

PROTECTION AGAINST BUTYL *p*-HYDROXYBENZOIC ACID INDUCED
OXIDATIVE STRESS BY *OCIMUM SANCTUM* EXTRACT IN MICE LIVER

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Abstract: Prime focus of the present investigation was to evaluate hepatoprotective potency of *Ocimum sanctum* (*O. sanctum*) aqueous extract against butyl *p*-hydroxybenzoic acid (butylparaben) toxicity in mice. Oral treatment of butylparaben (1320 mg/kg b.w./day) to mice for 30 days resulted in significant ($p < 0.05$) elevation in hepatic lipid peroxidation, which could be due to significant ($p < 0.05$) reduction in non-enzymatic (glutathione and total ascorbic acid) antioxidant contents and enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione transferase) antioxidants activities. Co-treatment of *O. sanctum* extracts in three different doses (100, 200 and 300 mg/kg b.w./day) resulted in significant ($p < 0.05$) reduction in butylparaben-induced hepatic changes. Oral administration of *O. sanctum* with butylparaben resulted in dose-dependent and significant ($p < 0.05$) reduction in lipid peroxidation as compared to butylparaben alone treated group. Similarly, all three doses of *O. sanctum* reduced butylparaben-induced changes in non-enzymatic and enzymatic antioxidants. The effect was significant ($p < 0.05$) and dose-dependent. All three doses of *O. sanctum* ameliorated butylparaben-induced changes, showing maximum protection at 300 mg/kg b.w./day dose. Results of present study indicate that butylparaben-induced hepatotoxicity involves its ability to induce oxidative stress, whereas antihepatotoxic effect of *O. sanctum* was mainly due to its antioxidative potency.

Key words: butylparaben, *Ocimum sanctum*, oxidative stress, hepatotoxicity

Parabens (*p*-hydroxybenzoic acid derivatives) are synthetic preservatives having long history of usage in various commodities due to its wide spectrum of antimicrobial activity, low cost and worldwide acceptance. Chemically, parabens are made up of six-member carbon ring having alkyl side chain. Butylparaben is butyl ester of *p*-hydroxybenzoic acid and is commonly used in cosmetics, personal care products, pharmaceuticals and beverages. Generally, metabolic fate of butylparaben involves its hydrolysis to parent acid by liver, kidney and skin esterases followed by conjugate formation with glycine and finally, its excretion in the form of *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide, *p*-hydroxybenzoyl glucuronide, and *p*-carboxyphenyl sulfate (1). Due to its long alkyl side chain, butylparaben is highly lipophilic in nature having high n-octanol/water coefficient, which allows its easy penetration and retention by fat tissues (2, 3). Studies reported the presence of unhydrolyzed butylparaben in patients suffering from breast tumors, indicating its accumulation in breast tissues (4). Butylparaben is known to mimic estro-

gen activity and is listed as endocrine disrupting chemical (5, 6). However, in comparison to reproductive organs, no sufficient research data is available on the effect of butylparaben on vital organs of mammals. Liver is the prime organ involved in detoxification of numerous xenobiotics by the process of biotransformation and is prone to be attacked by activated xenobiotic metabolites or free radicals generated by them.

Plants possessing medicinal properties are gaining a lot of attention for the remediation of various diseases and disorders. Health promoting effects of plants are primarily denoted by the presence of bioactive phytochemicals having nutritional and pharmacological properties (7). Herbs belonging to Lamiaceae family are found to possess strong antioxidative potency (8). *Ocimum sanctum* L. (*O. sanctum*) is a known Indian culinary herb and is well documented for its therapeutic values (9). In Ayurveda, whole herb had been used as preventive and curative drug due to synergistic interaction of various phytochemicals, which is generally not achieved in case of isolated compound treatment.

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Several *in vitro* studies using chemical models proved that crude extracts of *O. sanctum* are highly potent free radical scavengers at very low concentrations (10, 11). Studies have reported strong protective effect of *O. sanctum* extracts on various hepatotoxin models (ethanol, carbon tetrachloride, paracetamol) induced damages (12, 13). With these promising results of numerous research studies on *O. sanctum* extracts, they were selected to combat butylparaben hepatic toxication.

Present study deals with the evaluation of protective effect of *O. sanctum* aqueous extract on butylparaben-induced hepatotoxicity under *in vivo* conditions.

MATERIALS AND METHODS

Chemicals

Analytical grade butyl *p*-hydroxybenzoic acid was purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals used were of AR grade.

Ocimum sanctum extracts preparation

Fresh leaves of *O. sanctum* were collected from botanical garden of Botany Department of Gujarat University, Ahmedabad in the months of August–September 2008. Plant sample was authenticated and herbarium sample was preserved. Leaves were washed, shade dried and finely powdered for extraction. The aqueous polyphenols were extracted in distilled water according to the method of Bhargava and Singh with slight modification (14). Briefly, 5 g of leaf powder was mixed with 100 mL of double distilled water and the mixture was allowed to stand overnight for maximum extraction of polyphenols. Percolation of the extract was performed at room temperature in two stages. Collected filtrate was evaporated below 50°C to obtain final product in the form of residues which were stored under refrigerated conditions.

Experimental animals

Inbred adult healthy female Swiss strain albino mice weighing 30–35 g were obtained from Torrent Research Centre, Bhat, Gandhinagar-382 428, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 25 ± 2°C, 12 h light/dark cycle and relative humidity 50–55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols were approved by the

Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by Indian National Science Academy, New Delhi, India (1991).

Experimental design

Sixty animals were divided in six groups. Animals of group 1 received 0.2 mL olive oil/animal/day (olive oil was used to dissolve butylparaben) for 30 days and marked as vehicle control. Antidote control group (group 2) animals were given oral treatment of *O. sanctum* (300 mg /kg b.w./day). Based on the results of our earlier study (15), the high dose of butylparaben (1320 mg/kg b.w./day) was chosen further to evaluate hepatoprotective effect of *O. sanctum* aqueous extract. Animals of group 3 received butylparaben (1320 mg/kg b.w./animal/day) for 30 days. Animals of group 4, 5 and 6 were treated with butylparaben (1320 mg/kg b.w./animal/day) along with 100, 200 and 300 mg /kg b.w./day of aqueous *O. sanctum* extract (Table 1).

Animals were given treatment for 30 days and autopsied on 31st day. Livers were quickly isolated, blotted free of blood and used for determination of biochemical parameters.

Parameters studied

Lipid peroxidation levels

Lipid peroxidation (LPO) in liver homogenate was measured by estimating malondialdehyde (MDA) – intermediary product of lipid peroxidation by TBARS method as described by Devasagayam and Tarachand (16). The formed MDA was measured spectrophotometrically at 530 nm. The level of lipid peroxidation was expressed as nmoles of MDA formed/mg protein. Protein content was measured in liver by the method of Lowry et al. (17) using bovine serum albumin as a standard. Resulting blue color was measured at 540 nm.

Non-enzymatic antioxidants

Glutathione content (GSH) was determined by Ellman's reaction using 5'5'-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Moron et al. (18). The amount of reduced glutathione was measured at 412 nm on spectrophotometer and was expressed as µg of GSH/mg protein. Total ascorbic acid (TAA) content in the liver was estimated by the method of Roe and Kuether (19). TAA is oxidized to dehydroascorbic acid (DHA) by Norit reagent in the presence of TCA. This couples with 2,4-dinitro-

Table 1. Experimental protocol.

Groups	Treatment	No. of animals	Time of treatment (days)	Day of autopsy
1	Olive oil control (0.2 mL/animal/day)	10	30	31 st
2	Antidote control (300 mg/kg b.w./day)	10	30	31 st
3	Butylparaben (1320 mg/kg b.w./day)	10	30	31 st
4	Butylparaben (1320 mg/kg b.w./day) + OS (100 mg/kg b.w./day)	10	30	31 st
5	Butylparaben (1320 mg/kg b.w./day) + OS (200 mg/kg b.w./day)	10	30	31 st
6	Butylparaben (1320 mg/kg b.w./day) + OS (300 mg/kg bw/day)	10	30	31 st

Table 2. Effect of *O. sanctum* extract on butylparaben-induced changes in lipid peroxidation, non-enzymatic and enzymatic antioxidants in mice liver.

Parameters	Experimental groups					
	Vehicle control Group 1	Antidote control Group 2	Butylparaben High dose Group 3	Butylparaben High dose + OS100 Group 4	Butylparaben High dose + OS200 Group 5	Butylparaben High dose + OS300 Group 6
LPO	1.92 ± 0.04	1.86 ± 0.06	6.51 ± 0.08 ^{a*}	5.43 ± 0.02 ^{b*} (23.53)	3.32 ± 0.17 ^{b*} (69.50)	2.01 ± 0.09 ^{b*} (98.04)
GSH	11.26 ± 0.16	11.42 ± 0.22	4.94 ± 0.14 ^{a*}	5.93 ± 0.31 ^{b*} (15.66)	7.72 ± 0.26 ^{b*} (43.99)	10.11 ± 0.03 ^{b*} (81.80)
TAA	5.31 ± 0.09	5.42 ± 0.02	3.13 ± 0.09 ^{a*}	3.61 ± 0.09 ^{b*} (22.02)	4.16 ± 0.03 ^{b*} (47.25)	5.01 ± 0.04 ^{b*} (86.24)
SOD	3.72 ± 0.09	3.63 ± 0.03	1.23 ± 0.07 ^{a*}	1.83 ± 0.06 ^{b*} (23.79)	2.51 ± 0.02 ^{b*} (51.41)	3.27 ± 0.04 ^{b*} (81.93)
CAT	9.90 ± 0.02	9.72 ± 0.23	4.04 ± 0.03 ^{a*}	4.63 ± 0.02 ^{b*} (10.07)	5.84 ± 0.09 ^{b*} (30.72)	7.02 ± 0.04 ^{b*} (50.85)
GPx	3.12 ± 0.01	3.03 ± 0.05	1.26 ± 0.03 ^{a*}	1.81 ± 0.03 ^{b*} (29.57)	2.31 ± 0.06 ^{b*} (56.45)	2.83 ± 0.06 ^{b*} (84.41)
GR	2.23 ± 0.02	2.19 ± 0.06	1.15 ± 0.08 ^{a*}	1.32 ± 0.01 ^{b*} (15.74)	1.73 ± 0.02 ^{b*} (53.70)	2.04 ± 0.06 ^{b*} (82.41)
GST	2.31 ± 0.03	2.19 ± 0.04	1.17 ± 0.01 ^{a*}	1.53 ± 0.03 ^{b*} (31.58)	1.71 ± 0.01 ^{b*} (47.37)	2.03 ± 0.04 ^{b*} (75.44)

Results are expressed as the means ± SEM; n = 10, ^a as compared to vehicle control, ^b as compared to toxin treated (group 3). Level of significance *p < 0.05. Values in parenthesis indicate hepatoprotective index (HPI). No significant difference was noted between vehicle control and antidote control group. Units: LPO – nmoles MDA formed/mg protein/60 min, GSH – µg/mg protein, TAA – mg/gm tissue wt, SOD – U/mg protein, CAT – µmoles H₂O₂ consumed/mg protein/min, GPx – nmoles NADPH consumed/mg protein/min, GR – nmoles NADPH consumed/mg protein/min, GST – µmoles of CDNB conjugate formed/mg protein/min.

phenyl hydrazine in the presence of thiourea and sulfuric acid to yield a red colored complex which was read at 540 nm against blank. The TAA content was expressed as mg/g of tissue weight.

Enzymatic antioxidants

Activity of superoxide dismutase (SOD) was measured by the method of Marklund and Marklund (20). Superoxide radical formed reacts

with pyrogallol dye causing its autooxidation resulting in blue color which is read at 470 nm. Superoxide dismutase activity in liver tissue was expressed as U/mg protein. Catalase (CAT) activity in tissue was measured by the method of Sinha et al. (21) using hydrogen peroxide as standard substrate. Potassium dichromate-acetic acid reagent was added at the interval of 0, 15, 30 and 60 s to terminate the reaction. Resulting orange-yellow color was read at 590 nm. Catalase activity was expressed as $\mu\text{m H}_2\text{O}_2/\text{mg protein/min}$. The glutathione peroxidase (GPx) activity in the liver was assayed by modified method of Pagila and Valentine (22). The enzyme activity was expressed as units/mg protein/min, where 1 unit of GPx equals to nmoles NADPH consumed/mg protein/min. The glutathione reductase (GR) activity in liver was assayed by the method of Mavis and Stellwagen (23). The enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The decrease in absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated as nmoles NADPH consumed/mg protein/min. The liver glutathione S-transferase (GST) activity was assayed by the method of Habig et al. (24). The increase in absorbance was noted at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB). The enzyme activity was calculated as $\mu\text{moles CDNB conjugates formed/mg protein/min}$.

Hepatoprotective index

The liver protecting activity of the *O. sanctum* extract was expressed as hepatoprotective percentage (H) (25) which was calculated using the formula:

$$H = \left(1 - \frac{T - V}{C - V}\right) \times 100$$

where T is the mean value of plant extracts along with the butylparaben, C is the mean value of butylparaben alone, and V is the mean value of vehicle control animals.

Statistical analysis

The data were statistically analyzed using SPSS statistical software, version 16. The results were expressed as the mean \pm SEM. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) multiple comparison test. The level of significance was accepted with $p < 0.05$. Pearson's correlation analysis was used to find the correlation between dose administration and alteration in enzymatic and non-enzymatic parameters in the liver of mice.

RESULTS

Table 2 shows the effect of three doses of *O. sanctum* (OS100, OS200, OS300) on butylparaben-induced changes in oxidative stress parameters. Increase in hepatic LPO (group 3) was found to reduce to 271.61% (OS100), 165.8% (OS200), 100.65% (OS300) oral treatment of *O. sanctum*, which was 339.16% in case of butylparaben alone treatment. The reduction was significant ($p < 0.05$) and dose-dependent ($r = 0.99$). Protection denoted by *O. sanctum* against butylparaben-induced LPO was 23.53% (OS100), 69.50% (OS200) and 98.04% (OS300) as calculated by hepatoprotective index (Table 2). Recovery in the content of non-enzymatic antioxidants (GSH and TAA) was also achieved by *O. sanctum* treatment which was significant ($p < 0.05$) and dose-dependent (GSH $r = 0.99$, TAA $r = 0.99$) as compared to various control groups (group 1, 2). Butylparaben reduced levels of GSH (43.87%) and TAA (58.95%) in mice liver significantly ($p < 0.05$). Oral treatment of *O. sanctum* increased levels of GSH and TAA to 52.66%, 67.98% (OS100), 68.56%, 78.34% (OS200) and 89.79%, 94.35% (OS300), respectively. Hepatoprotective index calculated for GSH and TAA content was 15.66%, 22.02% (OS100), 43.99%, 47.25% (OS200) and 81.80%, 86.24% (OS300) as shown in Table 2.

Activities of enzymatic antioxidants were severely affected by butylparaben treatment, which were brought back to normal by contreatment of *O. sanctum* extract. Activities of hepatic SOD (33.06%) and CAT (44.44%) were reduced by butylparaben treatment and were found to increase significantly ($p < 0.05$) by various doses of *O. sanctum* in a dose-dependent manner ($r = 0.99, 0.99$). Percent recovery in SOD activity was 49.19% (OS100), 67.47% (OS200) 87.90% (OS300), which was 46.77% (OS100), 58.99% (OS200), 70.91% (OS300) in case of CAT activity. Hepatic protection, shown in Table 2, by three doses of *O. sanctum* for SOD and CAT was 23.79%, 10.07% (OS100), 51.41%, 30.72% (OS200) and 81.93%, 50.85% (OS300), respectively. Similarly, the protective effect of *O. sanctum* doses on the activities of GPx, GST and GR were also significant ($p < 0.05$) and dose-dependent ($r = 0.99, 0.99, 0.98$) as compared to high dose butylparaben intoxication. Treatment of butylparaben alone reduced activities of GPx, GST and GR to 40.38%, 50.7% and 51.56%, respectively. Increases in the GPx and GST activities were 61.22%, 66.23% (OS100), 74.04%, 74.03% (OS200), 90.70%, 87.88% (OS300), respectively, whereas GR activity was increased by 59.19%

(OS100), 77.57% (OS200) and 91.48% (OS300). Hepatoprotective index calculated for GPx and GST activity was 29.57%, 31.58% (OS100), 56.45%, 47.37% (OS200) and 85.41%, 75.44% (OS300), respectively, which was 15.74% (OS100), 53.70% (OS200) and 82.41% (OS300) in case of GR activity (Table 2).

DISCUSSION

Hepatoprotective effect of *O. sanctum* is principally due to its antioxidative potency. Butylparaben exerted toxicity in mice liver could be due to its ability to induce oxidative stress as reported with its parent acid (paraben) and analog (methylparaben) (26, 27). Butylparaben treatment in mice elevated levels of lipid peroxidation in liver upon 30 days treatment. *O. sanctum* doses significantly reduced levels of LPO in butylparaben intoxicated animals, which could be due to free radical scavenging effect of *O. sanctum* polyphenols as it was well correlated in our *in vitro* studies (unpublished data). Antiperoxidative effect of *O. sanctum* extracts were reported on various stress models by numerous researchers (28, 29). *O. sanctum* pretreatment also stabilizes the levels of tissue total sulfhydryl groups during reperfusion and responsible for prevention of reperfusion-induced LPO (30). Contents of non-enzymatic antioxidants of the hepatocytes were found to increase with cotreatment of *O. sanctum* extract, which were significantly reduced with butylparaben alone treatment. The effect was dose-dependent and significant. GSH – major nonprotein thiol in living organisms, was found reduced in butylparaben-treated animals, which was restored back by free radical scavenging and sulfhydryl (thiol) group protecting effects of plant extract. Reduction in GSH content with butylparaben treatment was also reported in isolated rat hepatocytes (31). Ascorbic acid content was also found to increase due to proton donating effect of *O. sanctum* extract sparing body's natural antioxidants from getting oxidized. *O. sanctum* extract-induced increase in non-enzymatic antioxidants had been reported by Ramesh and Satakopan (32). Increased level of glutathione could be the reason for reduction in LPO as in the presence of GSH, lipid peroxides are converted to less toxic alcohol derivatives rather than MDA (33).

Activities of enzymatic antioxidants (SOD, CAT, GPx, GR and GST) were found to reduce with butylparaben treatment for 30 days, which could be due to increased production of free radicals characterized by increased MDA content. These enzymes

are known to scavenge free radicals such as superoxide, hydroxyl and hydrogen peroxide, thus preventing damage caused by oxidative stress to the tissue (34). The present study shows that aqueous extract of *O. sanctum* significantly increases activities of enzymatic antioxidants in butylparaben intoxicated animals. Kusumaran et al. (35) showed the effect of *O. sanctum* extract on chemical carcinogens where *O. sanctum* leaves effectively increased GST activity and protected rats from deleterious effects of carcinogenesis. The levels of thiol groups plays vital role in maintaining structural and functional integrity of membranous and enzymatic proteins (36). This thiol spearing effect of *O. sanctum* extract could be the due to proton donating effect of extract, resulting in elevation of reduced glutathione and ascorbic acid levels ultimately maintaining enzyme protein structure and active site configuration. (37). Aqueous extract of *O. sanctum* is an excellent scavenger of superoxide, hydroxyl, nitrous oxide and DPPH radical under *in vitro* conditions (38). This could be the reason for *O. sanctum*-induced increase in enzymatic antioxidants. Antioxidative effect of *O. sanctum* extract was also responsible for its antiulcer and wound-healing property (39, 40). Increased activities of SOD, and catalase and GSH content along with simultaneous reduction in tissue LPO is principally responsible for protective effect denoted by *O. sanctum* extract against noise-stress and chronic restraint stress. *O. sanctum* mixed in rat diet was also found to increase activities of enzymatic antioxidants under diabetic conditions (41).

In conclusion, butylparaben oral administration caused alteration in oxidative stress marker (LPO) as well as enzymatic and non-enzymatic antioxidants in mice liver, which could be a principal mechanism responsible for its hepatotoxicity. *O. sanctum* aqueous extract reduced butylparaben-induced hepatic changes mainly due to its phytochemicals having antioxidative properties.

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