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Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7,12 dimethyl benz(*a*)anthracene-induced breast cancer in female Sprague Dawley rats

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Abstract

Breast cancer is one of the most common cancers in women of developed and developing countries. The optimum management of which requires a multidisciplinary approach including the use of certain biochemical and molecular markers. The effect of propolis along with paclitaxel on 7,12 dimethyl benz(*a*)anthracene (DMBA) induced experimental breast cancer was investigated in female Sprague Dawley rats. Female Sprague Dawley rats were divided into five groups of six animals each. Group I served as normal control animal. Group II animals received DMBA (20 mg in 0.5 ml sunflower oil and 0.5 ml of saline) i.p. to develop mammary tumor by the end of 90 days. Group III were breast cancer animals treated with 33 mg paclitaxel/kg body weight (bw) weekly once for 4 weeks. Group IV were breast cancer-bearing animals treated with 50 mg propolis/kg bw for 30 days. Group V were breast cancer-bearing animals treated with both paclitaxel and propolis as mentioned above. Administration of paclitaxel and propolis effectively suppressed breast cancer, which is revealed by the decrease in the extent of lipid peroxidation (LPO) with concomitant increase in the activities of enzymic antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) and non-enzymic antioxidants (reduced glutathione (GSH), Vitamin C and Vitamin E) levels when compared to breast cancer-bearing animals treated with either paclitaxel or propolis alone. From our results, we conclude that propolis is a potent antioxidant and, when given in combination with paclitaxel, offers maximum protection against DMBA induced mammary carcinogenesis.

Keywords: Propolis; Paclitaxel; DMBA; Breast cancer

Introduction

Breast cancer is the second most prevalent cancer worldwide and their incidence increases gradually (Parkin et al., 1997). Women who have stage IV breast cancer receive chemotherapy and/or hormonal therapy to destroy cancer cells and control the disease. The goal of chemotherapy is to shrink primary tumors, slow the tumor growth, and to kill cancer cells that may have spread (metastasized) to other parts of the body from the original tumor. Chemotherapeutic drugs elicit some toxicity towards normal cells also, that limits its usage.

Paclitaxel is an effective anticancer agent against a variety of human tumors, including non-small cell lung cancer (NSCLC), ovarian cancer, breast cancer, head and neck cancer, and melanoma (Rowinsky et al., 1990). Since the mid-1990s, paclitaxel has been widely used for locally advanced, metastatic and recurrent breast cancers and has demonstrated significant efficacy. Being a chemotherapeutic agent it has some side effects such myleosuppression that affects the immune system. Researchers are exploring ways to reduce the side effects of treatment to improve the quality of patients' lives, and reduce pain.

Propolis or bee glue is a resinous substance collected by bees from plant sources. More than 150 polyphenolic compounds like flavonoids, phenolic acid and their esters have been identified as constituents of propolis (Greenaway et al., 1991). In vivo and in vitro studies reveals the free radical scavenging (Pascual et al., 1994), antioxidant (Jaiswal et al., 1997), and antitumor (Scheller et al., 1989) and immunomodulatory (Ivanovska et al., 1995) activities of propolis. Biological actions of propolis are mainly due to its flavonoid content and reported to inhibit colon, tongue, skin and mammary cancers (Takino and Mochida, 1982). Ethanolic extract is found

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to be more effective in inhibiting mammary carcinogenesis than the aqueous extract (Kawabe et al., 2000). PM-3 (3-[2-dimethyl-8-(3-methyl-2-butenyl) benzopyran]-6-propenoic acid) isolated from propolis markedly inhibits the growth of MCF-7 human breast cancer cells (Luo et al., 2001). Reactive oxygen species (ROS) are involved in the initiation and progression of carcinogenesis. Moreover, the ROS-induced oxidative damage causes a decrease in the efficiency of antioxidant defense mechanism. In recent years, there has been a growing interest in studying the role played by lipid peroxidation (LPO) and antioxidant status. Proper balance between LPO and antioxidants should be maintained in the cell because of their potential importance in the pathogenesis of various pathologic diseases including cancer. Neoplastic cells may sequester essential antioxidants from circulation to supply the demands of growing tumor. The purpose of the present study is to evaluate the combined effect of paclitaxel and propolis against the 7,12 dimethyl benz(*a*)anthracene (DMBA)-induced mammary carcinogenesis. Therefore, the aim of our study was to assess the LPO and antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamin C and vitamin E in breast cancer-bearing animals and compare with the paclitaxel- and propolis-treated animals.

Materials and methods

Chemicals

7,12 Dimethyl benz(*a*)anthracene, reduced glutathione, dinitrophenyl hydrazine, bathophenanthroline and propolis were purchased from Sigma Chemical Company, USA. All the other chemicals used were of analytical grade.

Animals

Female Sprague Dawley rats aged between 50 and 55 days was procured from National Institute of Nutrition, Hyderabad and was housed in plastic cages. The animals were maintained under controlled environmental condition on alternative 12-h dark/light cycle. Commercial pelleted feed by Ms Hindustan lever Ltd., Mumbai under the name "Gold Mohr" and water ad libitum were given to animals. This research work on Sprague Dawley rats was sanctioned and approved by the institutional animal ethical committee (IAEC No. 02/001/01)

Experimental setup

The animals were divided in to five groups of 6 animals each. Group I animals served as control, Group II were animals treated with 20 mg of DMBA in 0.5 ml sunflower oil and 0.5 ml saline to induce breast cancer. After 90 days of tumor induction Group III animals were treated with paclitaxel (33 mg/kg body weight (bw)) once a week for 4 weeks. Group IV animals were treated with ethanolic extract of propolis (EEP; 50 mg/kg bw) for 30 days. Group V animals were treated with both paclitaxel and propolis (as in Group III and Group IV) after the induction of breast cancer.

After the experimental period, the animals were sacrificed by cervical decapitation. Breast and liver tissues were immediately excised. A 10% homogenate was prepared in Tris–HCl buffer 0.1 M pH 7.4 using Potter Elvehjem homogeniser with Teflon pestle. The tissue homogenates of breast and liver were used for the following parameters.

Biochemical analysis

Tumor weight was estimated according to the method of Geren et al. (1972). The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axes were measured with vernier caliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by 2.

Lipid peroxidation was analyzed by the method of Ohkawa et al. (1979) and total protein by the method of Lowry et al. (1951). The antioxidant enzymes superoxide dismutase was analyzed by Marklund and Marklund, 1974. The assay mixture contains 1.0 ml of pyrogallol–Tris–DETPA, 0.2 ml of tissue homogenate and 0.8 ml of H_2O. The rate of pyrogallol autoxidation is taken from the increase in absorbance at 420 nm. Catalase was assayed by the method of Sinha (1972). The reaction was started by the addition of 0.4 ml of hydrogen peroxide to the reaction mixture containing 1.0 ml of phosphate buffer and 0.1 ml of enzyme solution. The utilization of H_2O_2 by the enzyme was read at 620 nm. GPx activity was assayed by the method of Rotruck et al. (1973) with modifications. The reaction mixture in a total volume of 1.0 ml containing 0.2 ml of 0.4 mol/l phosphate buffer (pH 7.0), 0.2 ml of 0.4 mmol/l EDTA, 0.1 ml of 10 mmol/l sodium azide and 0.2 ml of tissue homogenate was incubated with 0.1 ml of H_2O_2 and 0.2 ml of glutathione for 10 min. Oxidation of glutathione by the enzyme was read at 420 nm.

The non-enzymic antioxidant reduced glutathione was analyzed by the method of Moron et al. (1979). 1.0 ml of homogenate was precipitated with 1.0 ml of TCA and the precipitate was removed by centrifugation. To 0.5 ml of supernatant, 2.0 ml of DTNB was added and the total volume was reached to 3.0 ml with phosphate buffer. The absorbance was read at 412 nm. Vitamin E was measured by the method of Desai (1984). To 0.5 ml of tissue homogenate, 0.5 ml of H_2O and 1.0 ml of TCA were added, mixed thoroughly and centrifuged. To 1.0 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37 °C for 3 h. Then, 1.5 ml of sulphuric acid was added and mixed well and the solution was allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm. Vitamin C was measured by the method of Omaye et al. (1979) in which 1.0 ml of homogenate and 1.0 ml of ethanol was added and mixed thoroughly. Then 3.0 ml of petroleum ether was added and shaken rapidly and centrifuged. 2.0 ml of supernatant was taken and evaporated to dryness. 0.2 ml each of bathophenanthroline, ferric chloride and *o*-phosphoric acid were added and the volume was reached to 3.0 ml with ethanol. The colour developed was read at 530 nm.

Table 1
Effect of paclitaxel and propolis on body and tumor weight of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Body weight (g)	180.00±17.00	135.00±13.00a***	160.00±15.00b**	155.00±15.00b*	170.00±13.00b***
Tumor weight (g)	–	7.03±0.59	3.72±0.31a***	5.51±0.47a***	3.37±0.32a***

Each value is expressed as mean±S.D. for six animals in each group.
Body weight: a, as compared with Group I; b, as compared with Group II.
Tumor weight: a, as compared with Group II.
Statistical significance: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was used followed by the Newman–Keuls multiple comparison test.

Results

Table 1 shows the body and tumor weight of animals in different experimental groups. There appears to be a significant ($p<0.001$) decrease in the body weight of the tumor-induced (Group I) animals, which was found to be increased in drug-treated groups. Paclitaxel- and propolis-treated Group V animals show a significant ($p<0.001$) increase in the body weight when compared with paclitaxel-treated Group III ($p<0.01$) or propolis-treated Group V ($p<0.05$) animals. Accordingly, the high tumor weight found in the Group II animals was also significantly ($p<0.001$) decreased on treatment with paclitaxel and propolis (Group V). Paclitaxel treatment (Group III) and propolis treatment (Group IV) also reduced the tumor burden significantly ($p<0.001$) when compared with Group II cancer-bearing animals.

Fig. 1 shows the levels of lipid peroxidation in the breast and liver tissues of various experimental groups. A highly significant increase ($p<0.001$) in the lipid peroxidation level was observed in the tumor-bearing rats (Group II) when compared with Group I control animals. This was significantly ($p<0.001$) reversed to near normal levels in EEP- (50 mg/kg bw) and paclitaxel- (33 mg/kg bw) treated animals (Group V) than the animals treated with paclitaxel (Group III) and EEP alone (Group IV) alone.

Figs. 2 and 3 depict the activities of enzymic antioxidants like SOD, CAT, GPx and non-enzymic antioxidants like GSH, vitamin C and vitamin E in the breast tissue of control and experimental animals. Group II cancer-bearing animals show a significant decrease ($p<0.001$) in the activities of these antioxidants when compared with Group I control animals. On treatment with paclitaxel, Group III animals showed a significant ($p<0.001$) increase in the levels of GSH and vitamin E, whereas the increase was significant at the level of $p<0.01$ in the case of SOD, CAT, GPx and at $p<0.01$ in the case of vitamin C. Propolis-treated Group IV animals showed a significant ($p<0.001$) increase in the activities of SOD, CAT and GSH, vitamin E whereas the increase was significant at the level of $p<0.01$ in the case of GPx and vitamin C. Combination therapy of paclitaxel and propolis in Group V animals restored ($p<0.001$) the activities and the levels of this antioxidant system to near normalcy in the breasts of cancer-bearing animals.

Figs. 4 and 5 depict the activities of enzymic antioxidants like SOD, CAT, GPx and non-enzymic antioxidants like GSH, vitamin C and vitamin E in the liver tissue of control and

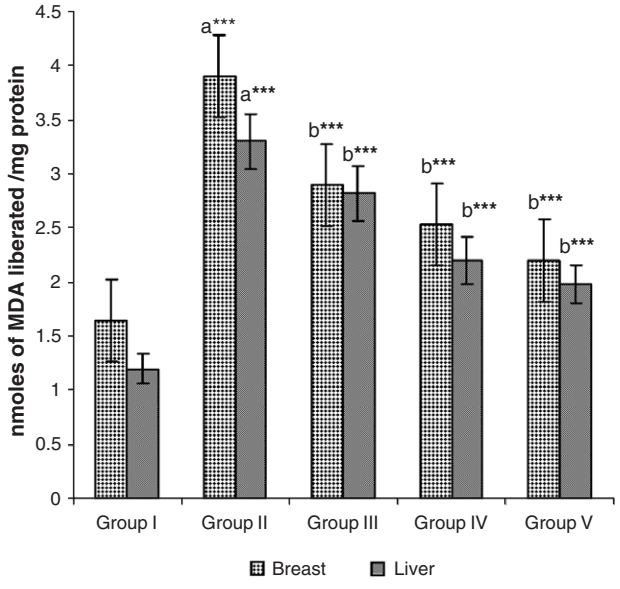

Fig. 1. Effect of paclitaxel and propolis on lipid peroxidation in the breast and liver of control and experimental animals. Each value is expressed as mean±S.D. for six animals in each group. a: as compared with Group I; b: as compared with Group II. Statistical significance: ***$p<0.001$.

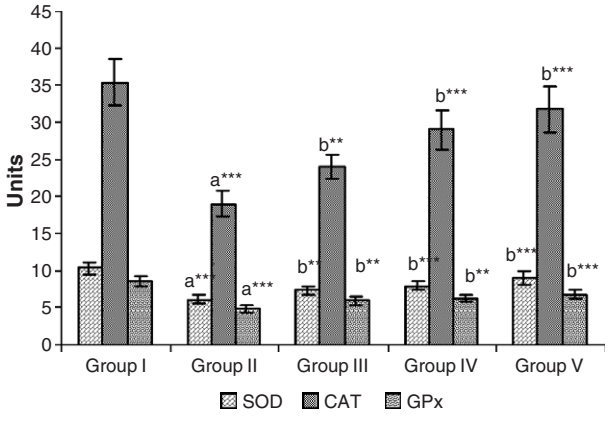

Fig. 2. Effect of paclitaxel and propolis on enzymic antioxidants in the breast of control and experimental animals. Units—SOD: units/min/mg protein. CAT: μmol of H_2O_2 liberated/min/mg protein. GPx: μmol of GSH oxidised/min/mg protein. a: as compared with Group I. b: as compared with Group II. Statistical significance: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

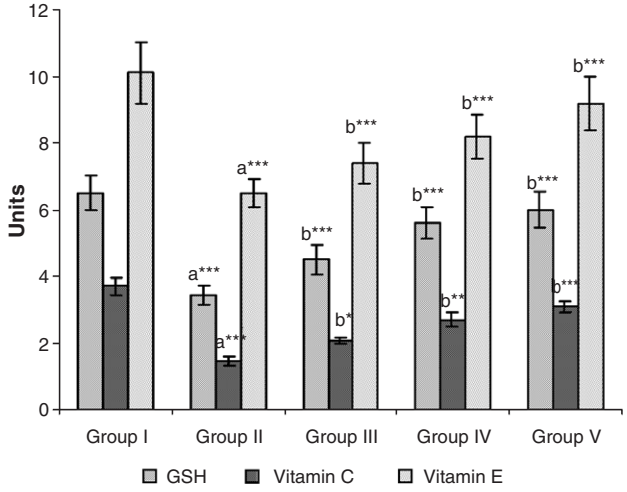

Fig. 3. Effect of paclitaxel and propolis on non-enzymic antioxidants in the breast of control and experimental animals. Each value is expressed as mean±S.D. for six animals in each group. Units—μg/mg protein. a: as compared with Group I. b: as compared with Group II. Statistical significance: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

experimental animals. Group II cancer-bearing animals show a significant decrease ($p<0.001$) in the activities of these antioxidants when compared with group I control animals. On treatment with paclitaxel, group III animals showed a significant ($p<0.001$) increase in the levels of SOD and GPS and in the levels of vitamin C and vitamin E, whereas the increase was significant at the level of $p<0.01$ in the case of catalase and GSH. Propolis administration showed a significant ($p<0.05$) increase in the activities of all the enzymic antioxidants and vitamin E where as the increase was significant at the level of $p<0.01$ in case of GSH and vitamin C. Combination therapy of paclitaxel and propolis in-group V animals restored ($p<0.001$) the activities and the

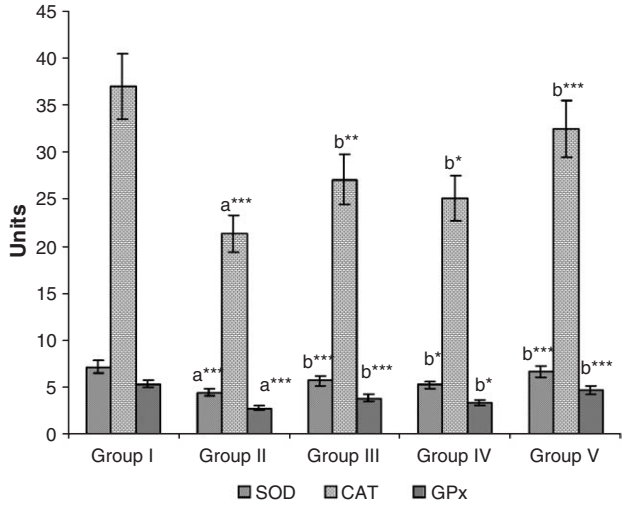

Fig. 4. Effect of paclitaxel and propolis on enzymic antioxidants in the liver of control and experimental animals. Each value is expressed as mean±S.D. for six animals in each group. Units—SOD: units/min/mg protein. CAT: μmol of H_2O_2 liberated/min/mg protein. GPx: μmol of GSH oxidised/min/mg protein. a: as compared with Group I. b: as compared with Group II. Statistical significance: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

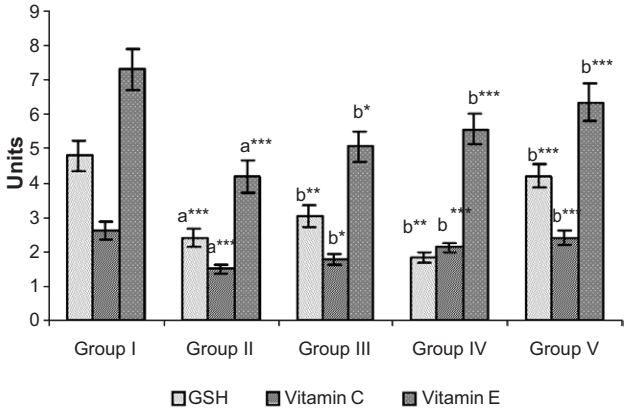

Fig. 5. Effect of paclitaxel and propolis on non-enzymic antioxidants in the liver of control and experimental animals. Each value is expressed as mean±S.D. for six animals in each group. Units—μg/mg protein. a: as compared with Group I. b: as compared with Group II. Statistical significance: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

levels of this antioxidant system to near normalcy in liver of cancer-bearing animals.

Discussion

Oxidative stress, especially lipid peroxidation is known to be involved in carcinogenesis (Trush and Kensler, 1991). Increased levels of lipid peroxidation products play a role in the early phases of tumor growth (Rice-Evans and Burdon, 1993). Faber et al. (1995) and Huang et al. (1999) have shown that the patients with breast cancer have higher MDA levels, which are regarded as indicators of lipid peroxidation when compared to controls. In the present study, an increase in the levels of lipid peroxidation was found in breast cancer-bearing animals and these were significantly reduced after treatment with paclitaxel and propolis.

Flavonoids inhibit lipid peroxidation at the initiation stage by acting as scavengers of superoxide anions and hydroxyl radicals (Torel and Cillard, 1986). It has been proposed that flavonoids terminate chain radical reactions by donating hydrogen atoms to the peroxy radical forming a flavonoid radical which in turn reacts with free radicals thus terminating the propagating chain (Afanas'ev et al., 1989; Robak and Grygleuski, 1988). Several research groups have reported that propolis in an extract form and their flavonoids constituent scavenges free radicals (Basnet et al., 1997; Shinohara et al., 2002).

Gonzalez et al. (1995) have reported that a single EEP injection reduces the increased serum lipid peroxide level in rats. It has been shown that various kinds of flavonoids are contained in propolis such as CAPE, quercetin that are known to inhibit LPO. Hence, our findings also suggest that EEP used in the present study exerts its antilipidperoxidative action by the flavonoid present in the extract.

Naturally there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that quench or scavenge them and protect the body against their deleterious effects (Kolanjiappan et al., 2002).

Hence, antioxidant status has been suggested as a useful tool in estimating risk of oxidative damage induced carcinogenesis.

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) constitute the enzymic antioxidant system, which scavenges ROS and lipid peroxidation. SOD is the only enzymes that disrupts superoxide radicals and are present in all cells with high amounts in erythrocytes (Beutlar and Gelbart, 1985). It protects the cells against superoxide- and hydrogen peroxide-mediated LPO. The malignant cells of different cancer types exhibit heterogeneity in the levels of oxidative stress, associated with various expression levels of SOD and other antioxidant enzymes. Decreased SOD activity was observed in various cancerous conditions (Van Driel et al., 1997; Selvendiran et al., 2003).

The enzymic antioxidant catalase is widely distributed in all tissues and catalyses the breakdown of hydrogen peroxide produced by tumor cells. The source of hydrogen peroxide is mainly SOD-mediated dismutation of superoxide radical, which is generated by various enzyme systems as well as by non-enzymic pathways. Several reports have cited decreased activities of SOD and catalase in various carcinogenic conditions (Thirunavukkarasu and Sakthisekaran, 2001; Floyd, 1982). In the present study, we have also observed a decline in SOD and catalase activities, which may be due to the increase in circulating lipid peroxides. This can result in accumulation of superoxide anion, a highly diffusible and potent oxidizing radical capable of traversing membranes causing deleterious effects at sites far from the tumor (Oberley and Buettner, 1979). The decreased activities of catalase found in the cancerous condition may be due to exhaustion of these enzymes in catalyzing the overproduction of hydrogen peroxide by the cancerous cells.

GPx is an equally important antioxidant, which reacts with hydrogen peroxide thus preventing intracellular damage caused by the same. Several studies have reported the decreased activities of GPx in various cancerous conditions. There was a decline in the activities of GPx in the present study, which may be due the altered antioxidant defense system caused by enormous production of free radicals in DMBA-induced carcinogenesis (Daniel and Joyce, 1983).

Neoplastic cells may sequester essential antioxidants from circulation to supply the demands of growing tumor (Ruddon, 1995). Vitamins C and E and reduced glutathione comprise the non-enzymic antioxidant system that protects the cells against free radicals and ROS. Antioxidant vitamins have a number of biological activities such as immune stimulation, scavenging the free radicals and alteration in metabolic activation of carcinogens (Van Poppel and Vanden Berg, 1997). They can utilize reactive oxygen metabolites, protecting biopolymers and reduce oxidative DNA damage (Halliwell, 1996). The major antioxidant that protects the cell is vitamin E. It is a potent oxygen radical scavenger that protects cells from carcinogenic chemicals by inhibiting LPO- and free radical-mediated consequences. In addition to its antioxidant properties vitamin E also functions as a biologic response modifier influencing the production of second messengers and products of arachidonic acid cascade which have profound effect on cell proliferation (Packer, 1995). Brigeluis-Flohe and Trabar (1999) have documented a positive correlation between vitamins E and C deficiency and LPO formation.

GSH is an important non-protein thiol which, in conjugation with GPx and GST, plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals (Obrador et al., 1997). GSH acts directly as a free radical scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radical. It also reduces peroxides and maintains protein thiols in the reduced state (Sies, 1986). Changes in the rate of cancer cell proliferation are accompanied by changes in their intracellular GSH levels and consequently these could be reflected in their antioxidant machineries (Navarro et al., 1999). The decrease in GSH levels in the present study may also be due to decrease in the substrate available for GSH synthesis.Flavonoids exhibit a wide range of biological effects including free radical scavenging and antioxidant activities (Hanasaki et al., 1994). Therapeutic activities of propolis depend mainly on the presence of flavonoids. The activities of enzymic and non-enzymic antioxidants were increased considerably on treatment with propolis. The antioxidative property of propolis is mainly due to its effect on GSH concentration. El-Khawaga et al. (2003) has also reported that ethanolic extract of propolis (EEP) enhances the levels of GSH and GST in cancer-bearing mice. These findings of elevated GSH levels by propolis suggest GSH dependent detoxification of free radicals. Propolis exerts antioxidant effects by reducing the concentration of lipid hydroperoxide and some of its compounds act as hydrophilic antioxidants and increase tissue concentration of vitamin C (Sun et al., 2000). Thus, the antioxidative property of propolis could very well be attributed to its flavonoid content (Pascual et al., 1994), which exerts its role through the enhancement of antioxidant system and acting as a free radical scavenger.

There is extensive evidence that administration of propolis for 14 days can increase the GSH level (El-Khatib et al., 2002). Since propolis is a complex mixture, its biological action in several instances are due not just to one compound but to a synergistic action of several components. In conclusion, the antioxidant properties of propolis could very well be attributed to their free radical scavenging activity against alkoxyl radicals. When given in combination with paclitaxel, it reduces the toxic side effects of the later by its immunomodulatory activity and improves the treatment strategy. Our data suggests that administration of EEP at a dose of 50 mg/kg bw with paclitaxel significantly decreases the toxic complications of chemotherapy and increases the levels of free radical scavenging enzymes.

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