

# Cytoprotective and immunomodulatory properties of Geriforte, a herbomineral preparation, in lymphocytes

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## Summary

The study was designed to determine the cytoprotective and immunomodulatory properties of Geriforte, an indigenous herbomineral compound, using lymphocytes as a model system. The possible involvement of free radicals and the ability of Geriforte to inhibit the oxidative process induced by tert-butylhydroperoxide (tert-BHP) was also investigated. The production of free radicals (evaluated by fluorescent probe fluorescein-diacetate), level of malondialdehyde (MDA, as index of lipid peroxidation), and levels of anti-oxidants – reduced glutathione (GSH) and glutathione peroxidase (GPx) were determined. There was an increase in cytotoxicity and apoptosis significantly in the presence of tert-BHP (100  $\mu$ M) over control. Addition of tert-BHP resulted in a marked increase in free radical production and MDA level with a concomitant decrease in GSH level in lymphocytes. Geriforte supplementation reduced cytotoxicity and apoptosis induced by tert-BHP. Further, Geriforte inhibited tert-BHP induced lipid peroxidation and maintained higher anti-oxidant levels. tert-BHP significantly inhibited the lymphocyte proliferation stimulated by mitogens (Lipopolysaccharide/Concanavalin A) and enhanced DNA fragmentation. Geriforte relieved the inhibitory effect of tert-BHP on lymphocyte proliferation and decreased DNA fragmentation appreciably. The results indicate that Geriforte possesses cytoprotective and immunomodulatory properties which could be due to its anti-oxidant activity.

**Key words:** Geriforte, herbomineral preparation, cytoprotection, immunomodulation, lymphocytes

## ■ Introduction

Living systems are able to regulate their defense mechanisms in response to oxidative damage. Free radicals and reactive oxygen species (ROS) generated in vivo are consumed by cellular and extracellular anti-oxidants to maintain homeostasis. However, imbalance in this equilibrium in favour of pro-oxidants, leads to oxidative stress which contributes to many disease processes including aging and cancer (Richards and Sharma, 1991).

The traditional medicine all over the world is nowadays reviewed by an extensive research on different plant species and their therapeutic principles. As plants

produce a lot of anti-oxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with anti-oxidant activity (Scartezzini and Speroni, 2000). Ayurveda, the Indian traditional health care system (ayus-life, veda-knowledge, meaning science of life), is the oldest medical system in the world which exploits the potential of various herbs as drugs. In recent years, the clinical importance of herbal drugs has received considerable attention. Geriforte, an indigenous herbomineral compound, is a mixture of several herbal extracts and minerals including amla (*Phyllanthus emblica* Linn), brahmi

(*Centella asiatica* Linn), asvagandha (*Withania somnifera* Dunal), haldi (*Curcuma longa* Linn), bhangra (*Eclipta alba* Linn), loh bhasma (iron oxide), jasad bhasma (zinc oxide), onion (*Allium cepa* Linn), garlic (*Allium sativum* Linn), grape (*Vitis vinifer* Linn), carrot (*Docus carota* Linn) and many other herbs (Grover et al., 1995). The preparation is being used as a restorative tonic to solve the problems of old age in India. It is reported to have health benefits including reduction in anxiety disorders (Upadhyaya et al., 1990) and age related enzymatic changes in liver and brain (Bardhan et al., 1985; Singh et al., 1994). It produces a better feeling of physical and mental well being, improves appetite and digestion, maintains positive nitrogen balance and cures menopausal symptoms (Bardhan et al., 1985).

Cells of the immune system are particularly sensitive to oxidative stress due to high level of polyunsaturated fatty acids in their plasma membranes (Meydani et al., 1995). Moreover, these cells are constantly exposed to ROS produced as part of their normal function. Therefore, immune cells form an excellent example for studying the ROS induced cellular damage by various insults. Keeping the above facts in view, the present study was designed to evaluate the cytoprotective and immunomodulating activities of Geriforte using lymphocytes as model system.

## Materials and methods

### Lymphocyte isolation

Sprague Dawley rats, weighing approximately 150–200 g were taken and killed by cervical dislocation. Splenocytes were isolated as described by Sairam et al. (1997). Briefly, spleens were aseptically removed and placed in sterile RPMI-1640 media. Single cell suspensions were prepared by disrupting the spleens into small pieces with scissors. Splenocytes were isolated by centrifugation at 3000 rpm and red cells were lysed by 0.84%  $\text{NH}_4\text{Cl}$ . Cells were washed twice with RPMI media and viability was determined by staining the cells with acridine orange and ethidium bromide (100  $\mu\text{g}/\text{ml}$  each). Later, splenocytes (viability > 90%) were suspended in RPMI 1640 media supplemented with 10% fetal calf serum and cell concentration was adjusted to  $10^6$  cells/ml.

### Drug preparation

Geriforte powder used in this study was obtained from Himalayan Drug company, Bangalore, India. Geriforte (450 mg) was suspended in 9 ml saline (50 mg/ml) and kept for 3–4 h at room temperature with intermittent shaking. The contents were centrifuged at 3000 rpm for 5 min to remove the particulate matter, supernatant was then collected and filter sterilized.

### Oxidative challenge of the lymphocytes with tert-butylhydroperoxide (tert-BHP)

Initial experiments were conducted by incubating lymphocytes with different concentrations (5, 10, 50, 100  $\mu\text{M}$ ) of tert-BHP in order to get a dose response relationship with regards to cytotoxicity.

To determine the cytoprotective activity of Geriforte, cells were incubated at 37 °C with 100  $\mu\text{M}$  tert-BHP in the absence or presence of different concentrations of Geriforte (10  $\mu\text{g}$ , 100  $\mu\text{g}$ , 500  $\mu\text{g}$ , 1 mg, 5 mg/ml) for 24 h in 96 or 24 well plates. Since the optimum concentration of Geriforte was found to be 1 mg/ml, all the experiments were performed at this concentration only.

### Determination of cytotoxicity and apoptosis

Apoptosis was studied morphologically using fluorescent dyes that intercalate DNA (Duke and Cohen, 1992). Acridine orange, a cationic dye enters only live cells and stains DNA green, allowing visualisation of chromatin pattern. On the contrary, ethidium bromide stains DNA orange, but is excluded by the live cells. Apoptotic cells have condensed chromatin that is uniformly stained by ethidium bromide. Dual staining allows enumeration of four populations: 1) Live-non-apoptotic (LNA) 2) Live-apoptotic (LA) 3) Dead-non-apoptotic (DNAP) 4) Dead-apoptotic (DAP). Briefly, after incubating the cells with different concentrations of tert-BHP and Geriforte (1 mg/ml), cells were stained with 4  $\mu\text{l}$  of staining solution (100  $\mu\text{g}/\text{ml}$  of acridine orange and 80  $\mu\text{g}/\text{ml}$  ethidium bromide) and then examined under fluorescent microscope. The percentage of live cells and apoptosis were calculated as follows:

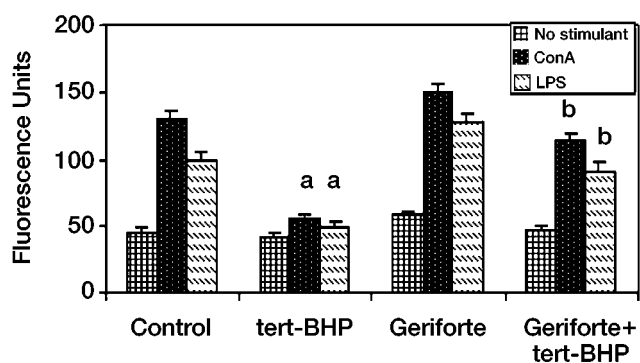
$$\% \text{ Live cells} = \text{LNA} + \text{LA} / \text{Total cells} \times 100$$

$$\% \text{ Apoptosis} = \text{LA} + \text{DAP} / \text{Total cells} \times 100$$

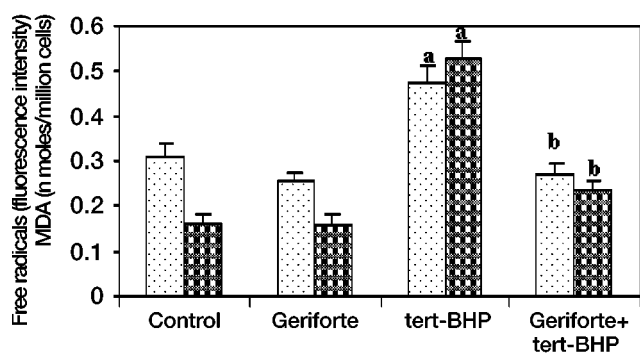
Cytotoxicity was studied using Neutral red as described by Sairam et al. (2000). Briefly, after incubating the cells with tert-BHP and Geriforte, 10  $\mu\text{l}$  neutral red (0.1%) was added to 200  $\mu\text{l}$  of incubation mixture and further incubated at 37 °C for 1 hr. After incubation, the cells were centrifuged, washed with saline three times. 200  $\mu\text{l}$  of ethanol and acetic acid (50:1) solution was added to the pellet to release the neutral red and absorbance was read at 570 nm using ELISA reader.

### Evaluation of cell proliferation

Cell proliferation experiments were carried out in 96 well microplates using Lipopolysaccharide (5  $\mu\text{g}/\text{ml}$ ) and Concanavalin A (6  $\mu\text{g}/\text{ml}$ ) as mitogens for stimulating B and T lymphocytes respectively. Briefly the cells were incubated along with Geriforte and sublethal concentration of tert-BHP (5  $\mu\text{M}$ ) with or without Concanavalin A/Lipopolysaccharide (Con A/LPS) for 72 h. Later, the culture plates were centrifuged for 10 min at  $300 \times g$  and the culture medium was removed. The cells were washed three times by sterile saline to remove the



**Fig. 1.** Effect of Geriforte on lymphocyte proliferation in the presence/absence of tert-BHP. Values are mean  $\pm$  S.D.  $p < 0.001$ . a – vs control ; b – vs tert-BHP.



**Fig. 2.** Effect of Geriforte on tert-BHP induced free radical production and MDA in lymphocytes. Values are mean  $\pm$  S.D.  $p < 0.001$ . a – vs control ; b – vs tert-BHP.  $\square$  MDA,  $\blacksquare$  Free radicals.

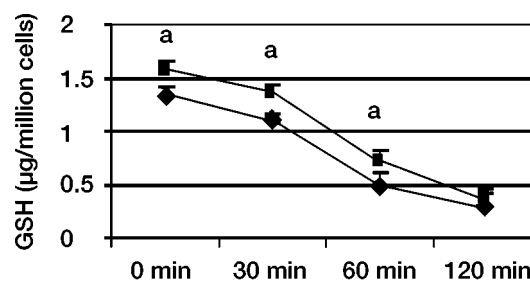
serum. Later, 200  $\mu$ l of Hoescht reagent (5  $\mu$ g/ml) was added to the wells and the fluorescence was measured using an excitation and emission filters of 355 and 460 respectively (Blaheta et al., 1991).

**Determination of free radical production, lipid peroxidation**

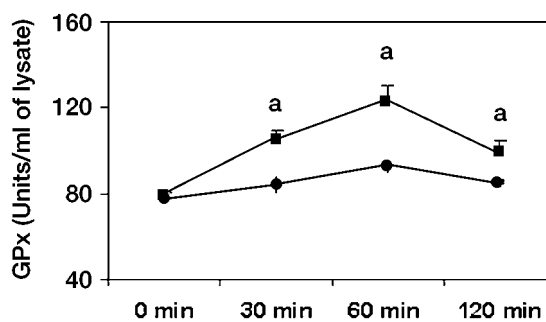
After incubating the cells with tert-BHP and Geriforte overnight, the cells were lysed with lysis buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). The production of free radicals was determined by using 2,7-Dichlorofluoresceindiacetate (DCFDA) as described by Cathcart et al. (1983). Lipid peroxidation was determined by measuring malondialdehyde (MDA) by the method of Dousset et al. (1983).

**Determination of GPx and GSH**

Lymphocytes were incubated at 37  $^{\circ}$ C with 100 $\mu$ M tert-BHP in the presence or absence of Geriforte (1 mg/ml)



**Fig. 3.** Effect of Geriforte on GSH level during oxidative stress induced by tert-BHP in lymphocytes. Tert-BHP was added to the control cells and the cells supplemented with Geriforte, the cells were lysed at 0, 30, 60 and 120 min and GSH was estimated. Values are mean  $\pm$  S.D.  $p < 0.05$ , a – vs control.  $\blacklozenge$ –Control,  $\blacksquare$ –Geriforte



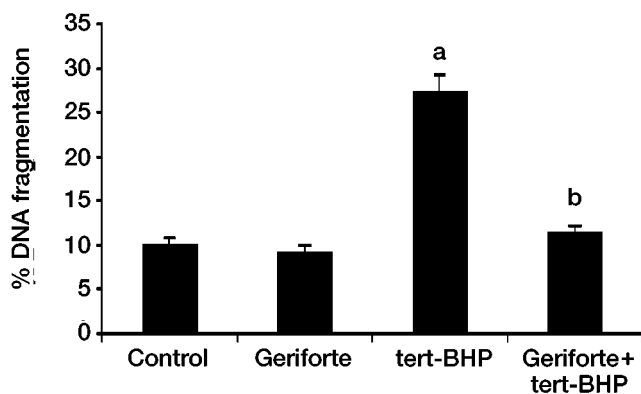
**Fig. 4.** Effect of Geriforte on GPx level during oxidative stress induced by tert-BHP in lymphocytes. Tert-BHP was added to the control cells and the cells supplemented with Geriforte, the cells were lysed at 0, 30, 60 and 120 min and GPx activity was determined. Values are mean  $\pm$  S.D.  $p < 0.01$ , a – vs control.  $\blacklozenge$ –Control,  $\blacksquare$ –Geriforte.

for different time intervals (30, 60, 120 min). The cells were lysed with lysis buffer at 0, 30, 60 and 120 min and GPx activity was determined in cell lysates using RANDOX kit following the instructions of the manufacturer. The reduced glutathione (GSH) level in the cells was determined fluorimetrically by the method of Burchill et al. (1978).

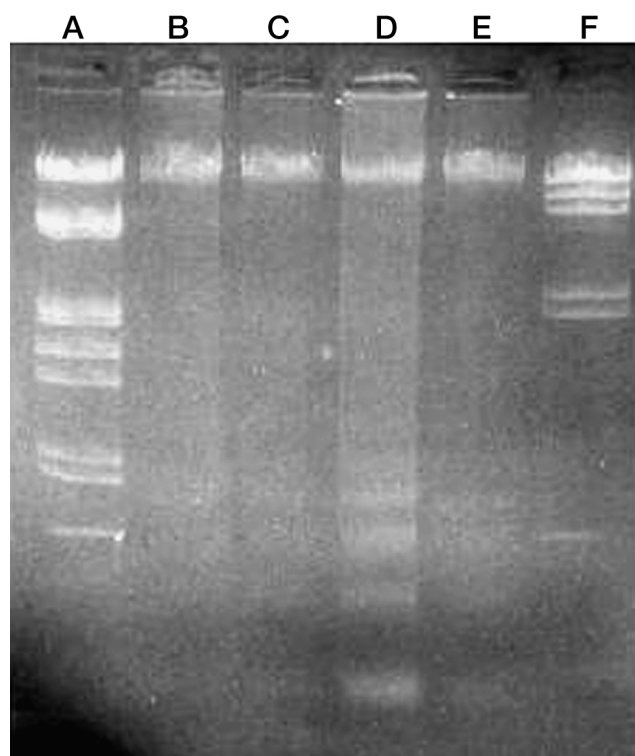
**DNA fragmentation analysis**

After incubating the cells with tert-BHP and Geriforte overnight, cells were pelleted down and washed with saline three times. The cells were lysed by adding 250  $\mu$ l of lysis buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and incubated for 30 min. The samples were then centrifuged at 27000g for 30 min. The supernatant, pellet and uncentrifuged lysate were assayed for DNA content using DAPI (4,6,-diamidino-2-phenylindole) reagent spectrofluorometrically (Brunk et al., 1979). Briefly, 20  $\mu$ l of the sample

was added to 2.0 ml DAPI reagent (100ng/ml DAPI in 10 mM Tris, pH 7.4, containing 100 mM NaCl) and the fluorescence intensity was measured at 450 nm with excitation at 362 nm. The percentage of DNA fragmentation was calculated as the ratio of the DNA content of the supernatant obtained at 27000 g to the total DNA in the lysate (Wyllie, 1980).



**Fig. 5.** Effect of Geriforte on tert-BHP induced DNA fragmentation in lymphocytes. Values are mean ± S.D.  $p < 0.01$ , a – vs control ; b – vs tert-BHP.



**Fig. 6.** Agarose Gel Electrophoresis depicting DNA fragmentation induced by tert-BHP. Lane A – Mol. Wt. Marker (Hind III and EcoR I double digest); Lane B – Control; Lane C – Geriforte; Lane D – tert-BHP; Lane E – Geriforte + tert-BHP; Lane F – Mol. Wt. Marker (Hind III digest).

**DNA isolation and agarose gel electrophoresis**

The cells (500 µl) after incubation were collected in 1.5 ml microfuge tubes and DNA was isolated by the method of Laird et al. (1991). Briefly the cells were lysed by adding 500 µl lysis buffer (100 mM Tris HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml proteinase K). The cells were digested at 5 °C for 3–4 h. DNA was purified by phenol:chloroform extraction, precipitated by adding two volumes of cold ethanol. Agarose gel (1.8%) electrophoresis was carried at 70 V for 1 hr to study the DNA fragmentation pattern. Eco RI- Hind III double digest and Hind III digest of λ-phage DNA were used as molecular size markers.

All the experiments were conducted thrice on different occasions and data were analysed using student’s t-test.

**Results**

**Cytotoxicity and apoptosis**

The cytoprotective effect of Geriforte on tert-BHP induced cytotoxicity and apoptosis is shown Table 1. Tert-BHP was found to be sub-lethal at 5 µM concentration (data not shown) and thereafter the cytotoxicity increased with increase in its concentration. There was a significant increase in % apoptosis in the presence of tert-BHP compared to control. However, Geriforte inhibited tert-BHP induced cytotoxicity and apoptosis appreciably.

**Table 1.** Effect of Geriforte on Cytotoxicity and apoptosis induced by tert-BHP (Values are mean ± SD).

Groups	% Live	% Apoptosis
Control	92 ± 8	2 ± 0.2 (0)
Geriforte (1mg/ml)	87 ± 7	1 ± 0.1 (0)
tert-BHP (100 µM)	55 <sup>a</sup> ± 4	30 <sup>a</sup> ± 0.2 (10)
tert-BHP (50 µM)	59 <sup>a</sup> ± 4	23 <sup>a</sup> ± 0.2 (11)
tert-BHP (10 µM)	71 <sup>a</sup> ± 6	18 <sup>a</sup> ± 0.1 (9)
Geriforte + tert-BHP (100 µM)	88 ± 7	10 <sup>ab</sup> ± 0.1 (2)
Geriforte + tert-BHP (50 µM)	89 ± 7	12 <sup>ab</sup> ± 0.2 (2)
Geriforte + tert-BHP (10 µM)	91 ± 8	10 <sup>ab</sup> ± 0.1 (1)

Values in paranthesis indicate dead apoptotic cells  $p < 0.01$ ; a – vs Control; b – vs tert-BHP

### Lymphocyte proliferation

To determine the effect of Geriforte on lymphocyte proliferation during oxidative stress, experiments were conducted using 5  $\mu$ M tert-BHP (as higher concentrations resulted in cytotoxicity). There was significant inhibition of mitogen (Con A/LPS) induced lymphocyte proliferation when the cells were treated with tert-BHP as compared to the control cells (Fig. 1). However, Geriforte relieved the inhibitory effect of tert-BHP on lymphocyte proliferation and the proliferation was more or less similar to that of control cells.

### Free radical production and MDA

As Fig. 2 shows, tert-BHP resulted in significant increase in free radical production (measured by using DCFDA) which was also evident by increase in MDA level ( $p < 0.01$ ). Treatment of the cells with Geriforte inhibited tert-BHP induced increase in free radical production and MDA level appreciably ( $p < 0.01$ ).

### GSH and GPx levels

There was a steady decrease in GSH content with time, when the cells were incubated with or without Geriforte in the presence of tert-BHP. However, its level was higher throughout in the presence of Geriforte compared to control cells (Fig. 3). There was no appreciable change in GPx activity with time in the absence of Geriforte whereas treatment with Geriforte along with tert-BHP, resulted in significant increase in this enzymatic activity with time (Fig. 4).

### DNA fragmentation analysis

When the cells were exposed to tert-BHP only, significant increase in DNA fragmentation was observed (Fig. 5). However when Geriforte was added along with tert-BHP, there was considerable decrease in DNA fragmentation. Agarose gel electrophoresis of DNA isolated from cells also showed the presence of typical DNA ladder characteristic of apoptosis in the presence of tert-BHP. The DNA ladder was not observed when the cells were exposed to Geriforte along with tert-BHP (Fig. 6).

## Discussion

Oxidative stress is an outcome of the overproduction of oxidants that overwhelms the anti-oxidant capacities of the cell. Anti-oxidants protect against excess ROS and free radicals, that can damage lipid, protein and DNA resulting in wide variety of clinical disorders. In recent years, the anti-oxidants in natural products have received

considerable attention and it is now becoming more obvious that a combination of different anti-oxidants can collectively produce a much better beneficial effect than taking a single compound. Geriforte is a mixture of several herbal extracts and minerals, commonly advocated for arresting the aging process and has also been found beneficial in reducing anxiety and stress (Bardhan et al., 1985; Upadhyaya et al., 1990). Here we report the anti-oxidant, cytoprotective and immunomodulating properties of Geriforte using lymphocytes as the model system.

If the anti-oxidant status of lymphocytes is enhanced by Geriforte supplementation, they would be expected to show increased resistance to oxidative damage when challenged in vitro with an oxidant such as tert-BHP. Tert-BHP, an organic hydroperoxide, is a useful model compound for the study of mechanisms of oxidative cell injury (Altman et al., 1994). Organic hydroperoxides form as a result of oxygen addition to alkyl radicals and/or by hydrogen atom abstraction from peroxy radicals. Tert-BHP may then decompose to other alkoxy and peroxy radicals that accelerate lipid peroxidation chain reactions (Baker and He, 1991). This decomposition is aided by metal ions and their complexes. In the present study, addition of tert-BHP to lymphocytes, induced a marked increase of free radical production, as evaluated by the fluorescent probe DCFDA (Fig. 2). This was further confirmed by increase in MDA and decreased GSH levels in the cells treated with tert-BHP. This shows that depletion of intracellular anti-oxidant levels by tert-BHP was due to increased free radical production. Several studies have shown that exposure to tert-BHP results in oxidative stress, producing free radicals which in turn results in increased MDA and reduced intracellular GSH levels (Palozza et al., 1996; Latour et al., 1999; Amoroso et al., 1999; Dubuisson et al., 2000; Suzuki et al., 2000). Incubating the cells along with Geriforte significantly arrested the free radical production induced by tert-BHP. This was also evident from the decreased level of MDA and higher GSH and GPx levels in the presence of Geriforte as compared to tert-BHP treated cells. GPx is an important anti-oxidant enzyme which plays a key role in the elimination of  $H_2O_2$  and lipid hydroperoxides by the oxidation of GSH. Enhanced levels of GSH and GPx suggest Geriforte's effectiveness in combating the pro-oxidative state to which the body gets exposed to, due to various reasons including drug toxicity, diseases and aging. Singh et al. (1994) and Pathania et al. (1998) also reported anti-oxidant properties of Geriforte in rats and mice.

The lymphocyte proliferation is a sensitive test, being used as a potential biomarker for toxic exposures (Snyder and Valle, 1991). In the present study, we determined the ability of Geriforte to modulate the lymphocyte proliferation by inhibiting the same by tert-BHP. tert-BHP inhibited the lymphocyte proliferation

stimulated by both LPS and ConA significantly over control. Further it was observed that Geriforte prevented the inhibitory effects of tert-BHP (a known potent oxidizing agent) on lymphocyte proliferation which could be attributed to its anti-oxidant activity.

Cytotoxicity (cell viability) and apoptotic studies showed that there was a significant increase in the percentage of dead and apoptotic cells (showing condensed chromatin) in the presence of tert-BHP. Further, there was an enhanced DNA fragmentation in the presence of tert-BHP and our results fall in confirmation with earlier studies (Sestilli et al., 1998; Aherne et al., 2000). Agarose gel electrophoresis of DNA isolated from lymphocytes also showed the presence of typical DNA ladder, characteristic of apoptosis in the presence of tert-BHP. Interestingly, Geriforte supplementation reduced the cytotoxicity and DNA damage significantly.

tert-BHP induced DNA damage may be triggered by two mechanisms: first iron metabolism plays a key role that leads to free radical formation and second thiol oxidation followed by activation of a calcium dependant endonuclease, which can lead to DNA strand breaks (Latour et al., 1995; Aherne et al., 2000). Sestilli et al. (1998) and Aherne et al. (2000) reported that iron ions are involved in tert-BHP induced DNA strand scission, and iron chelators (1,10-phenanthroline and deferoxamine mesylate) suppress tert-BHP induced DNA damage and cytotoxicity both whereas radical scavenging anti-oxidants (butylated hydroxytoluene) prevent only the latter response. In our study, we observed that Geriforte protected the cells both against DNA damage and cytotoxicity, induced by tert-BHP. Whether, ability of Geriforte to protect the cells against tert-BHP injury is due to iron chelation needs to be evaluated.

In conclusion, the results of this study demonstrate that Geriforte inhibits tert-BHP induced cytotoxicity and apoptosis. Further, Geriforte prevented the inhibitory effects of tert-BHP on lymphocyte proliferation induced by both LPS and Con A. The ability of Geriforte to inhibit the free radical production (induced by tert-BHP) and maintain higher antioxidant levels may be responsible for the observed cytoprotective activity.

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