Trakia Journal of Sciences, Vol. 10, Suppl. 1, 17-24, 2012

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

A COMPARATIVE EVALUATION OF AN ANTIOXIDANT OF NATURAL ORIGIN DERIVED FROM *SILYBUM MARIANUM* CHARACTERIZED BY *IN VITRO* ASSAYS AND ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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ABSTRACT

Purpose: - Estimation of Antioxidant and radioprotective efficacy of silymarin: An *in vitro* study
Methods: - The study shows the efficacy of silymarin as an antioxidant by calculating ABTS, DPPH, EPR studies and radioprotective efficacy was performed by analysis of Micronuclei count.
Results: - The value of free radical scavenging activities of silymarin against DPPH (>65%) and ABTS (22%) were evaluated. Based on the calculated g values, the strong EPR signal stability shows the antioxidant activity of silymarin. Reduction in micronucleus count was seen at 25µg/ml with 2Gy γ-irradiation in HEK cells.
Conclusions: - Silymarin has the capacity of minimizing the deleterious of radiation. Due to its non-toxic nature and safety in humans, silymarin could be used as a radiation countermeasure agent (RCM) agent during nuclear/radiological emergencies.

Key words: Antioxidant, Radioprotection, Radiation, Micronuclei, HEK cells.

INTRODUCTION

Among the environmental stresses, both ionizing and non-ionizing radiation have been reported to induce free radicals. Free radicals including reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-), hydroxyl radicals (HO·), hydrogen peroxide (H_2O_2) and peroxyl radicals (RCOO·) are active oxygen components that are often generated during aerobic conditions (1). Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms (2). They are short lived known to cause damage to cellular components including lipid, DNA, protein, carbohydrate and other biological molecules (3). In recent years, antioxidants have been subjected to many studies that have related their consumption to a reduction in the incidence of oxidative damage related to radiation. Therefore, attention has been focused on the use of antioxidant-specially natural herbal origin antioxidant for reduction in the radiation-induced free radical and improvement of human health (4). Kidney is one of the most vital organs of human body. Radiation induced kidney problems is a common symptoms of acute radiation. Silymarin, derived from *Silybum marianum (L.) Gaertner* (common name Milk thistle) worldwide belongs to the family

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Asteraceae/Compositae. It contains mainly flavonolignans with small amounts of flavonoids and other polyphenolic compounds is widely used as a hepatoprotectant and as a supportive therapy of liver disorders (5) (6). Flavonoids are phenolic compounds of plant origin in which antioxidant properties have been attributed (7). This may be attribute due to their ability to scavenge free radicals and to chelate metal ions (8). Silymarin is a mixture of four structural components: silybin, silidianin, silychristin and Silipide (9) (10) and it was first isolated by using reversed phase-HPLC method (11). Silybin has structural similarity with steroidal hormones and thereby acts in protein synthesis (12). Such potential was attributed to its ability to maintain the cell fluidity (13), to the hepatocyte Ca^{2+} content (14), to enhanced protein and DNA synthesis, and to its anti-inflammatory ability (15), and ability to modulate the hepatic detoxification machinery (16). Silymarin interferes with promotion and progression of cancer. It shows direct anticancer effects against prostate, breast, and ectocervical tumor cell line of the active constituents of silymarin (17) (*i.e.,* silibinin) is known for its radioprotective efficacy on Plasmid and cellular DNA (18).

Keeping in view the multifarious properties of silymarin, the present study was done using standard procedures to evaluate the radioprotective properties of silymarin with a view to elucidating the mechanism of action.

MATERIAL AND METHODS

Herbal Material

The milk thistle powder was obtained from Wuxi Gorunjie Technology Co. Ltd, Jiangsu, China. The sample was stored in a cool and dry place away from strong light and heat as per the prescribed information of the manufacturer.

Irradiation

A ^{60}Co gamma source (Gamma cell 5000, Board of Radiation and Isotope Technology, Mumbai, India) at a dose rate of 1.4 kGy/h was used as source of irradiation in the liposomal based membrane protection assay and HEK cells were grown in 35 mm petri dishes, and irradiated using ^{60}Co gamma chamber (Bhabhatron-II Telecobalt unit – BARC, Mumbai, India; Dose rate of 1.4 Gy/min) at room temperature. Fresh air was circulated in the chamber throughout the course of irradiation.

DPPH radical scavenging activity

The free radical scavenging activity using DPPH was evaluated (19). The inhibitory effect of different concentrations of silymarin on DPPH was measured by recording the absorbance of the reaction mixture at 517 nm, continuously for 5 min.

ABTS radical scavenging activity

The ABTS diammonium salt radical cation decolourization test is also a radical scavenging test. (20). The reaction mixture contained ABTS with varying concentrations of silymarin. The absorbance was measured at 734 nm against PBS as control.

Micronuclei reduction

HEK cells, treated with different concentrations of silymarin, were washed twice with in phosphate buffer saline (PBS, pH 7.2; Himedia, India) and fixed in 70% ethanol at 4°C for 2-4h. Fixed cells were stained with 10μg/ml Propidium iodide (Sigma, USA) in phosphate buffer ($Na_2HPO_4.2H_2O$, 0.5% tween-20 and 0.1M Citric acid) final pH 7.4 for 30min in dark at room temperature (21). After washing the excess stain with distilled water, the slides were observed under fluorescence microscope (Olympus BX60, Japan) using UV excitation filter. A total of 1000 cells in duplicates were scored per group.

Electron paramagnetic resonance spectroscopy analysis

For all EPR measurements an X-band EMXmicro, EPR spectrometer (Bruker, Germany) equipped with standard Resonator were performed. Quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (i.d. 3mm) that was placed in the EPR cavity. All EPR experiments were performed at room temperature (18-230C), air conditions and relative humidity 40 %, were carried out in triplicate and repeated. Spectral processing (g-value calculation, spectra intensity) were performed using Bruker WIN-EPR and SimFonia software.

Direct EPR spectroscopy study of Silymarin powder and ethanol solution form were recorded before and after 2hr of irradiation in the range of light-waves from 290 nm to 320 nm using Transilluminator - 4000 (Stratagene, USA). EPR settings were the follows: center field 3513,5 G, sweep width 120 G, modulation amplitude 10.00 G, microwave power 1.28 mW, gain 2 x 103, time constant

40.96 ms, sweep time 61.44 s, 10 scans per sample. EPR spectrum of the irradiated powder was recorded at the following EPR settings: center field 3513,5 G, sweep width 120 G, modulation amplitude 10.00 G, microwave power 1.28 mW, gain 2 x 103, time constant 40.96 ms, sweep time 61.44 s, 1 scans per sample.

RESULTS

Antioxidative property of Silymarin by using DPPH and ABTS scavenging activity

The primary basis of free radical mechanism was evaluated using ABTS and DPPH free radical scavenging activity models. In the former case, silymarin exhibited 22% activity at 100ng/ml and increased in a concentration-dependent manner **(Figure 1b),** while in the latter case, the activity was found to be 67%. Concluding the DPPH scavenging activity, silymarin exhibited significantly ($p < 0.05$) higher activity as compared to ascorbic acid, which was used as standard **(Figure 1a)**. A difference of more than 30% activity in free radical modulation was observed in both models with higher activity in the DPPH model revealing its broad spectrum action against various free radicals.

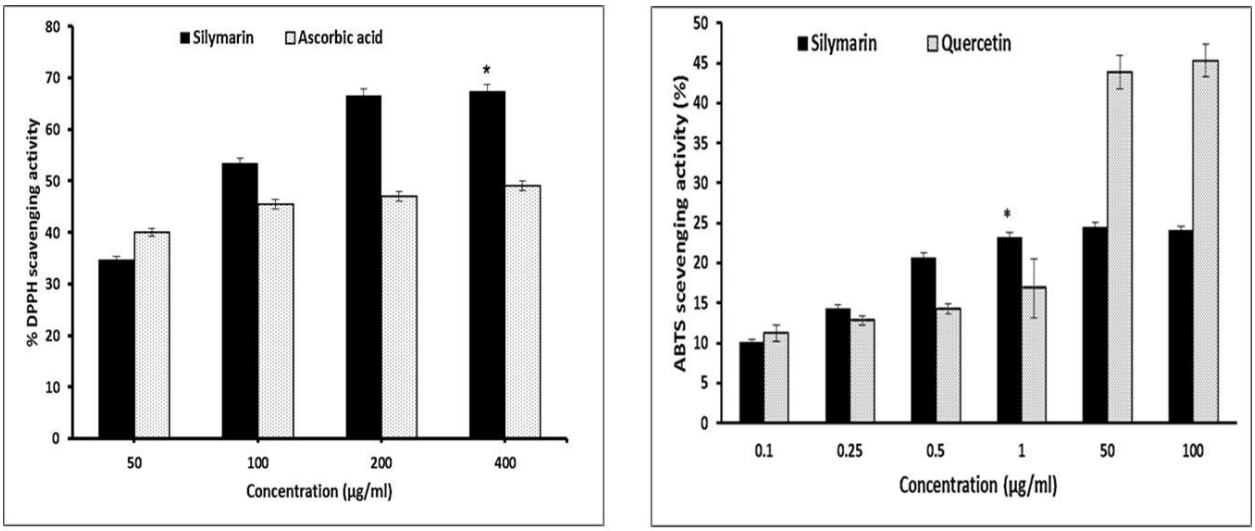


Figure 1(a). Percentage of DPPH scavenging activity in comparison to the standard ascorbic acid
(b). Percentage of ABTS scavenging activity in comparison to the standard quercetin

Radioprotective efficacy of silymarin by calculating Micronuclei frequency

Radioprotective ability of silymarin was estimating micronuclei frequency using HEK cells. The micronuclei (22) formation frequency in HEK cells was evaluated after 2Gy of γ-irradiation or in combination with silymarin (25µg/ml). 2Gy γ-irradiation significantly increased the MN formation in cells as compared to control ($p < 0.05$), as well as in silymarin alone group also at all concentrations. Pre-treated irradiated silymarin also resulted in decrease in MN formation as compared to irradiated group. Silymarin alone (25µg/ml) decreased MN frequency by nearly three-times as compared to irradiated control, exhibiting its nontoxic nature. Silymarin elicited maximum reduction (69%) in the radiation-induced MN induction at 72h time-point **(Figure 2)**. Maximal reduction in micronuclei was observed at an optimum dosage of 25µg/ml and optimal pre-treatment time of 0.5h.

Electron paramagnetic resonance spectroscopy

Radiation degrades the membrane of the cells, the wall of the cells, and so facilities a more efficient release of the extractable substances (23). EPR spectra registered in the non-irradiated powder and and after 2h- UVB- radiation of Silymarin are presented on **Figure 5**.

As is seen the EPR spectra registered in non-irradiated powder studied samples exhibited almost a symmetrical single EPR spectral line with g values - 2.00597± 0.0002 at 3513 G magnetic field, intensity of the EPR signal DI/N-1.826 (in arbitrary units). After 2 hours UVB-irradiation, EPR spectra registered in powder samples of Silymarin exhibited EPR singlet signal with g values - 2.00586± 0.0002 at the same magnetic field and intensity of the signal DI/N- 1.431, correspondingly **(Figure 3)**.

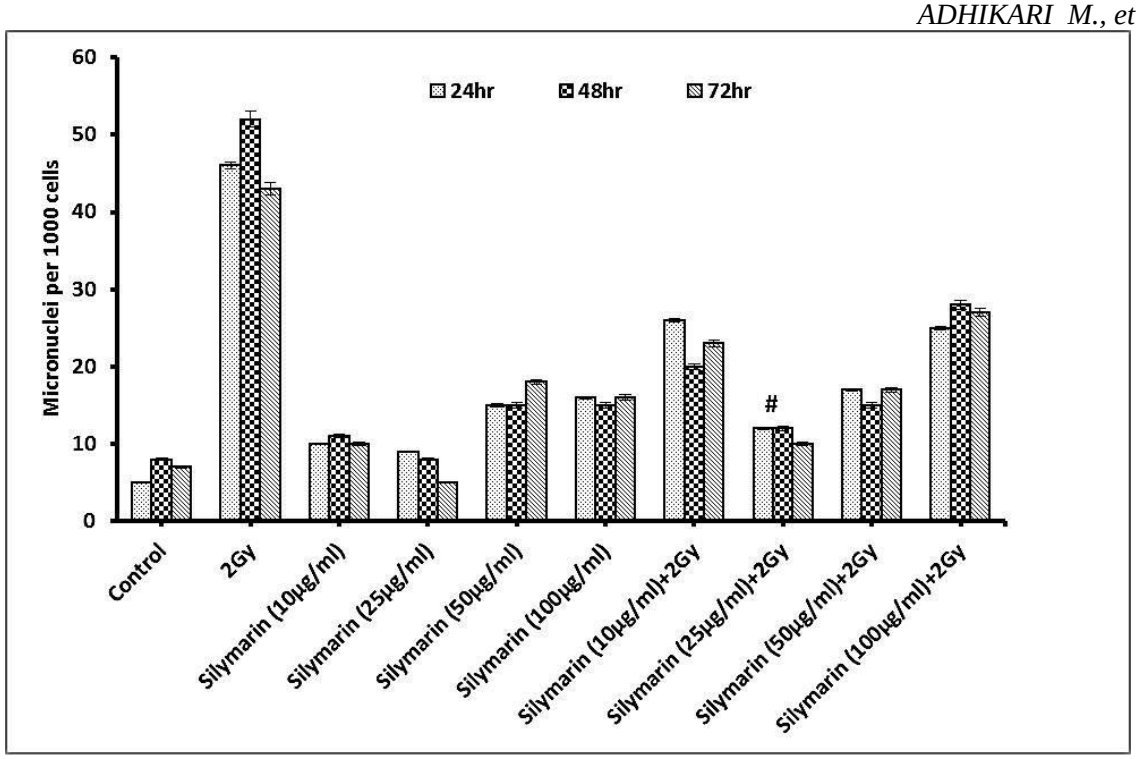
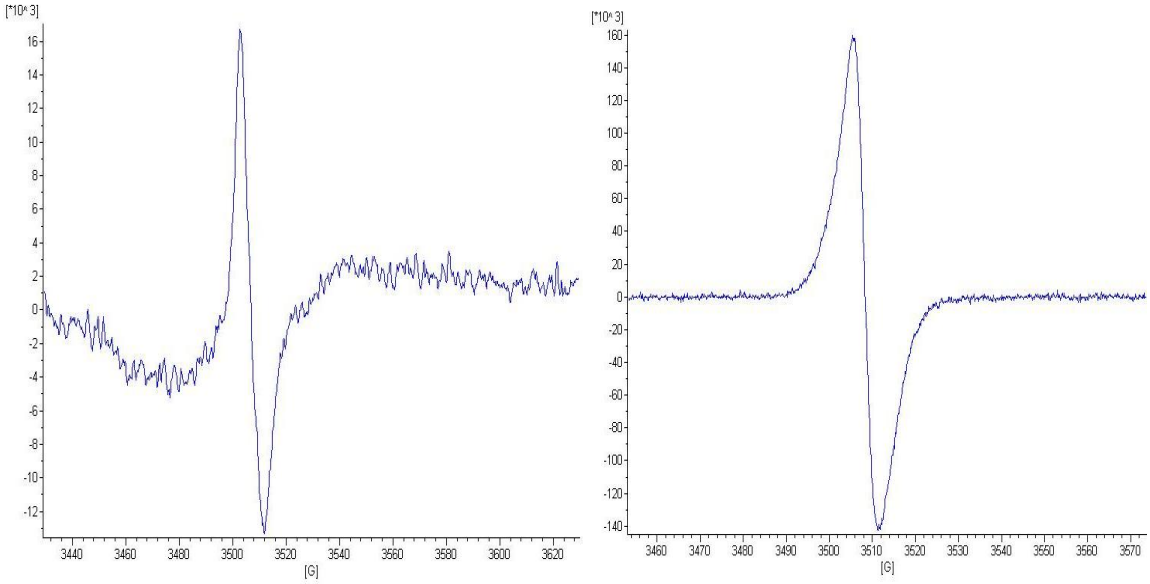


Figure 2. Micronuclei frequency calculated in HEK cells per 500 cells. Pre-treated silymarin group significantly showing decline in MN frequency as compared to Irradiated group in all time-points. Results are expressed as mean of replicates (in thrice) from three independent experiments. *Significant ($p < 0.001$) as compared to unirradiated control at various time point



(a) (b)

Figure 3. EPR spectrum of silymarin in powder form before (a) and after 2h of irradiation (b).

EPR spectra registered in the solution form before and after 2h- radiation of silymarin are presented on **Fig. 4.**

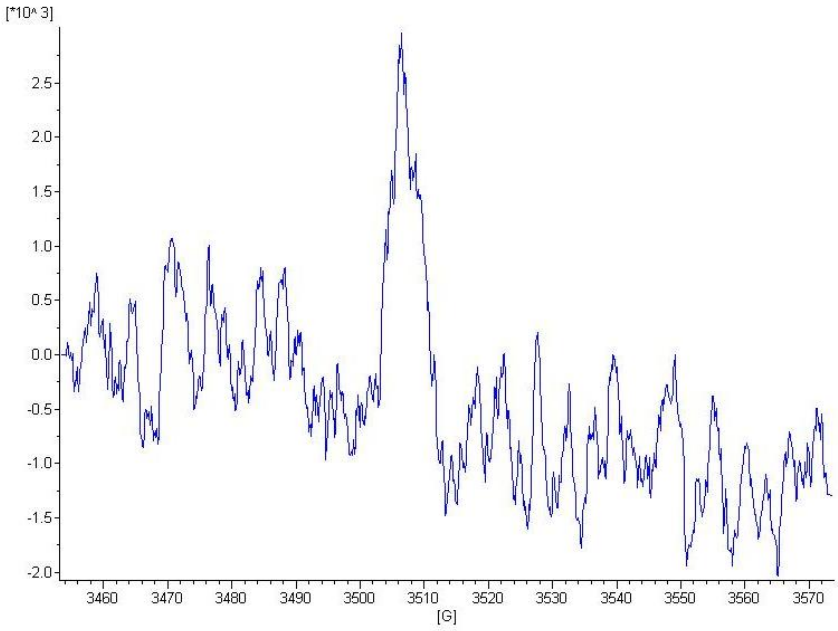


Figure 4. EPR spectrum of silymarin in solution form before irradiation.

As is seen the EPR spectra registered in non-irradiated powder studied samples exhibited almost a symmetrical single EPR spectral line with g value - 2.00597± 0.0002 at 3513 G magnetic field, intensity of the EPR signal DI/N- 1.826 (in arbitrary units). After 2 hr irradiation, EPR spectra registered in s powder samples of silymarin exhibited EPR singlet signal with g values - 2.00586± 0.0002 at the same magnetic field and intensity of the signal DI/N- 1.431, correspondingly (**Figure 4**).

Moreover, the intensities of the EPR signals, their g values and the presence of high numbers of polyphenols in the structure registered in powdered or in solution form of silymarin were not affected by the irradiation ($p > 0.05$) which means that the radical structure presenting in silymarin is rather stable to the radiation treatment.

It is well known that the stable radical DPPH can react with antioxidants that possess functional groups donating ·H and/or with free radicals and these interactions might be written as:

$$DPPH^{\cdot} + AH = DPPH\text{-}H + A^{\cdot} \text{ or } DPPH^{\cdot} + R^{\cdot} = DPPH\text{-}R$$

It was also reported that a number of natural antioxidants and synthetic compounds possessing phenolic groups in their structures could generate unstable radicals after oxidation (24).

Results obtained from determination of the DPPH radical scavenging capacity of silymarin, after 2h of irirradiation by the EPR spectroscopy technique are present on **Figure 4**. After addition, the corresponding tested samples concentration no changes in the shape of the DPPH spectrum was found but relative intensity of the spectrum was decreased. Maximum scavenging ability for non-irradiated silymarin solution form (83.48% ± 6,503%) was observed at the highest concentration (50 μl/mI) as compared to the maximum activity of irradiated form (81,87 ± 6,3781%) at the same concentration.

It was established that before irradiation the percent of the scavenged DPPH radicals statistical significant increased when the concentration of silymarin increased ($p < 0.05$). The same dependence was demonstrated for the irradiated samples (**Figure 5**).

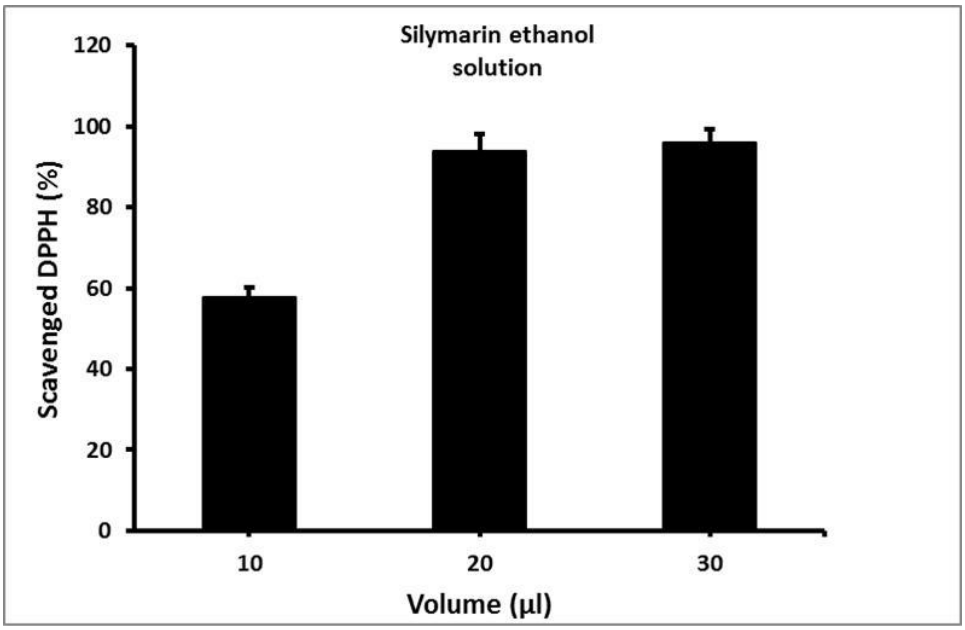


Figure 5. The percent of DPPH radical scavenging capacity of Silymarin ethanol solution after 5Gy irradiation at different concentrations.

For every studied concentration of silymarin, either before or after irradiation, there was not found statistical significant difference in the percent of the scavenged DPPH radicals. This result was in accordance with our finding that the free radical structure presenting in silymarin was not affected by radiation treatment for the period of 2hr and probably the same structure was involved in the reaction with DPPH.

DISCUSSION

The continued quest to develop ideal radioprotective agents has, with reference to designing appropriate modifiers of radiation response, led to an enhanced understanding of free radical biology. This led to the screening of various natural plant products as bioactive constituents for the development of future radioprotective formulations. The focus of the present study was to evaluate the antioxidant and radiomodulatory potential of silymarin.

An imbalance of generation of ROS with respect to inactivation caused by internal/external effects/stimuli or by radiation stress may lead to oxidative stress. The underlying basis of free radical mechanism was evaluated using ABTS and DPPH free radical scavenging activity models (**Figure 1**). A difference of more than 30% activity in free radical modulation was observed in both models with higher activity in the DPPH model revealing its broad spectrum action against various free radicals.

In order to determine radiation-induced damage on critical normal tissues, we selected HEK cells and check radiation-induced DNA damage of Micronuclei (22) assay with combinations of radiation and silymarin at different dose concentration (10-100µg/ml). Gamma radiation known to induce genotoxic damage to DNA, chromosomes, and the nucleus. Micronuclei frequency used as a biological dosimetry now days for scoring dicentric chromosomes in the field of radiation protection (25). The current study revealed that 25µg/ml concentration of silymarin is most appropriate dose as it demonstrates the non-toxic nature of silymarin towards HEK cells. In the current study, irradiation (2Gy) enhanced the frequency of MN in HEK cells significantly in comparison to control. Pre-irradiation (0.5h) treatment with silymarin substantially countered this upsurge in MN frequency thereby shielding them against radiation-induced DNA damage. By the present EPR in vitro spectroscopy studies, we have demonstrated that silymarin behaves in vivo as a good antioxidant. Moreover, after irradiation its antioxidant activity increases, presumably due to the generation of considerably stable free radicals in the chemical composition, confirmed by the EPR spectra registered.

ACKNOWLEDGMENT

Authors are grateful to Dr. W. Selvamurthy, Chief Controller, Research and Development (Life Sciences and International Cooperation) and Dr. R.P. Tripathi, Director, INMAS for support in multifarious ways. Thanks are also to Department of Science and Technology, Delhi, India for providing resources under the Indo-Bulgarian Joint Collaborative Project.

REFERENCES

1. Halliwell B. and Gutteridge J. M. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219: 1-14, (1984).
2. Apel K. and Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55: 373-399, (2004).
3. Martin K. R. and Barrett J. C. Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signaling versus high-dose toxicity. Hum Exp Toxicol 21: 71-75, (2002).
4. Liu F. and Ng T. B. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sci 66: 725-735, (2000),Zheng W. and Wang S. Y. Antioxidant activity and phenolic compounds in selected herbs. J *Agric Food Chem* 49: 5165-5170, (2001).
5. Ball K. R. and Kowdley K. V. A review of Silybum marianum (milk thistle) as a treatment for alcoholic liver disease. *J Clin Gastroenterol* 39: 520-528, (2005).
6. Kren V. and Walterová D. Silybin and silymarin - new effects and applications. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 149: 29-41, (2005).
7. Bors W., Heller W., Michel C. and Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* 186: 343-355, (1990).
8. Saran M. and Bors W. Radical reactions in vivo - an overview. *Radiation and Environmental Biophysics* 29: 249-262, (1990).
9. Valenzuela A. and Garrido A. Biochemical bases of the pharmacological action of the flavonoid silymarin and of its structural isomer silibinin. *Biol Res* 27: 105-112, (1994).
10. Wagner H., Hörhammer L. and Münster R. [On the chemistry of silymarin (silybin), the active principle of the fruits from Silybum marianum (L.) Gaertn. (Carduus marianus L.)]. *Arzneimittelforschung* 18: 688-696, (1968).
11. Kim N., Graf T. N., Sparacino C. M., Wani M. C. and Wall M. E. Complete isolation and characterization of silybins and isosilybins from milk thistle (Silybum marianum). *Org Biomol Chem* 1: 1684-1689, (2003).
12. Kosina P., Kren V., Gebhardt R., Grambal F., Ulrichová J. and Walterová D. Antioxidant properties of silybin glycosides. *Phytother Res* 16 Suppl 1: S33-39, (2002).
13. Muriel P. and Mourelle M. The role of membrane composition in ATPase activities of cirrhotic rat liver: effect of silymarin. *J Appl Toxicol* 10: 281-284, (1990).
14. Farghali H., Kameniková L., Hynie S. and Kmonicková E. Silymarin effects on intracellular calcuim and cytotoxicity: a study in perfused rat hepatocytes after oxidative stress injury. *Pharmacol Res* 41: 231-237, (2000).
15. Dehmlow C., Murawski N. and de Groot H. Scavenging of reactive oxygen species and inhibition of arachidonic acid metabolism by silibinin in human cells. *Life Sci* 58: 1591-1600, (1996).
16. Baer-Dubowska W., Szaefer H. and Krajka-Kuzniak V. Inhibition of murine hepatic cytochrome P450 activities by natural and synthetic phenolic compounds. *Xenobiotica* 28: 735-743, (1998).
17. Kakkar P., Das B. and Viswanathan P. N. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* 21: 130-132, (1984).
18. Tiwari P., Kumar A., Ali M. and Mishra K. P. Radioprotection of plasmid and cellular DNA and Swiss mice by silibinin. *Mutat Res*: (2009).
19. Cuendet M., Hostettmann K., Potterat O. and Dyatmiko W. Iridoid Glucosides with Free Radical Scavenging Properties from Fagraea blumei. *Helvetica Chimica Acta* 80: 1144-1152, (1997).
20. Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26: 1231-1237, (1999).
21. Schmid W. The micronucleus test. *Mutation research* 31: 9-15, (1975).
22. Puri S. C., Nazir A., Chawla R., Arora R., Riyaz-Ul-Hasan S., Amna T., Ahmed B.,

Verma V., Singh S., Sagar R., et al. The endophytic fungus Trametes hirsuta as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. (2006).

23. Thariat J., Collin F., Marchetti C., Ahmed-Adrar N. S., Vitrac H., Jore D. and Gardes-Albert M. Marked difference in cytochrome c oxidation mediated by HO• and/or O2•− free radicals in vitro. *Biochimie* 90: 1442-1451, (2008).
24. Mason R. P., Hanna P. M., Burkitt M. J. and Kadiiska M. B. Detection of oxygen-derived radicals in biological systems using electron spin resonance. *Environ Health Perspect* 102 Suppl 10: 33-36, (1994).
25. Vral A., Fenech M. and Thierens H. The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure. *Mutagenesis* 26: 11-17, (2011).