IJPT
1735-2657/07/61-15-21
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 6:15-21, 2007

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RESEARCH ARTICLE

Exploring the Effect of *Cytisus Scoparius o*n Markers of Oxidative Stress in Rats

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Received May 12, 2006; Revised January 27, 2007; Accepted February 15, 2007

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

Antioxidant activity of aerial part of *Cytisus scoparius*, Link (Family – Leguminosae) was studied both by *in vitro* and *in vivo* models. Different concentrations of different extracts of chloroform, ethyl acetate, methanol and hydroalcoholic extracts of plant were investigated for *in vitro* antioxidant activity using the thiocyanate method. The hydroalcoholic extract exhibited highest inhibitory activity on peroxidation, over other organic extracts and IC50 value (30.2 µg/ml) of the hydroalcoholic extract was found to be less than the standard, α-tocopherol (IC50 value of 66.1µg/ml). The hydroalcoholic extract was further evaluated for in vivo antioxidant activity, such as ferric reducing ability of plasma (FRAP), thiobarbituric acid reactive substance (TBARS), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). A significant ($p < 0.05$) rise in FRAP level was observed in the hydroalcoholic extract. The extract showed significant ($p < 0.05$) reduction of TBARS and increase in SOD enzyme level in liver and kidney. The extract treatment at higher dose only caused a significant ($p < 0.05$) increase in the level of the catalase in liver and kidney. However, there was no significant effect on TBARS, SOD, and CAT in the heart. In reduced glutathione, the extract did not cause significant changes in liver, heart and kidney. These results suggested that hydroalcoholic extract of *Cytisus scoparius* had significant antioxidant activity, which supports the claim about the plant to be used as antioxidant in Indian system of medicine.

Keywords: *Cytisus scoparius, Antioxidant, Free radical scavenging, Lipid peroxidation*

Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanism is an important factor that has been implicated in various diseases like atherosclerosis, cancer, diabetes mellitus, myocardial infarction, Alzheimer's disease and also in ageing [1]. However, the physiological system has a series of defense mechanism including antioxidant enzymes-SOD (superoxide dismutase), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase and other free radical scavengers, β-carotene, α- tocopherol, ascorbic acid, α-lipoic acid and glutathione to protect the cell against cytotoxic reactive oxygen species (ROS) [2]. There is evidence that antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is an increasing interest in the protective biochemical function of natural antioxidants contained in vegetables, fruits and medicinal herbs [3-4].

Cytisus scoparius Link (Family- Leguminosae) is a well known medicinal plant and is found growing wild as a garden escape at elevation of 7000 – 8000 ft. The green twings of plant collected before flowering, are used either fresh or after drying, as a heart tonic and diuretic in dropsy [5]. In large doses they are emetic and purgative. In ethnomedicine, this plant is used for diuretics, hypnotic and sedative [6], diabetes [7] and liver disease [8]. Pharmacological studies have confirmed its uterine stimulant effect [9], antispasmodic activity [10] and estrogenic effect [10]. The plant *Cytisus scoparius* contains the flavone 6´´ O acetyl scoparin [11], flavonals namely rutin, quercetin, quercitrin, iso rhamnetin and kaempferol [12] and some isoflavones like genistin and sarothamnoside [13]. Three carotenoids of chrysanthemaxanthin, xanthophyll and xanthophyllepoxide are also reported to be present in the plant [5]. Quinolizidine alkaloids namely spartein, sarothamine and lupanine [14] and isoquinoline alkaloids such as tyramine and hydroxyl tyramine [15] have also been isolated from this plant. Benzenoid compounds like phenyl ethanol and cresol [16] have also been reported to be present in the plant. Generally, plants containing flavonoids have been reported to possess strong antioxidant properties [17].

In this respect flavonoids and other polyphenolic compounds have received the greatest attention [18-20]. Based on these reports our studies have been designed to examine the plant extract of *Cytisus scoparius* for *in vitro* and *in vivo* antioxidant activities using different experiment models, so as to establish its claim of being used as antioxidant in traditional medicine.

MATERIALS AND METHODS

Plant material

Aerial parts of *Cytisus scoparius* was collected in Nilgiri hills, Tamilnadu, India and authenticated. Voucher specimen (SNPS-011/ 2003-2004) of this plant material has been retained in the School of Natural Product Studies (SNPS), Jadavpur University, India.

Preparation of extracts

Preparation of chloroform, ethyl acetate and methanol extracts

The aerial part of *Cytisus scoparius* was dried at room temperature and reduced to coarse powder. This powder (500 g) was successively extracted with chloroform, ethyl acetate and methanol for 48 hours. The solvents were completely removed by rotary evaporator and the extracts were concentrated, dried (yield 4.6 % w/w, 3.8 % w/w and 3.0 % w/w respectively) and stored in vacuum desiccators. Further the extracts were used for antioxidant studies.

Preparation of hydroalcoholic extract

The crude powder (500 g) was extracted with hydroalcoholic mixture (ethanol-water, 7:3). The solvent was completely removed by rotary evaporator and the extract was concentrated, dried (yield 3.8 % w/w) and stored in vacuum desiccators for further use.

Chemicals

All chemicals were analytical grade and chemicals required for all biochemical assays were obtained from Sigma chemicals co, USA.

Assay of in vitro antioxidant activity

The antioxidant activity of different extracts of chloroform, ethyl acetate, methanol, hydroalcoholic extracts were determined according to the thiocyanate method. Briefly, 500 µL of all extracts at different concentration (25, 50, 100 and 200µg) were mixed with 2.5 mL of 0.02M linoleic acid emulsion (contains equal weight of tween-20 in phosphate buffered saline, pH 7.4) and the final volume was adjusted to 5 mL with phosphate buffered saline in a test tube and incubated in darkness at 37°C. The amount of peroxide was determined by measuring absorbance at 500 nm after coloring with 0.1 mL of $FeCl_2$ (0.02M) and 0.1 mL thiocyanate (30%) at intervals during incubation [21-22]. The solutions without added extracts were used as blank samples. α-Tocopherol was used as a reference compound.

Test animals and Groups

Wistar albino rats (200 –250 g) of either sex were maintained under standard environmental conditions and had free access to feed and water. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee. Wistar albino rats of either sex were divided into three groups of six animals each. Group 1 served as control and was given the vehicle alone (sodium carboxy methyl cellulose, 0.3% w/v). Groups 2 and 3 received hydroalcoholic extract of *Cytisus scoparius* orally at 250 and 500 mg/kg body weight, respectively. The treatments were given for 14 days and on 15^{th} day of the experiment, all the animals were sacrificed by decapitation. The heart, liver and kidney were removed, washed in cold saline and stored in liquid nitrogen for further biochemical studies.

Ferric reducing ability of plasma (FRAP) assay

Total plasma antioxidant capacity was measured according to the FRAP method [23]. The blood samples were collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 7, 14 days of treatment. Briefly, 3 mL of freshly prepared and warm (37°C) FRAP reagent [1 mL (10 mM) of 2,4,6 tripyridyl-s- triazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM $FeCl_2.6H_2O$, 10 mL of 0.3 M acetate buffer (pH 3.6)] was mixed with 0.375 mL distilled water and 0.025 mL of test samples. The absorbance of developed colour in organic layer was measured at 593 nm. The temperature was maintained at 37°C. The readings at 180 seconds were selected for the calculation of FRAP values. Ferrous sulphate ($FeSO_4.7H_2O$) was used as a standard for calibration and the data was expressed as nM Fe^{2+}/Liter.

Biochemical estimation of markers of oxidative stress

Preparation of rat heart, liver and kidney homogenate

Tissue homogenate was prepared in a ratio of 1 g of wet tissue to 10 times (w/v) 0.05M-ice cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. 0.2 mL of sample homogenate was used for estimation of thiobarbituric acid reactive substance (TBARS). The remaining part of the homogenate was divided into two parts. One part was mixed with 10% trichloro acetic acid (1:1), centrifuged at 5000*g* (4°C, for 10 minutes) and the supernatant was used for GSH estimation. The remaining part of the homogenate was centrifuged at 15,000 *g* at 4°C for 60 minutes and the supernatant was used for superoxide dismutase, catalase and protein estimation [24].

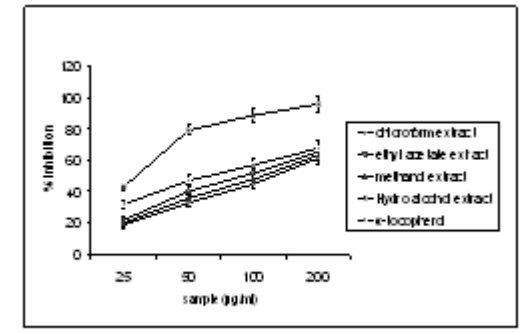

Fig 1. Percentage inhibition of lipid peroxidation using the standard (α- tocopherol) and using different doses of extracts [chloroform, ethyl acetate, methanol and hydroalcohol (7:3 ratios)] of Cytisus scoparius in the linoleic acid emulsion. Results are mean ± s.d of five parallel measurements.

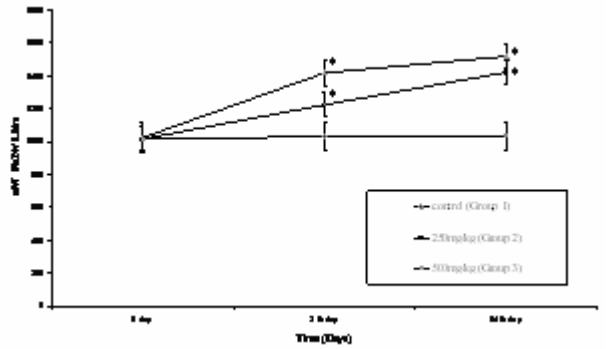

Fig 2. Changes in rat total antioxidant capacity of plasma (FRAP) measured by Fe2+ equivalent after administration of hydroalcoholic extract of Cytisus scoparius values are mean ± s.d (n=6). *P< 0.05

Estimation of thiobarbituric acid reactive substance (TBARS)

Lipid peroxide was measured by the method of Liu *et al* [25]. Acetic acid 1.5 mL (20%) pH 3.5, 1.5 mL thiobarbituric acid (0.8%) and 0.2 mL sodium dodecyl sulphate (8.1 %) were added to 0.1 mL of processed tissue samples, then heated at 100°C for 60 minutes. The mixture was cooled and 5 mL of n-butanol-pyridine (15:1) mixture and 1 mL of distilled water was added and vortexed vigorously. After centrifugation at 4000 *rpm* for 10 minutes, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer and concentration of TBARS was expressed as nM/g tissue.

Estimation of superoxide dismutase (SOD)

SOD activity was analyzed by the method described by Kakkar *et al* [26]. Assay mixture contained 0.1 mL of sample, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 mL of phenazine methosulphate (186 µM), 0.3 mL of nitroblue tetrazolium, 300 µM and 0.2 mL of nicotinamide adenine dinucleotide reduced disodium salt (NADH, 750 µM). Reaction was started by addition of NADH. After incubation at 30° C for 90 seconds, the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 mL of n- butanol. Mixture was allowed to stand for 10 minutes, centrifuged and the butanol layer was separated. Colour intensity of the chromogen in the butanol layer was measured at 560 nm, spectrophotometrically and concentration of SOD was expressed as Units/mg of protein.

Estimation of catalase (CAT)

Catalase activity was measured by the method of Aebi [27]. Supernatant (0.1 mL) was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as Units/mg of protein.

Estimation of reduced glutathione (GSH)

Reduced glutathione was measured according to the method of Ellman [28]. Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 mL of this supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5'5-dithio, bis (2-nitrobenzoic acid) and 0.4 mL double distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 minutes. The concentration of reduced glutathione was expressed as µg/g tissue.

Estimation of protein

Protein concentration was estimated according to the method of Lowry *et al* [29]. Using bovine serum albumin as a standard.

Behavioural and toxic effects

The acute oral toxicity study was evaluated in mice according to the method of Litchfield and Wilcoxon [30]**.** Five groups of 10 mice were administered p.o., 250, 500, 1000, 2000 and 4000 mg/kg, B/W of the *Cytisus scoparius* extract, while one group with the same number of mice served as a control. The animals were observed continuously for one hour for any gross behavioural changes, symptoms of toxicity and mortality if any and intermittently for the next 6h and then again, 24h after dosing with plant extract.

Statistical analysis

The data were expressed as mean ± s.d, which for biochemical and physiological parameters were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnet-t-test using the SPSS statistical software for comparison with control group. $P \leq 0.05$ was considered as significant.

RESULTS

Assay of in vitro antioxidant activity

The antioxidant activity of different extracts (chloroform, ethyl acetate, methanol and hydroalcohol) of *Cytisus scoparius* was determined by the thiocyanate

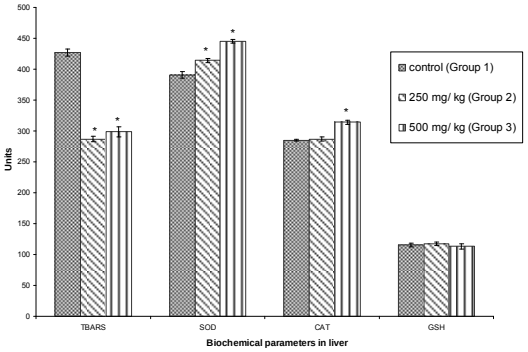

Fig 3. Changes in rat liver thiobarbituric acid reactive substances (TBARS; nM/g wet wt), superoxide dismutase (SOD; 10-1 U/mg protein), catalase (CAT; 10-1 U/ mg protein) and reduced glutathione (GSH; 101 µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius*. Values are mean ± s.d. (n=6). *P< 0.05 (one way analysis of variance) compared with control.

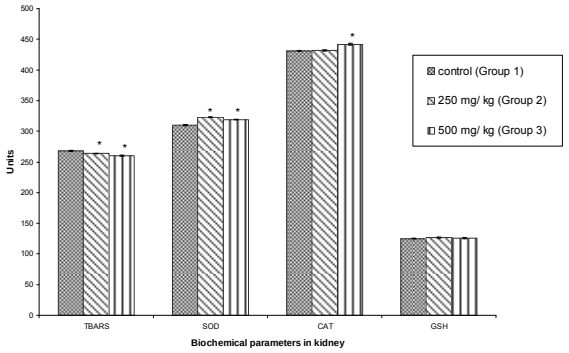

Fig 4. Changes in rat kidney thiobarbituric acid reactive substances (TBARS; nM/g wet wt), superoxide dismutase (SOD; 10-1 U/mg protein), catalase (CAT; 10-1 U/mg protein) and reduced glutathione (GSH; 10-1 µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius*. Values are mean ± s.d. (n=6). *P< 0.05 (one way analysis of variance) compared with control.

method. The effects of various amounts of extracts on peroxidation of linoleic acid emulsion are shown in Fig 1. All extracts showed inhibition of lipid peroxidation (LPO) effect and the relative antioxidant potencies for different extracts of the plant were hydroalcoholic extract > ethyl acetate extract > methanol extract > chloroform extract. Less antioxidant activity was observed for chloroform extract at all concentrations. The ethyl acetate and methanol extracts showed comparable lipid peroxidation effects with that of the standard, α – tocopherol and the IC_{50} values for ethyl acetate and methanol extracts were 94.1 µg/mL and 109.2 µg/mL, respectively. All concentrations of hydroalcoholic extract values showed higher antioxidant activity than the other extracts and that of standard α – tocopherol and IC_{50} values were found to be 30.2 µg/mL and 66.1 µg/mL for hydroalcoholic extract as well as standard.

Assay of in vivo antioxidant activity

As maximum lipid peroxidation inhibition was found with the hydroalcoholic extract of *Cytisus scoparius* in the *in vitro* study. So, only the hydroalcoholic extract was used for the *in vivo* studies.

Ferric reducing ability of plasma (FRAP) assay

The total antioxidant capacities measured as FRAP in the plasma of rat after administration of hydroalcoholic extract of *Cytisus scoparius* over a period of 14 days is shown in Fig 2In control group, there was no significant change in FRAP value on days 7 (1032 nM Fe^{2+}/Litre) and 14 (1034 nM Fe^{2+}/Litre) when compared with zero day (1015 nM Fe^{2+}/Litre). However, in groups 2 and 3 on days 7 (1224 and 1416 nM Fe^{2+}/Litre, respectively) and 14 (1419 and 1518 nM Fe^{2+}/Litre, respectively) there was a significant (p <0.05) increase in FRAP value as compared with zero day. Maximum enhancement was obtained in group 3 after 14 days of treatment.

Estimation of TBARS

The effect of various doses of hydroalcoholic extract of *Cytisus scoparius* on the lipid peroxidation and endogenous antioxidants of liver, heart and kidney of rats is shown in Fig 3, Fig 4 and Fig 5, respectively. A significant ($p < 0.05$) decrease in TBARS concentration in liver (287 nM/g and 299 nM/g wet wt tissues) and kidney (264 nM/g and 260 nM/g wet wt tissue tissues) for group-2 and group-3 when compared with control group (427 nM/g and 268 nM/g wet wt tissue) was observed. There was no change in TBARS and endogenous antioxidant levels in heart tissue in the treated groups 2 and 3 (312 nM/g and 306 nM/g wet wt tissue) compared with control group (300 nM/g wet wt tissue).

Estimation of SOD

The administration of hydroalcoholic extract caused no significant decrease at 250 mg/kg body weight (39.5 units/mg of protein) and 500 mg/kg body weight (46.9 units/mg of protein) in the level of SOD in heart ($p < 0.05$), when compared with control (47.5 units/mg of protein). However, the level of SOD in the kidney and liver of the treated rats was not dose related and found to be significantly increased ($p < 0.05$) at the two doses of 250 mg/kg (32.3 units/mg of protein & 41.4 units/mg of protein) and 500 mg/kg (31.9 units/mg of protein & 44.5 units/mg of protein) when compared with control (31.0 and 39.1 units/mg of protein). The results are presented in Fig 3, Fig 4 and Fig 5.

Estimation of CAT

The treatment of hydroalcoholic extract to normal rats for 14 days induced a dose dependent increase in the level of catalase in the liver and kidney, however decrease in the heart. The results are significantly increased at 500 mg/kg body weight dose of the treatment ($p < 0.05$), respectively for liver (31.4 units/mg of protein) and kidney (44.2 units/mg of protein), when compared with control group (28.5 and 43.1units/mg of protein). However, there was no significant change in the endogenous antioxidant levels in heart tissue. The results have been presented in Fig 3, Fig 4 and Fig 5.

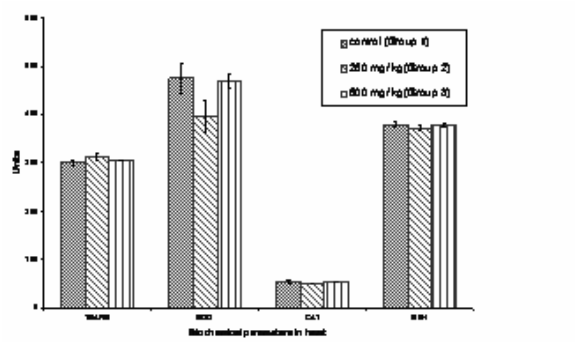

Fig 5. Changes in rat heart thiobarbituric acid reactive substances (TBARS; nM/g wet wt), superoxide dismutase (SOD; 10-1 U/mg protein), catalase (CAT; 10-1 U/mg protein) and reduced glutathione (GSH; µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius*. Values are mean ± s.d. (n=6). $^{*}P< 0.05$ (one way analysis of variance) compared with control.

Estimation of GSH

There was no significant change of reduced glutathione level in liver (1.17 mg/g and 1.13 mg/g wet wt of tissues), heart (373 µg/g and 378 µg/g wet wt of tissues) and kidney (12.7 µg/g and 12.55 µg/g wet wt of tissues) for groups 2 and 3, as compared with control group of liver, heart and kidney (1.15 mg/g, 380 µg/g and 12.5 µg/g wet wt of tissues, respectively).

Behavioural and toxic effects

In the toxicity study, no morality occurred within 24h with the five doses of plant extract tested. The LD_{50}, was therefore, greater than 4000mg/kg p.o., in mice, it may be considered relatively safe (Data not shown).

DISCUSSION

The reactive oxygen species formed may cause cellular and subcellular damage by peroxidation of membrane lipids, by denaturing cellular proteins and by breaking DNA strands, disrupting cellular functions [1]**.** Aerobic cells are endowed with extensive antioxidant defense mechanisms including both low molecular weight scavengers, which counteract the damaging effects of toxic oxygen species [31-32] and endogenous antioxidant enzyme such as superoxide dismutase which converts the superoxide free radical anion to hydrogen peroxide. Catalase is capable of scavenging the hydrogen peroxide radical, which is formed during various biochemical and metabolic reactions. The glutathione is involved in many important cellular functions, ranging from the control of physico chemical properties of cellular proteins and peptides to the detoxification of free radicals [33]. However, when the balance between these species and antioxidants is altered, a state of oxidative stress results, possibly leading to permanent cellular damage.

In the present study, *in vitro* antioxidant activities of chloroform, ethyl acetate, methanol and hydroalcoholic extracts of *Cytisus scoparius* were determined by using the thiocyanate method. From this method, the amount of peroxides formed in emulsion during incubation was determined spectrophotometrically by measuring absorbance at 500 nm. High absorption is an indication of high concentration of peroxides formed. Therefore, low absorbance indicates high antioxidant activity [21]. All extracts and standard of α – tocopherol were showed to inhibit the formation of peroxides at various concentrations. However, maximum inhibition of peroxidation was found with hydroalcoholic extract over the other organic extracts. The FRAP test measured total antioxidant capacity determined by non-enzymatic antioxidants. Several methods have been developed to assess the total antioxidant capacity of serum or plasma because of the difficulty in measuring each antioxidant component separately in the serum or plasma [34]. One of these is FRAP, which measures the reduction of ferric to ferrous iron in the presence of water soluble exogenous antioxidants [23]. The significant (p<0.05) increase in FRAP level after oral administration of hydroalcoholic extract indicates the presence of bioavailable antioxidants in the plant. Increased total antioxidant capacity in plasma following consumption of the plant extract was also associated with the decreased lipid peroxidation in *in -vitro* and *in- vivo* experiments.

The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major reactive oxygen species that function in concert to induce LPO (lipid peroxidation) of cell membrane lipids. The toxic peroxidative products cause wide spread cellular injury [35]**.** The present study showed the depletion in the lipid peroxidation as observed by significant decrease in the TBARS level of the liver and kidney in the treated groups, but there was no change of TBARS level in heart compared with control. SOD metabolizes the superoxide radical anion. It is an effective defense of the cell against endogenous and exogenous generation of superoxide [36]**.** The ROS scavenging activity of SOD is effective only when it is followed by the action of CAT and GPX, because of the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which is more toxic than oxygen derived free radicals and required to be scavenged further by CAT and GPX [37]**.** The administration of hydroalcoholic extract of 500 mg/kg B/W significantly increased the level of catalase in liver and kidney and the level of SOD in the liver. This shows the antioxidant nature of the extract. Generally, results for the kidney have shown fewer changes in antioxidant activity compared to liver [38]. However, decrease in the level of SOD and CAT was observed in the heart, which could explain the present observation. Reduced glutathione is a protective molecule against chemical induced cytotoxicity [39]. GSH metabolism plays a vital role in many biological processes; such as the detoxification of xenobiotics. The hydroalcoholic extract has been found to increase GSH level in liver and kidney. However, a decrease in the level of GSH was observed in the treated groups in the heart.

However, long-term administration of hydroalcoholic extract did not show significant result in decrease of GSH levels in the organs, indicating a protective antioxidant effect.

CONCLUSION

It can be concluded that, the hydroalcoholic extract of *Cytisus scoparius* had significant antioxidant activity. The antioxidant action of plant extract may be attributed to the presence of known flavonol, which provides maximum conjugation with free radical species, thus reducing the number of free radicals available as well as oxidative stress-related diseases of major organs such as liver, kidney and heart. Further investigation could be done on the isolation and identification of antioxidative components in *Cytisus scoparius*.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to University Grants Commission (UGC), India; Department of Biotechnology (DBT), Govt of India; Department of Science and Technology (DST), Govt of India for providing financial assistance through research projects to the School of Natural Product Studies, Jadavpur University. Financial assistance from All India Council for Technical Education, Government of India, New Delhi is gratefully acknowledged.

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