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ORIGINAL RESEARCH ARTICLE

IN VIVO ASSESSMENT OF POSSIBLE TOXICITY RISKS OF GREEN TEA EXTRACT IN MICE

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Abstract: There is a general belief that herbs are safer than pharmaceuticals because they are natural. But the fact is, in low amounts, they may be ineffective while in the right amounts, they may prove beneficial. Their use in high quantities and over a prolonged period may be injurious to health. Experiments were conducted to determine the oral lethal dose (LD50). The GTE dose ranges of 100 to 5000 mg/kg body weight was administered orally to the mice and the treated groups were observed continuously for 10 days (acute toxicity). The effects on the weight changes and some biochemical parameters were evaluated. Increase in the body weight of the GTE treated groups was normal. The plasma concentrations of urea, creatinine and SGOT (Serum glutamic oxaloacetic transaminase) were not much changed as compared to controls. The levels of GSH (Reduced Glutathione), SOD (Superoxide Dismutase) and CAT (Catalase) were found slightly increased in the animals that received the GTE tested doses, but the increase was not significant. Hence the acute toxicity value (LD50) of the GTE was determined to be more than 5000 mg/kg body weight, indicating that it poses no risk to general health of treated animals. But still not more than four cups a day of highly concentrated (up to 3% of green tea extract) may be consumed.

Key words: Lethal Dose, Green tea extract, Acute toxicity, Biochemical parameters.

INTRODUCTION

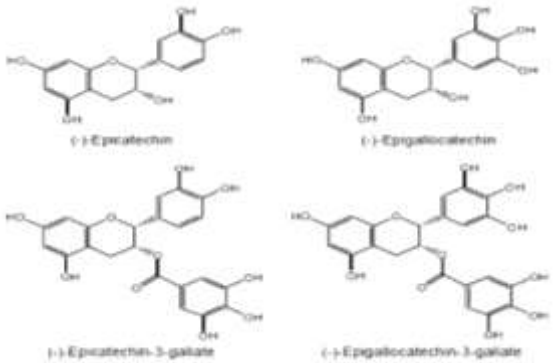
Scientific research has demonstrated that many traditionally used herbal medicines are potentially toxic and some are even mutagenic and carcinogenic [1]. The toxicity benchmarks for herbal drugs, therefore, depend on the purity of herbs, presence of any toxic constituents of herbs, bioavailability of its constituents in body, and its reported adverse effects [2]. Acute toxicity is the toxicity that manifests itself immediately or within 14 days following exposure of animals or humans to single doses of chemicals (xenobiotics) by ingestion, inhalation or through the skin. Acute toxicity tests are generally carried out in rodents and occasionally in rabbits. Their current purpose is to identify a clearly toxic but a sub-lethal dose and to provide a rough guide for dose selection in subsequent repeated dose toxicity tests. Here Green tea extract is used to assess its possible toxicity risk in mammals (mice). Tea is the second-most consumed beverage in the world (water is the first) and has been used medicinally for centuries in India and China. The tea shrub (genus *Camellia*, family Theaceae) [chromosome number (2n=30)] is a perennial evergreen with its natural habitat in the tropical and sub-tropical forests of the world. Several catechins are present in significant quantities; epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) [3] [Figure 1].

MATERIALS AND METHODS

Chemicals

0.1 M Tris-HCL buffer (Sigma-Aldrich); Glycine-NaOH (50mmol/L, pH 10) (Gibco, Invitrogen); Adrenaline (1mmol/L) (Gibco, Invitrogen); ASAT kit-Crest Biosystems Pvt Ltd.; Picric acid (Sigma-Aldrich); Sodium hydroxide (Sigma-Aldrich); Creatinine (Sigma-Aldrich); Urea reagent, (Gibco, invitrogen); Diacetyl monoxime reagent (Gibco, Invitrogen); Standard urea (Gibco, Invitrogen); TCA-25% Solution (Sigma-Aldrich); TCA-5% Solution (Sigma-Aldrich); Phosphate buffer Solution (pH.8) 0.2M (Sigma-Aldrich); Dithiobis (2-nitrobenzoic acid) DTNB-0.6mM (Sigma-Aldrich); Reduced glutathion (GSH) (Sigma Aldrich); Sodium pyrophosphate buffer (0.025M, pH 8.3) (Sigma-Aldrich); Phenazine methosulphate (PMS) (186µM) (Sigma-Aldrich); Nitroblue tetrazolium (NBT) (300µM) (Sigma-Aldrich); NADH (780µM) (Sigma-Aldrich); Glacial acetic acid(Sigma-Aldrich); n-butanol (Sigma-Aldrich); Potassium phosphate buffer (50mM, pH 6.4) (Sigma-Aldrich); Phosphate buffer: 0.067 M (pH 7.0) (Sigma-Aldrich); Hydrogen peroxide (2mM) in phosphate buffer (Sigma-Aldrich).

Figure 1: Main catechin components of Green tea polyphenols



*Figures Courtesy: National Centre for Complementary and Alternative Medicine, May 2009. http://nccam.nih.gov/health/index.htm

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Preparation of Green tea leaf extract (GTE)

Green tea was collected from the farms of Kangra (Himachal Pradesh, India). Green tea extract (GTE) was prepared according to Khan *et al.,* (2009) [4], by adding 30g green tea leaves to 500ml of boiling water, which is steeped for 15–20 min. The resulting infusion was cooled to room temperature and then filtered. The tea leaves were steeped a second time with 500ml of boiling water and filtered again, and the two filtrates were combined to obtain 3% green tea extract (3g tea leaves/100 ml water). This dietary treatment reflected a daily consumption of 3 cups of green tea by an adult human weighing 70kg. The resulting clear solution is similar to tea brews consumed by humans. The extract was poured into the animals feeding bottles. The mice were supplied with freshly prepared tea extract every morning.

Animals and experimental design

Animals Swiss albino mice (*Mus musculus* L.) 35-50g, 10-12 weeks old were procured from Lucknow (U.P.), India and were grouped in five different polypropylene cages (four animals/group, total 20 mice). The animals were fed *ad libitum* and allowed to adjust to the new environment for one week before starting the experiment, at a mean temperature of 25°C. Permission was granted for experimentation by the departmental ethical committee. During the experiments, maximum care was taken to minimize animal suffering and, in addition, the number of rats used was kept at minimum. Animals were randomly divided into five experimental groups each of four animals as follows:

1. **Group I (control):** Animals were given standard diet and tap water.
2. **Group II:** Animals were given standard diet and green tea extract (GTE). This Group was given 100 mg/kg body weight GTE instead of drinking water within a repeated dose for 10 days to determine the dose dependent effect.
3. **Group III:** Animals were given standard diet and 1000 mg/kg body weight GTE instead of drinking water within a repeated dose for 10 days to determine the dose dependent effect.
4. **Group IV:** Animals were given standard diet and 2500 mg/kg body weight GTE instead of drinking water within a repeated dose for 10 days to determine the dose dependent effect.
5. **Group V:** Animals were given standard diet and 5000 mg/kg body weight GTE instead of drinking water within a repeated dose for 10 days to determine the dose dependent effect.

Measurement of body weight and daily fluid intake

Fluid intake was recorded daily within the 10 day period. Animals drank from graduated water bottles and at the same time each day, the decrement in the amount of fluid consumed over 24 hours was measured by subtracting the day's reading from that of the previous day. End-of-treatment weights were used for weight analysis.

Acute toxicity study

The mice were observed throughout the ten days treatment, and all signs of toxicity, their latencies and deaths were recorded. Behavior and other physiological activities of all the above five groups of mice were noted.

Preparation of Specimens

The animals were starved overnight for 12 hours before the blood was collected. After the experimental period of 10 days, blood was collected from the orbital sinus of mice under ether anesthesia. This blood was centrifuged at 3000 rpm at 4°C for five minutes to obtain the blood serum. Serum was placed into a -20°C freezer until the time of the assay.

All animals were sacrificed by cervical decapitation under light ether anesthesia on the eleventh day. Immediately after sacrifice, the liver was dissected out, washed in the ice-cold saline, and the homogenate was prepared in 0.1 M Tris-HCL buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for the assay of marker enzymes. The supernatant was placed into a -20°C freezer until the time of the assay.

Biochemical Parameters investigated

Plasma was used for the determination of the concentration of urea, creatinine and serum glutamic oxaloacetic transaminase (SGOT). Liver homogenate was used for the determination of the levels of Glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT) using appropriate reagents and kits supplied by manufacturers. Optical density measurement was done using Spectrophotometer (Beckman Coulter, U.S.A.).

- **Estimation of Serum Creatinine:** Alkaline picrate method by Brod and Sirota, 1948 [5] was used to measure serum creatinine.
- **Estimation of Serum Urea:** The method described by Marsh *et al.,* 1965 [6] was used for the assay.
- **Estimation of Serum Glutamate-Oxaloacetate-Transaminase (SGOT) (EC 2.6.1.1):** Levels of serum glutamic oxaloacetic transaminase (SGOT) were determined using Rietman and Frankel's method adopting ASAT kit by colorimetric method. Values were expressed as IU/L One international unit (IU/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions. Principle: Samples of serum were incubated with

either DL-aspartate and α ketoglutarate for SGOT determination. The oxaloacetic acid so formed was then reacted with 2,4dinitrophenylhydrazine to form an adduct which absorbs light at 505 nm.

- **Estimation of Reduced Glutathione (GSH):** The method described by Moron *et al.*, (1979) [7] was used for the assay.
- **Estimation of Superoxide Dismutase (SOD) (EC 1.15.1.1):** SOD was assayed according to the method of Kakkar *et al.*, (1984) [8].
- **Estimation of Catalase (CAT) (EC 1.11.1.6)** Catalase activity was assayed following the method of Luck (1974) [9].

Statistical analysis

Student's t test was used to determine the significance of the differences between the groups. Data were expressed as mean ± SE. Differences between the control and the tea extract treated groups of mice were analyzed using Chi-square test, Kruskal-Wallis tests when applicable. The P-value of ≤0.05 was considered to be significant.

RESULTS

The results of this study are tabulated below. The acute oral toxicity of the Green tea extract was very low in mice. No deaths and no other signs or symptoms of toxicity were observed up to highest test dose tested (5000 mg/kg of body weight). Except for hyperactivity and excessive urination, no other adverse effect was noted. There were no deaths of animals at any dose in 10 days. No negative effect was seen on body weight gain percentage in all the experimental groups of mice (Table 1 and 2).

Table 3 is a summary of the results of the effects of the Green tea extract administration on the biochemical parameters of mice after the experimental period of 10 days. The plasma concentrations of urea, creatinine and SGOT were not much changed as compared to controls. The findings reveal that Green tea extract results in slight decrease in urea and creatinine levels but these changes were not significant (Figure 2 and 3). SGOT concentration was found only slightly increased in the groups treated with GTE, but the values too were not significantly different from the control (Figure 4). The levels of GSH, SOD and CAT were found slightly increased in the animals that received the GTE tested doses. No significant increase was observed in these values. (Figure 5, 6 and 7).

Table 1: Daily fluid intake and percentage change in body weight of all the groups of mice

Groups (Each group has five mice, n=4) Dose of GTE	Parameters	
	Fluid intake (ml/100g/day)	Increase in body weight (%)
Control	13.70 + 2.93	30.56 + 17.20
GTE 100mg/kg bw	10.23 + 1.22	31.02 + 16.19
GTE 1000mg/kg bw	8.33 + 0.15	31.67 + 16.14
GTE 2500mg/kg bw	8.00 + 1.2	30.88 + 15.17
GTE 5000mg/kg bw	7.50 + 1.6	30.86 + 16.14

All values are mean are mean of 4 determinations of each group of mice + SEM. GTE: Green tea extract; bw: Body weight.

Table 2: Acute toxicity study of Green tea extract in all the groups of mice (For a period of 10 days)

Doses of GTE mg/kg body weight of the animal	Number of animals in a group	Number of animals dead
0 (Control)	4	0
100	4	0
1000	4	0
2500	4	0
5000	4	0

GTE: Green tea extract.

Table 3: Effect of daily administration of the Green tea extract for 10 days on biochemical profiles of the control and the treated mice groups.

Parameters	Treatment groups				
	Control	GTE 100mg/kg	GTE 1000mg/kg	GTE 2500mg/kg	GTE 5000mg/kg
Urea (mg/dl)	46.9 ± 1.8^{a}	44.80 ± 2.3^{a}	40.8 ± 1.3^{a}	38.23 ± 1.48^{a}	36.4 ± 1.3^{a}
Creatinine (mg/dl)	0.61 ± 0.02^{b}	0.58 ± 0.2^{b}	0.56 ± 0.5^{b}	0.55 ± 0.2^{b}	0.53 ± 0.18^{b}
GSH (µg/mg protein)	8.34 ± 0.34^{c}	8.52 ± 0.95^{c}	9.03 ± 0.50^{c}	9.77 ± 0.34^{c}	10.67 ± 0.54^{c}
SGOT (IU/L)	144.8 ± 0.06^{d}	144.9 ± 0.12^{d}	145.2 ± 0.08^{d}	145.6 ± 0.06^{d}	145.8 ± 0.35^{d}
SOD (Unit/mg protein)	15.0 ± 0.56^{e}	16.2 ± 0.94^{e}	17.61 ± 0.33^{e}	18.3 ± 0.84^{e}	18.90 ± 0.65^{e}
CAT (Unit/ mg protein)	84.0 ± 2.11^{f}	84.5 ± 2.30^{f}	84.8 ± 1.33^{f}	85.0 ± 3.1^{f}	85.7 ± 3.3^{f}

Results are mean of 4 determinations of each group ± SEM. Values bearing different superscripts in the same row, are significantly different (P< 0.05). SGOT: serum glutamic oxaloacetic transaminase; GSH: Reduced Glutathione; SOD: Superoxide Dismutase; CAT Catalase.

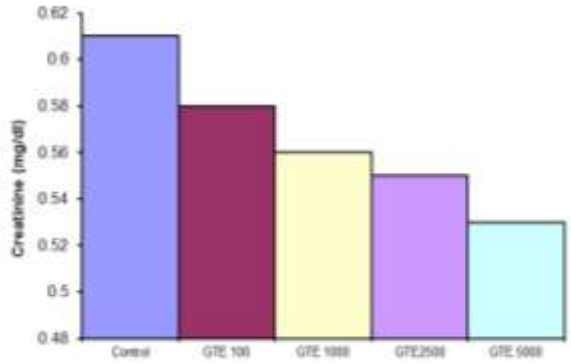

Figure 2: Effect of Green tea extract (GTE) on blood Creatinine levels of mice

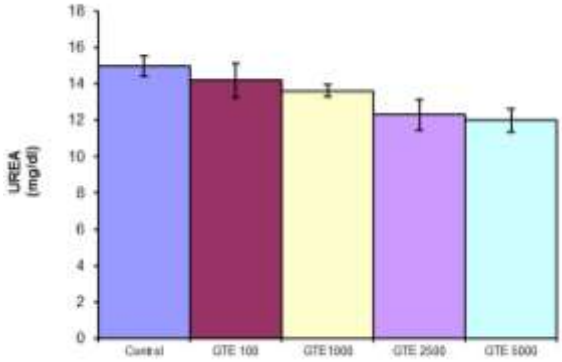

Figure 3: Effect of Green tea extract (GTE) on blood Urea levels of mice

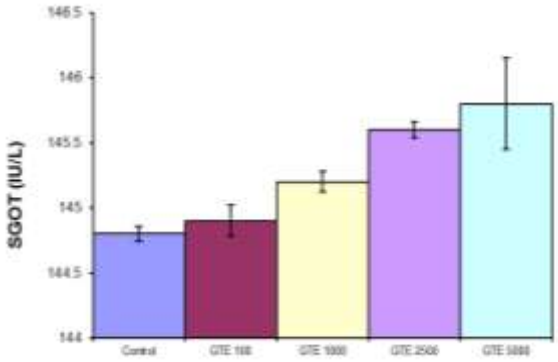

Figure 4: Effect of Green tea extract (GTE) on SGOT enzyme levels of mice

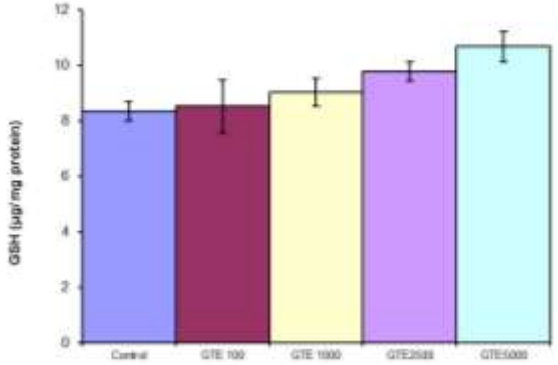

Figure 5: Effect of Green tea extract (GTE) on GSH enzyme levels of mice

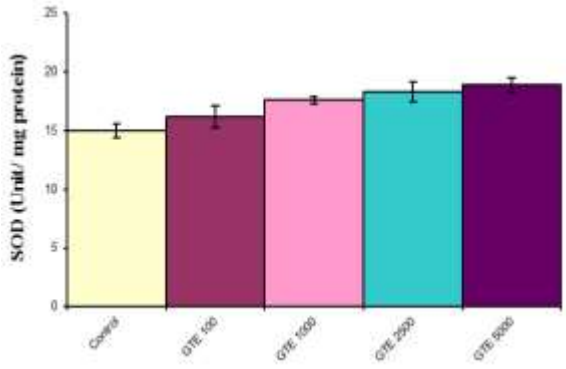

Figure 6: Effect of Green tea extract (GTE) on SOD enzyme levels of mice

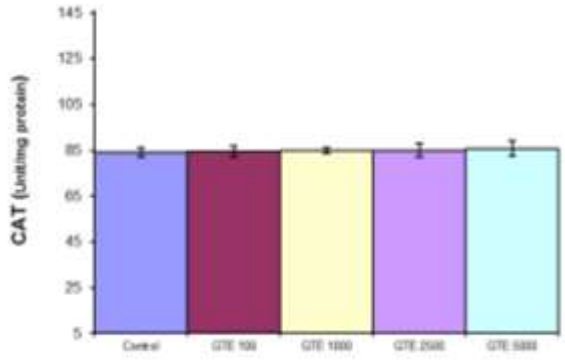

Figure 7: Effect of Green tea extract (GTE) on CAT enzyme levels of mice

DISCUSSIONS

Phytomedicine research is gaining more attention as people are relying heavily on herbal medicine, which occasionally has been shown to be less toxic and cheaper, compared to synthetic orthodox medicine. Among such phytomedicines are the dietary plants with proven antioxidant properties, which plays significant role in the reduction of the incidence of cancer and free radical–related complications such as diabetes, cardiovascular and liver diseases. This however, involves the interactions of the dietary antioxidants with one or more biomolecules at the cellular or molecular level or both, leading to the induction of certain enzymes, as well as inhibition or inactivation of some key enzymes. This study thus investigates the possible toxicity risks of Green tea extract in mice. The body weight changes serve as a sensitive indication of the general health status of animals. However weight gains were observed in all animals administered with GTE upto 5000mg/kg/day. Thus, it can be stated that even at this much concentration GTE, did not interfere with the normal metabolism of animals. In addition, the diets were well-accepted by animals treated with GTE, suggesting that it did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals (Table 1).

In oral acute toxicity studies, no untoward clinical signs were observed in the mice at all the doses of GTE studied (100, 1000, 2500 and 5000mg/body weight). There were no changes in the nature of stool, urine and eye color. No mortality was observed at all dose levels from the critical 24 hours post administration to the end of the tenth day. Orally, 5000 mg/kg of GTE was well tolerated in mice even after 10 days. Hence the LD50 was estimated to be <5000 mg/kg (orally) (Table 2).

Elevations of biochemical parameters such as plasma or serum urea and creatinine are considered reliable for investigating drug-induced nephrotoxicity in animals and man [10]. Elevated levels of uric acid and creatinine have been reported as a constant finding in lead toxicity. Urea is a waste product which is produced in the liver, dissolved in blood (in a concentration of 2.5-7.5mM), and excreted by the kidneys. Urea also plays a very important role in protein catabolism, removal of toxic ammonia from the body, and the counter-current system which allows for re-absorption of water and critical ions in the nephrons. Therefore marked decrease in serum urea and creatinine, as noticed in this study, confirms an indication of no functional damage to the kidney. Urea level can be increased by many other factors such as dehydration, antidiuretic drugs and diet, whilst creatinine is more specific to the kidney, since kidney damage is the only significant factor that increases serum creatinine level [11]. Therefore, a decrease in urea and creatinine levels noticed in this study are a classical sign that the kidney was not affected by GTE administration (Table 3).

The anti-oxidant enzymes are the marker enzymes for the liver malpractice and malnutrition. Liver uses transaminase enzymes SGOT and SGPT to metabolize amino acids and to make proteins. When liver cells get damaged SGOT and SGPT leak into blood stream. The SGOT level remained almost normal after Green tea extract treatment indicating normal level of insulin secretion (Table 3). SGOT and SGPT levels act as indicator of liver function, hence normal SGOT levels indicate normal functioning of liver.

Glutathione (GSH) constitutes the first line of defense against free radicals and other oxidant species. The liver homogenate reduced glutathione (GSH) content is presented in Table (3) Figure Green tea extract treatment in mice causes a slight increase in their reduced glutathione content when compared with normal control group. This may be due to the high content of phenolic compounds in GTE that played a significant role on the anti-oxidation potential of the tea.

Superoxide dismutase is a metalloprotein and is a first enzyme involved in the antioxidant defense by lowering the steady state of oxygen radical. Catalase is a hemeprotein, localized in the peroxisomes or the microperoxisomes, which catalyses the decomposition of hydrogen peroxide to water and oxygen and thus protect the cell from oxidative damage produced by hydrogen peroxide. Under normal physiological conditions, a delicate balance exists between the rate of formation of H_2O_2 via dismutation of O^-_2 by SOD activity and the rate of removal of H_2O_2 by CAT and glutathione peroxidase. Therefore any impairment in this pathway will affect activities of other enzymes in the cascade [12]. The present study indicates that GTE treatment resulted in slightly increased SOD and CAT activities. Results from this study suggest that treatment with GTE may have exerted an oxidative effect due to its cytotoxic and hence increasing levels of SOD and CAT activities.

According to El Daly 2011 [13], ingestion of the higher dose (3% GTE) revealed depletion of glycogen in hepatocytes cytoplasm, in a histochemical study. It may possibly induced instability of metabolic enzymes related to glycogen storage. The mechanism by which green tea extract induces trouble in carbohydrate metabolism may be attributed to mitochondrial toxicity and reactive oxygen species (ROS) formation by tea catechins which induced cytotoxic effects [14].The repeated ingestion of highly concentrated up to 3% green tea extracts has its harmful effect on the liver tissues that are subjected to variable hazards due to hepatotoxicity from tea extract polyphenols. So it can be allowed to ingest GTE beverage in a low concentration and for one or two times a day is good for the hepatic tissues [13].

CONCLUSION

According to toxicity classes of Hodge and Sterner (2005) [15], any compound with oral LD50 (rat) of 5000mg/kg or more should be considered as practically harmless. This could be attributed the fact that, the tea preparation is rich in polyphenols, which possess antioxidant potential [16]. In conclusion, the results showed that the acute toxicity of the Green tea extract is low and no evidence was found that it poses risks to general health of treated animals. But still not more than four cups a day of highly concentrated tea (up to 3% green tea extract) must be consumed.

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