standard antioxidant for all these in vitro assays. Total polyphenols and flavonoid contents were also estimated. Tested plant extracts were found to exhibit dose dependent antioxidant activities. All three plant were observed to be relatively more protective at lower concentrations. Pro-oxidative or less protective efficacy of AI was observed in different tissue systems at higher concentration used. All three test drugs were found to be anti-peroxidative. However, higher concentration showed decreased efficacy and/or harmful effects. Significant amount of polyphenols and flavonoids, present in the test drugs supported their antioxidative property.***

I. INTRODUCTION

Hyperglycemia is already known as one of the factors for increased oxidative stress. Increased level of tissue free radicals is also believed to induce diabetes mellitus (DM)[1]. A number of prior investigations also showed that the oxidative stress and DM both mutually influence each other, resulting in numerous pathological complications[2,3]. Obviously, free radical scavengers are likely to treat such abnormalities. As, herbs are rich source of polyphenols, flavonoids, saponins etc which serve as natural and safer protecting agents[4,5] for years, numerous herbs have been in use as anti-hyperglycemic and anti-oxidative agents[1,6,7]. Supporting data showed that, so far more than 800 plants have been reported for their antioxidative and antidiabetic activities[8].

Although, herbs are supposed to be safe and for long have been used as home remedy or without any prescribed dose, in recent analysis, more than 900 herbs have been known to cause several side effects including hepatotoxicity, renal failure, cardiovascular dysfunction and allergic reactions[2,4]. The studies with some plants also showed that lower doses were protective but the same herb at higher doses were observed to be toxic[5]. Whole, literature is available on some plants that possess antioxidative activity[7,9] but effects of their higher concentrations were not known[6]. Hence, there was a need to establish scientific evidences regarding safety concern of these routinely used antidiabetic plants.

Before going for preclinical analysis, it was important to test their efficacy, first by in vitro assays[3,10-12]. Because of the fact that, in vitro study permits an enormous level of simplification of system under study[6,13-15]. Therefore, in this investigation, the concentration dependent effects of three plant extracts were measured using different free radical scavenging assay systems, although, every method has its limitations, antioxidative efficacy was determined using various antioxidant assay methods[8]. Here we emphasized to understand dose specificity and free radical specific nature with special attention to possible undesirable effect(s) of test drugs, if any.

II. MATERIAL AND METHODS

All paragraphs must be indented as well as justified, i.e. both left-justified and right-justified.

A. Chemicals and plants

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA; while carboxy methyl cellulose, sodium nitroprusside, phosphoric acid and thio-barbituric acid (TBA), Bovine serum albumin (BSA), Folin-Ciocalteu reagent and other chemicals were of experimental grade. 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.

B. Animals

Healthy in-bred Wistar rats (150-200 gm) were housed in polypropylene cages under constant temperature (27±2°C) and photo-schedule (14 h light and 10 h dark). They were provided with commercial rodent feed (Golden Feeds, New Delhi, India) ad libitum and had free access to boiled drinking water. 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. The approval of departmental ethical committee (Reg. No.-779/PO/Ere/S/03/CPCSEA) for handling and maintenance

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for experimental animals was also obtained before starting the experiments.

C. Preparation of poly/mono herbal drug extracts

The extracts of herbs were prepared using 20 volume alcoholic solutions. In brief, 10 g powdered herbs 200 ml ethanol (70%) was added and then solutions were kept for 24 hours at room temperature[9]. The extracts were vacuum dried to obtain in fine powder form[16-18]. These powders were dissolved in DW for oral administration to the experimental animals.

D. Determination of total polyphenols and flavonoids

Total polyphenols of the test extract was measured by the protocol of Leontowicz et al[19] using Folin-Ciocalteu. The absorbance was measured against the prepared blank at 765 nm in comparison with standard of known concentrations of gallic acid. The results were expressed in mg gallic acid equivalent / 100 g dry weight of the extract.

Total flavonoids were also determined following the method of Leontowicz et al[19] with some modification as followed in our laboratory earlier[9,12]. OD was taken against the prepared blank at 510 nm in comparison to standards prepared similarly with the known concentrations of quercetin. The results were expressed in mg quercetin equivalent/ 100 g dry weight of the extract.

E. In vitro free radical scavenging assays

The antioxidative activity of drugs were determined in vitro by different free radical scavenging assays in which graded concentrations of test drugs were added with assay mixtures.

The 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay - 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 a drug was expressed in % inhibition [% scavenging = (control OD – sample OD/ control OD) X 100], as done earlier[9,12,13].

Nitric Oxide (NO) radical scavenging assay - This assay was performed following the protocol of Wang et al[20]. 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. The NO scavenging activity (%) was measured with respect to control.

Reducing power assay- The ferric ion reducing power of the extracts was determined by the method of Lim et al[21]. 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 FeCl3 (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[8,14]. The assay is based on the principle that the excessively generated free radicals may cause peroxidation of membrane lipids. And among many peroxidation products, Malondialdehyde (MDA) is one of the major products of LPO and when this is heated (at 100℃) with thiobarbituric acid (TBA) it produces pink color complex. 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 this study.

III. RESULTS

In all assays, drugs exhibiting more than 50% free radical scavenging potential were considered to be safe and effective. In DPPH assay, AI and IR showed highest protective activity at a concentration of 125 mg/ml, while OS showed maximum DPPH free radical scavenging potential at 15.25 mg/ml. It was clearly seen that higher concentrations of all three herbs were less effective considerably than standard. In NO assay, higher concentrations of AI were observed to be ineffective, while for OS and IR they were found to be less effective. These outcomes were also significantly ($P<0.001$) less effective than the standard. Differential effects were again observed in reducing power assay where higher concentrations of all three test herbs were seem to be less effective than their respective lower concentrations ($P<0.001$, for all) and also to the standard antioxidants ($P<0.001$).

In liver tissue homogenate, FeSO4 induced LPO was increased by 262.24%, than that of control which was observed to be decreased in tubes treated with different concentrations of drugs ($P<0.001$). Since, the LPO values were consistently increased over 30 mg/ml for all the tested drugs (results not shown), these were considered to be toxic. OS and IR were found to be effective at the 30 mg/ml concentration ($P<0.001$) but no significant differences were seen in case of AI at 30 mg/ml of concentration. Similarly, in

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kidney tissue, OS and IR, exhibited significant decrease in FeSO4-induced LPO at all studied concentrations. On the other hand, AI was observed to exert pro-oxidative effects at 40 mg/ml of drug dose and above this concentration.

Isolated erythrocytes showed 122.02% increased in LPO upon FeSO4 treatment as compared to untreated cells. In case of OS and IR, out of five drug concentrations, 5 and 10 mg/ml were found to be significantly ($P<0.001$) protective against FeSO4 treatment; while, for AI, at 20 and 30 mg/ml drug concentrations no protective activities were seen; instead, 30 mg/ml concentration was observed to be associated with increased LPO ($P<0.05$) than only FeSO4 treated cells. As observed in Table 7, all three drugs were observed to posses flavonoids and polyphenolic compounds in measurable quantities.

IV. DISCUSSION

Due to enormous beneficial health effects, antioxidants have received much attention of medical sciences. The obtained results revealed that herbs exhibited different degrees of antioxidative/ antiperoxidative potential depending on their concentrations, assay and the tissue system used. In vitro free radical scavenging activity of individual herbs is also in accordance with the earlier investigations[12,21,22]. In fact, the present findings mainly emphasizes on the lesser antioxidative / protective effects of the test drugs at higher concentrations, as also found earlier with some other herbs[13,15].

DPPH is a stable free radical, which is believed to be a suitable model compound for the analysis of antioxidative potential of drugs[20,21]. Our findings also revealed that IR and OS possess a significant nitrogen radical scavenging potential which is comparable to the standard antioxidant. Few reports on nitrogen free radical scavenging potential of herbs have been documented earlier[19,23]. The obtained results indicate safer action of the lower doses of test drugs, and higher concentrations of almost all test drugs were considerably less effective[14,18].

Since, NO is an important cellular signalling molecule, that serves as a fundamental component of nervous, immune and other physiological systems, its presence is thought be required for various biological purposes. However, it's over production was found to cause many pathological conditions including DM[17,19]. NO directly oxidizes iron-containing proteins such as ribonucleotide reductase, aconitase, guanylate cyclase, sulfhydryl groups that affect their activities[23]. NO radical scavengers are observed to be effective therapeutic agents against oxidative damage[22.24]. Interestingly, in the present study, all the test extracts were found to possess noteworthy NO scavenging potency, which clearly revealed their protective potential against NO free radical generation. Furthermore, according to Gutierrez et al[25] in in-vivo system, oxidative stress via NO radical, severely damages β-cells of pancreas and leads to DM and the test drugs having better NO inhibition efficacy can be considered as highly effective in vivo antioxidant[4-6,18]. But, here drugs were found to exhibit lesser inhibition at higher drug concentrations that might reveal their negative health effects at higher doses.

As, mentioned earlier, hydroxyl free radicals are extremely active bio-molecules and are essentially required for normal functioning of macrophages and microglial cell. But their excessive generation may cause DNA damage[26]. To examine the hydroxyl radical scavenging activity of test drugs, reducing power assay was performed and the obtained data suggested that the protective effects of drugs show adverse effects at concentration, similar to be previous reports[5,8-12].

In above mentioned different assay systems, tested drugs showed diverse range of antioxidative action, indicating that different herbs serve through different pathways. As mentioned elsewhere, the possible mode of action of the test drugs could be either through their direct free radical scavenging action or through chelating ferric/ ferrous ions in the reaction mixture. Interestingly, the test drugs were able to scavenge both types of radicals[26]. Thus, the present findings may provide supplementary information for further research[12,14,21].

Excessively formed free radicals may steal electrons from the lipids in the plasma membrane, resulting cell damage. Such oxidative lipid degradation is referred as LPO. The products of LPO are highly damaging and may cause cancer, mutation or other abnormalities[7,10]. For years FeSO4 is known as potent oxidizing agent which forms ferryl-perferryl complex by Fenton reaction that leads to oxidative stress. We also found a higher TBARS level in FeSO4 treated tissue homogenates and in isolated erythrocytes which was consistent with previous findings[10,12,20].

In both liver and kidney tissue homogenates, all extracts were found to be effective against FeSO4 induced oxidative stress. Earlier also, in vitro protective effects of OS and IR extracts were documented[14-16,22]. However, the present work is the first one that revealed possible negative impact of herbs on different tissues. Particularly at higher concentration our findings also emphasized that the protective effects were also tissue specific.

Erythrocytes serve as oxygen carriers and are constantly exposed to oxidative injury; further the presence of redox active hemoglobin molecules and PUFA rich membrane render them weaker against free radical attack, as observed in DM patients[9,11]. Preventive applications of natural antioxidants in the prevention of such oxidative stress-related diseases may prove to be an effective therapy. The free radical scavenging / inhibitory potential of herbal extracts can be explained on the basis of the earlier reports, which

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demonstrated that the plant derived antioxidative activity might have been mediated through an inhibition of hydroxyl radical generation, reduction of total amount of ferrous/ferric ions available for chain reaction and/or metal chelation activities which may block the reaction at any instant[16-19,24].

Reviewing all these findings it appears that protective efficacy of the test drugs are method specific. Numbers of in vitro methods have been applied in different systems, because there is no simple universal method available by which antioxidant potency can be assessed accurately and quantitatively [13,16]. Therefore, for more accurate results, different free radical scavenging assays have been employed time to time[20-13,25]. Interestingly we found more or less positive effects with all the test drugs.

The total polyphenolics and flavonoids content of each test drug, justified the free radical scavenging strengths of the test extratcs[19,21-23]. Reports regarding direct free radical scavenging and oxidation resistance activities of polyphenols have been documented[14,16] as also observed here. On the basis of earlier researches the less protective /harmful/ peroxidative effects of test drugs can be explained. It has been seen that at higher concentrations polyphenolic and/or flavonoids compounds undergo various polymerization reactions and spatial conformations that may modify the reactivity of molecules[20-23,26].

In conclusion, the present results clearly demonstrated that test drugs exhibited concentration dependent protective effects. These results also suggested that different test drugs may exhibit differential activities in different assay systems. Significant inhibition of DPPH oxidation, NO formation and decrease in Induced LPO in biological system by test indicate their possible use as natural and safe antioxidants. However, in all assay systems, lesser protective efficacy at higher drug concentrations may point out towards their regulated and systematic use. However, studies by in vitro methods may not be considered as conclusive without further in vivo studies. Obviously, more investigations are required using more parameters and in in vivo systems.

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