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IMMUNOSTIMULATORY POTENTIAL OF N-BUTANOLIC FRACTION OF HYDROALCOHOLIC EXTRACT OF *COSTUS SPECIOSUS* KOEN. RHIZOME

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Keywords:

Costus speciosus Koen, Immunomodulatory activity, hydroalcoholic extract.

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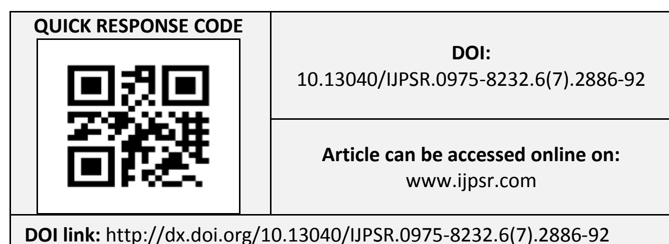
ABBREVIATIONS:

Sheep red blood cells (SRBCs), Optical density (OD), Low dose test extract (LDTE), High dose test extract (HDTE), Nephalometric Turbidity Unit (NTU), Humoral antibody (HA) titer, Delayed type hypersensitivity (DTH).

ABSTRACT: The immunomodulatory potential of n-butanolic fraction of rhizome extract (hydroalcoholic) of *Costus speciosus* was evaluated. For this different models like estimation of serum Immunoglobulin level, carbon clearance test, delayed type hypersensitivity reaction and humoral antibody were used. In delayed type hypersensitivity reaction and humoral antibody titer , mice were immunized by injecting 20% sheep red blood cells (SRBCs) suspension in sub planter region of paw and intraperitonially, respectively. All the Data was analyzed by one way ANOVA followed by Dunnet's test at *p<0.05, **p<0.01, ***p<0.001. Serum immunoglobulin level was raised from normal 58.4 ± 2.47 NTU up to 73.083 ± 2.367 and 147.416 ± 2.36 NTU in Low dose test extract (100 mg/kg) and High dose test extract (200 mg/kg) respectively. Phagocytic index of animals in control group was found to be 1.9 ± 0.196 and was raised upto 3.00 ± 0.067 and 4.77 ± 0.710 in Low dose test extract (100 mg/kg) and High dose test extract (200 mg/kg) respectively. In humoral antibody titer reaction button formation took place at 53.33± 6.76 and at 106.66 ± 13.49 in Low dose test extract (100 mg/kg) and High dose test extract (200 mg/kg) respectively. In Low dose test extract (100 mg/kg BW) there was increase in DTH response after 48 hours. In High dose test extract (200 mg/kg) there was significant increase in DTH reaction after 24 and 48 hours. This study showed that n-butanolic fraction of hydroalcoholic extract of *Costus speciosus* Koen rhizome possessed immunostimulatory activity.

INTRODUCTION: Several herbal preparations are used to enhance the body's immune status. Many plants constituent have unique ability to modulate immune system e.g. saponins, glycosides, polysaccharides alkaloid, flavonoids, sterols and sterolins.



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The advantage of using saponins based adjuvant is, they modulate all component of immune system, improve antibody production at low doses [1]. *Costus speciosus* Koen. (Keu, Crape ginger) is an Indian ornamental plant which belongs to the family Costaceae [2]. *Costus speciosus* rhizomes are rich in saponins. Actions of *Costus speciosus* are astringent, purgative, depurative, anti-inflammatory, anthelmintic, antivermin, maggoticide, antifungal, smooth-muscle-relaxant activity, cardiotonic activity and antispasmodic, CNS-depressant, diuretic and hydrocholeretic activities [3].

MATERIAL AND METHODS:

Chemicals:

Ethanol, n-butanol (71-36-3), EDTA, sodium chloride (7647-14-5), CMC, zinc sulphate (7446-20-0), barium chloride (10326-27-9), and sulphuric acid (7664-93-9) were purchased from CDH, pvt ltd, New Delhi.

Plant material:

Semi-dried rhizomes of *Costus speciosus* was procured and authenticated from VHCA herbals, Karnal, Haryana.

Extraction and fractionation:

Semi-dried rhizomes were further shade dried and coarsely powdered. 100 g of the powdered drug was defatted with 230 ml of petroleum ether at 50 ºC – 55 ºC for 4 hours. It was further extracted with 50% ethanol at 65-75ºC for 18 hours using soxhlet apparatus. The extractive was suspended in water and shaken with n-butanol saturated with water using separating funnel in 1:1. N-butanolic layer was taken out. N-butanolic fraction was concentrated under vacuum using rotary vacuum evaporator, dried and weighed [4].

Quantitative estimation of saponin:

Saponin content was determined by the method of Obadoni and Chuko (2001). About 2.0g of each extract was mixed with 100ml of 20% ethanol, and were incubated in a water bath at 55oC for 4hrs with stirring. The mixture was filtered and the extract was reextracted with 200ml of 20% ethanol. The combined extract was concentrated to 40ml in a water bath at 90°C. The concentrate was then transferred into a 20ml separatory funnel and 20ml diethyl ether was added, and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded, the purification process was heated and 60ml of n-butanol added. The combined n-butanol extracts were washed twice with 10ml of 5% aqueous NaCl, and the remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight, and the percentage saponin content was calculated [5]. The procedure was repeated thrice.

Animals:

Wistar albino rats weighing 150-200 gm and swiss albino mice weighing 22-30 gm were procured. The animals were housed under standard laboratory condition with temperature (25 ±1ºC), 12/12 h light dark cycle and fed with standard diet and water *ad libitum*. All the animals were acclimatized for a week before conducting the study. The experimental protocol was approved by CPCSEA. CPCSEA No. 711/02/a/CPCSEA.

Preparation of 20% v/v SRBC suspension:

The blood was collected from jugular vein of a healthy sheep from the Meerut local slaughterhouse, India, in a mixture of 0.5% EDTA and 0.9% of sodium chloride solution and preserved at a temperature of 2–8 °C. On the day of immunization, the blood sample was centrifuged at 4000 rpm for 10 min and then washed three times, to remove plasma, with 0.9% sodium chloride solution. The SRBC (20% v/v) suspension was then prepared in 0.9% sodium chloride solution [10].

Experimental protocol:

***In – vivo* Carbon clearance test:**

Animals (Wistar albino rats) were divided into three group. Each group contained six animals. Group I served as control and received 1% CMC (p.o), group II and group III received test extract low dose (100 mg/kg) and high dose (200 mg/kg) respectively from day 0 to day 7. On day 7, all the animal of entire group was treated with injection of Indian ink dispersion (0.3 ml per 30 g) via tail vein.

Blood samples were withdrawn by retro-orbital puncture and 50µl of this blood samples is taken in EDTA solution (125mM, 5µl) at interval of 0 and 15 minutes. Blood samples were added to 2 ml of 0.1% sodium carbonate to lyse the erythrocytes. Absorbances of samples were taken at 660 nm using Shimadzu UV-1601 spectrophotometer. After 15 min of blood collection animals were sacrificed and livers and spleen were collected and weighed. Rate of carbon clearance (K) and Phagocytic index (α) were calculated by using following formula.

$$\text{Rate of carbon clearance (K)} = \frac{\log OD_0 - \log OD_{15}}{T2 - T1}$$

$$\text{Phagocytic index } (\alpha) = \frac{K^{1/3} \times \text{body wt of animal}}{\text{Liver wt+spleen wt}}$$

Where OD_0 is the log absorbance of blood at 0min; OD_{15} is log absorbance of blood at 15 min; T2 is the last time point of blood collection; T1 is the

first time point of blood collection. Rate of carbon clearance and phagocytic index of treated group animals were compared with the control group animals [6, 7].

Effect on serum immunoglobulins:
Animals (Wistar albino rats) were taken for the study. They were divided into three groups, each group contained six rats. Group I was taken as control group and animals in this group were treated with 1% CMC (p.o). Animals in group II and group III received test extract low dose (100 mg/kg, p.o) and high dose (200 mg/kg, p.o). All the treatments were done up to 21 days. Blood samples were collected after 6 hours of the last dose and serum was separated. Serum was then analyzed for immunoglobulins level.

For each serum sample to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution were prepared. To each, 0.1 ml of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acids. The turbidity was expressed in NTU [8, 9].

Humoral antibody titre:
Mice were divided into three groups containing six animals each. Each animal were then immunized with 20% SRBC (0.1 ml) intraperitonially. Group I (control) was treated with 1% CMC. Group II and group III served as test group and received low dose (100 mg/kg, p.o) and high dose (200 mg/kg, p.o) for seven days. Blood samples were collected from individual animals by retro-orbital puncture on day 8 and the serum was separated. Two-fold dilution of 50µl sera (heat inactivated at 56 °C for 30 min) was performed in normal saline. Serial dilution (taking 50µl of the aliquot) was performed in 50µl normal saline medium into 96 well micro-titer plates. The fresh, SRBC (1.0%; 25µl) suspension was dispensed into each well and mixed thoroughly. The plates were then incubated at room temperature for 2h and examined for button formation. The reciprocal of the dilution, just before the button formation, was observed and values were calculated[10].

Delayed type hypersensitivity response:
Mice were divided into three groups containing six animals each. Group I (control) was treated with 1% CMC. Group II and group III served as test group and received low dose (100 mg/kg, p.o) and high dose (200 mg/kg, p.o) for seven days orally on day 0 and continued till day 7 of challenge. On 7th day the thickness of right hind foot pad was measured using digital vernier caliper. The animals were then challenged by injecting SRBCs suspension in right hind foot pad and foot pad thickness was measured at 0, 24 and 48 h after the challenge. The difference between pre and post challenge foot pad thickness expressed in mm was taken as a measure of DTH response and the mean value obtained for treatment groups were compared with that of control group. The data obtained was subjected to statistical analysis[11].

RESULTS: Table 1 presents Phytochemical screening of n-butanolic fraction of hydroalcoholic extract of *Costus speciosus* Koen rhizome which shows presence of saponins, glycosides and carbohydrates.

TABLE 1: PHYTOCHEMICAL SCREENING OF HYDROALCOHOLIC EXTRACT OF COSTUS SPECIOSUS KOEN RHIZOME.

S.No.	Phytoconstituents	N-butanolic fraction of Hydroalcoholic extract of *Costus speciosus* Koen rhizome
1	Alkaloid	-
2	Glycoside	+
3	Saponin	+ +
4	Flavanoids	-
5	Protein	-
6	Tannins	-
7	Carbohydrate	+
8	Volatile oil	-
9	Triterpenoid	-

(+) Present (-) Absent

Table 2 presents percent saponin content of n-butanolic fraction of hydroalcoholic extract of *Costus speciosus* Koen rhizome. The saponin content was found to be 12.93 ± 0.324.

TABLE 2: PERCENT SAPONIN CONTENT OF N-BUTANOLIC FRACTION OF HYDROALCOHOLIC EXTRACT OF *COSTUS SPECIOSUS* KOEN RHIZOME

S.No	Extract	Saponin (%)
1	n-butanolic fraction of hydroalcoholic extract of *Costus speciosus* Koen rhizome.	12.93 ± 0.324

In case of carbon clearance test results showed that HDTE (200 mg/kg body weight) was effective in clearing the inorganic carbon particle and hence showed significant (at * p<0.05, **p<0.01, ***p<0.001) effect in carrying out the phagocytosis. Treatment of animal with LDTE (100 mg/kg BW) does not effectively phagocytose the carbon particle and hence had lower phagocytic index when compared with control. Phagocytic index of animals in control group was found to be 1.9 ± 0.196. Phagocytic index of animals in LDTE and HDTE was found to be 3.00 ± 0.067 and 4.77 ± 0.710 (**Table 3, Fig. 1**).

TABLE 3: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON PHAGOCYTIC INDEX.

Treatment	Dose (mg/kg body weight)	Phagocytic index
Control (1% CMC)	-	1.9±0.196
LDTE	100 mg/kg	3.00±0.067^{ns}
HDTE	200 mg/kg	4.77 ±0.710***

Results are expressed as mean ± SEM, (n=6), analysed by one way ANOVA followed by Dunnet's test. * P<0.05, **p<0.01, ***p<0.001

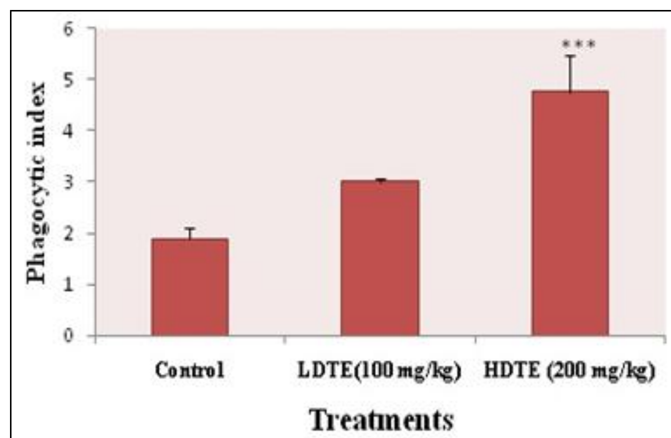

FIG. 1: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON PHAGOCYTIC INDEX. Results are expressed as mean ± SEM, (n=6), analysed by one way ANOVA followed by Dunnet's test. *p<0.05, **p<0.01, ***p<0.001 when Arthritic control group compared with other treated groups.

There was elevation of serum immunoglobulins level of animals in both LDTE and HDTE groups when compared to animals in control group. However, the effect was highly significant (at * p<0.05, **p<0.01, ***p<0.001) in case of HDTE (200 mg/kg BW) and slightly less significant in case of LDTE (100 mg/kg body weight) indicating immunostimulatory effect of the drug. Serum immunoglobulins level of animals in normal control group was found to be 58.4 ± 2.47 NTU. LDTE and HDTE raised serum immunoglobulins level up to 73.083 ± 2.367 and 147.416 ± 2.36 respectively (**Table 4, Fig. 2**).

TABLE 4: EFFECT OF COSTUS SPECIOSUS KOEN ON SERUM IMMUNOGLOBULIN LEVEL OF ANIMALS

Treatment	Dose (mg/kg of body weight)	Serum Ig level (NTU)
Control (1% CMC)	-	58.4± 2.47
LDTE	100	73.083 ± 2.549**
HDTE	200	147.416 ± 2.367***

Results are expressed as mean±sem, (n=6), analysed by one way ANOVA followed by Dunnet's test. * P<0.05, **p<0.01, ***p<0.001 when control group compared with other treated groups

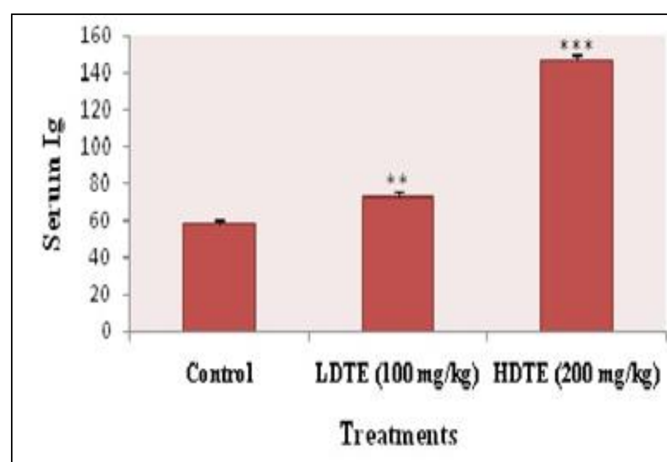

FIG. 2: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON SERUM IG LEVEL OF EXPERIMENTAL ANIMALS. Results are expressed as mean ± SEM, (n=6), analysed by one way ANOVA followed by Dunnet's test. * P<0.05, **p<0.01, ***p<0.001 when control group compared with other treated groups.

HA titer was estimated by interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. These antibodies bind to antigen and neutralize it or facilitate its elimination by cross linking to form latex that is more readily ingested by phagocytic cells. The end point of this reaction is button formation which indicates antigen-antibody reaction. At LDTE (100 mg/kg BW) button formation took place at 53.33± 6.74 and the effect of HDTE (200 mg/kg BW) was highly significant,

button formation took place at 106.99 ± 13.49 (**Table 5, Fig. 3**).

TABLE 5: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON HA TITRE VALUE.

Treatment	Dose (mg/kg body weight)	HA titre
Control (1% CMC)	-	9.66±2.84
LDTE	100	53.33± 6.746**
HDTE	200	106.667 ± 13.492***

Results are expressed as mean±sem, (n=6), analysed by one way ANOVA followed by Dunnet's test. P<0.05, **p<0.01, ***p<0.001 when Arthritic control group compared with other treated groups.

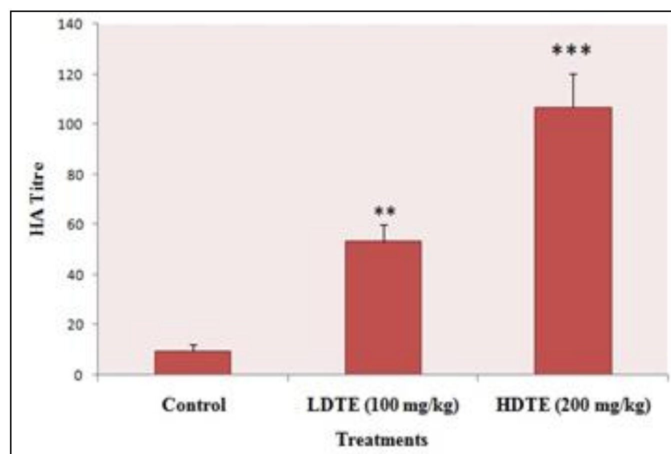

FIG.3: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON HA TITRE OF EXPERIMENTAL ANIMALS. Results are expressed as mean ± SEM, (n=6), analysed by one way ANOVA followed by Dunnet's test. *p<0.05, **p<0.01, ***p<0.001 when Arthritic control group compared with other treated groups.

Delayed type hypersensitivity reaction was estimated by measurement of paw thickness. An increased in paw oedema thickness was calculated after +24 and +48. In our study, foot volume was enhanced after treatment with n-butanolic fraction of hydroalcoholic extract of *Costus speciosus* suggesting enhancement of cell mediated immune by SRBC.

LDTE (100 mg/kg BW) DTH response is not significant after 24 hours but after 48 hours there was increase in DTH reaction. Treatment of animals with HDTE (200 mg/kg BW) there was significant increase in DTH reaction (at * p<0.05. **p<0.01, ***p<0.001) after 24 and 48 hours (**Table 6, Fig.4**).

TABLE 6: EFFECT OF COSTUS SPECIOSUS KOEN EXTRACT ON DTH REACTION.

Treatment	Dose (mg/kg of body weight)	DTH (24 hrs)	DTH (48 hrs)
Control(1% CMC)	-	0.113±0.077	0.011± 0.048
LDTE	100	0.363± 0.045*	0.383± 0.048***
HDTE	200	0.696± 0.066***	0.753± .090***

Results are expressed as mean±sem, (n=6), analysed by one way ANOVA followed by Dunnet's test. * P<0.05, **p<0.01, ***p<0.001 when Arthritic control group compared with other treated groups

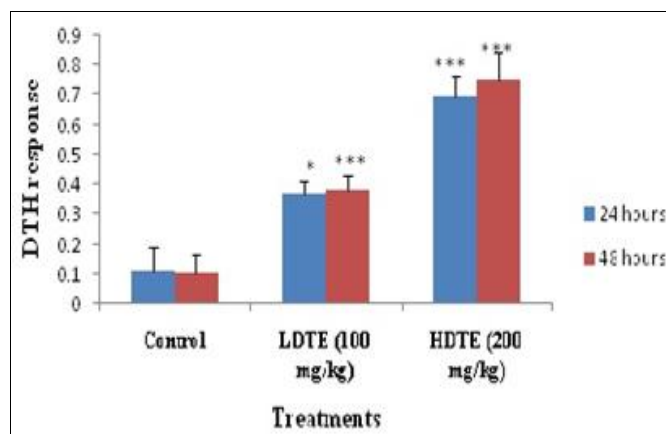

FIG.4: EFFECT OF *COSTUS SPECIOSUS* KOEN EXTRACT ON DTH RESPONSE. Results are expressed as mean ± SEM, (n=6), analysed by one way ANOVA followed by Dunnet's test. * P<0.05, **p<0.01, ***p<0.001 when Arthritic control group compared with other treated groups.

DISCUSSION: Different models were used to evaluate the immunomodulatory activity of *Costus speciosus.* Zinc sulphate turbidity test was used for rough estimation of Immunoglobulins. Immunoglobulins are antibodies that react specifically with the antigen. The zinc sulphate turbidity test is used to gain a rough estimation of the amount of immunoglobulins present in the serum. Zinc sulphate causes precipitation of the immunoglobulins making the solution cloudy[9]. The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis. When ink containing colloidal carbon is injected intravenously via tail vein, the macrophages engulf the carbon particles of the ink. Rate of clearance of (carbon particles) ink from blood is known as phagocytic index[12].

SRBCs suspension is given in subplanter region in Delayed type hypersensitivity reaction because this model is simply based on the fact that whenever

any antigen is injected subplanter region it will cause edema due to inflammation. Inflammation is generally seen by inducing paw edema. In case the extract is immunostimulatory, edema (inflammation) will progress due to accumulation of macrophages, leucocytes and other immune cells. The DTH response directly correlated with T-lymphocytes therefore increased the effect on cell mediated immunity. Antigens sensitized T-lymphocytes to convert lymphoblasts and secrete lymphokines, attracting more scavenger cells such as macrophages and basophils and induction becomes apparent within 0-48 h in rats[13].

The HA titer was other model used for evaluation of immunomodulatory activity. SRBCs suspension was administered i.p. in this model because it is based upon antigen-antibody reaction which is seen as button formation as end result. This button formation takes place because, first the antigen (SRBCs suspension) was administered i.p. for development of antibody, specifically for this antigen (SRBCs suspension) and the basis of the result is, first the serum containing antibody was taken in ELISA plate and a antigen was added to it , so that due to antigen-antibody reaction button formation took place. It involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. These antibodies bind to antigen and neutralize it or facilitate its elimination by cross linking to form latex that is more readily ingested by phagocytic cells[14].

The test involves the preparation of double dilutions of serum samples and the addition of constant amount of the SRBC. If the serum contains antibodies to the SRBC, there will be agglutination because of the formation of antibody bridges with the neighboring erythrocytes and these settle at the bottom as latex. Unagglutinated red blood cells appear in the well bottom as a button. Treatment of animals both with LDTE (100 mg/kg BW) and HDTE (200 mg/kg BW) significantly increased HA titer in dose dependant manner[15].

The immunostimulatory activity of *Costus speciosus* Koen may be contributed to presence of saponins. Though, the exact mechanism of immune stimulatory action of saponins has not been completely understood but many theories have been put forward. Saponins reportedly induce production of cytokines like interleukins and interferons that might mediate its immune stimulant action[16].

CONCLUSION: Estimation of serum Ig level, phagocytic index, DTH response and HA titer was used as a model for evaluation of immunomodulatory activity. The findings suggested that both at low doses and high doses the *Costus speciosus* Koen rhizome extract elevated serum Ig level and phagocytic index, DTH reaction, HA titer in dose dependant manner i.e. the effect at higher dose (200 mg/kg BW) were found to be highly significant than lower dose (100 mg/kg BW) which produce less significant effects.

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