



INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND DEVELOPMENT (IJPRD)

Platform for Pharmaceutical Researches & Innovative Ideas

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THE PROTECTIVE ROLE OF BENINCASA HISPIDA ON DICLOFENAC SODIUM INDUCED HEPATOTOXICITY IN ALBINO RAT MODEL

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ABSTRACT

The objective of the present investigation was to study the protective role of aqueous extract of pulps of *Benincasa hispida* (BH) on diclofenac sodium- induced hepatotoxicity model in adult male albino rats. Hepatotoxicity in rats was caused by diclofenac sodium at a dose of 10 mg- /ml/kg body weight. Hepamerz (L-ornithine, L- aspartate) was administered orally as standard hepatoprotective agent for 14 consecutive days prior to diclofenac sodium treatment at a dose of 10mg- /ml/kg body weight. This drug has many side effects. These side effects have prompted the scientific world for the search of alternative herbal remedies of liver damage. In our country BH is easily available, cheap and has no side effects. The aqueous pulp extract of BH was administered orally to rats daily for 14 days before diclofenac sodium treatment. The biochemical parameters were investigated. The results indicated that biochemical changes produced by diclofenac sodium were restored to normal by aqueous extract of pulps of BH. The aqueous pulp extract of BH showed significant hepatoprotective effect through the modulation of antioxidant - mediated mechanism by altering serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) and lipid peroxidation (LPO) levels - against diclofenac sodium - induced hepatotoxicity model in rats.

Key words: *Benincasa hispida*, diclofenac sodium, Hepamerz, hepatotoxicity.

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INTRODUCTION

Diclofenac sodium is a NSAID (non steroidal anti-inflammatory drug) that has been used as anti-inflammatory, analgesic, and antipyretic agent in 120 countries since its introduction in Japan in 1974^[1]. It is currently the eighth largest selling drug and the most frequently used NSAID in the world. Diclofenac is a potent inhibitor of cyclo-oxygenase enzyme activity as well as lipo-oxygenase enzyme pathway with the release and reuptake of arachidonic acid. It is used in the United States for long term symptomatic treatment of rheumatic arthritis, osteo-arthritis and ankylosing spondylitis. Diclofenac is highly effective in relieving dysmenorrhoea in most women and also in the treatment of uncomplicated urinary tract infections^[2].

This widely used drug has some adverse effect, like hepato-toxicity, elevation of transaminase activity, loss of body weight, myocardial infarction, renal failure and gastrointestinal ulcer^[3].

Liver is an important organ for detoxification and disposition of endogenous substances. It is continuously and widely exposed to xenobiotics, hepatotoxins, and chemotherapeutic agents that lead to impairment of its functions^[4]. Diclofenac is a member of the arylalkanoic group of NSAIDs. Early reports about diclofenac showed that it caused a rise in liver function tests^[5]. Since 1983 five reports of hepatitis caused by diclofenac have been published, including that of a 55 year old French woman, who died with fulminant hepatitis after a three week course of diclofenac^[6, 7, 8, 9, 10].

On the other hand Hepamerz (L-ornithine, L-aspartate) has been reported as a liver-protective drug which corrects liver toxicity^[11]. But it has many side effects like vomiting, nausea, headache, etc. These side effects have prompted the scientific world for the search of alternative herbal remedies of liver damage. A number of Indian medicinal plants have been used for thousands of years in the traditional system of medicine (Ayurveda). These plants have been used for the management of hepatitis and liver damage. Some of these plants have already been reported to possess strong antioxidant activity. *Benincasa*

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hispidata (BH) pulp contains vitamin-E, beta-carotene, flavonoids and flavonols. It has no side effects. The BH tree is like annual vines, has thick, furrowed stems with coarse hairs and triangular and irregularly lobed leaves. The fruits and seeds of BH possess a number of pharmacological properties and uses: anthelmintic^[12], laxative, tonic, diuretic, aphrodisiac, antiperiodic, in haemoptysis, other internal haemorrhages, in insanity, epilepsy and other nervous disorders^[13]. Some of the important compounds reported to have been isolated from BH are triterpenes, sterols and glycosides^[14] and volatile oils.

Thus, the present study was undertaken to determine the protective role of BH pulp extract on diclofenac sodium-induced hepatotoxicity with the possible involvement of antioxidants.

ACTUAL MATERIALS AND METHODS

Collection of Plant Materials

The fruits of BH were purchased from the local market and the identity of the plant was authenticated by the Botanical Survey of India, Howrah, and kept in the Department – of Physiology, Katwa College, University of Burdwan.

Preparation of Aqueous Extract from the Pulp of BH

The pulp of BH fruit was used throughout the experimental study. The fruits of BH were cut into pieces, sun-dried and ground with the help of an electrical grinder to get a free-flowing powder. This powder was subjected to extraction with water (1:3) at room temperature for 48 hours. The extract obtained was filtered through Whatman filter paper and vacuum dried at 40–50°C to get a dry powder, which was dissolved in double-distilled water for final use^[15].

Chemicals

Diclofenac sodium and Hepamerz were obtained as gift samples from Krishnath College (University of Kalyani), Berhampore, Murshidabad, West Bengal, India.

Animals Used and Maintenance

Thirty-six male Holtzman strain adult albino rats of age approximately 120 days and weighing 250--300 g were used in the following studies. The animals were individually housed and maintained under standard laboratory conditions with natural dark and light cycle (approximately 12--h light/10--h dark cycle) and room temperature ($27\pm 1^{\circ}\text{C}$) and constant humidity (60%) in accordance with the 'Institutional Ethical Committee'- rules and regulations. Food and water were freely available except during testing. Drinking water was supplied ad libitum. Animals were randomly divided equally into six groups of six each, - as follows: control group, diclofenac sodium treated experimental group, BH treated control group, Hepamerz treated control group, BH pretreated diclofenac sodium treated experimental group and Hepamerz pretreated diclofenac sodium treated experimental group. The control group was kept in the laboratory condition for 42 (28+14=42) days. A dose of 10 mg-/ml/kg body weight of diclofenac sodium was given orally through orogastric cannula daily for 28 days to each of the 2nd, 5th and 6th group of the animals. The dose was standardised in our laboratory. In the 4th and 6th groups, i.e., Hepamerz treated control group and Hepamerz pretreated diclofenac sodium treated experimental group, a dose of 5 ml of liver protective drug Hepamerz (L--ornithine, L--aspartate) was administered for 14 days. After that, diclofenac sodium at a dose of 10mg- /ml/kg body weight was administered orally for about 28 days. Body weights of the rats were recorded everyday and maintained in the laboratory throughout the experimental period [15].

Biochemical Estimations

Collection of serum

Rats were sacrificed by cervical dislocation after 28 days of diclofenac sodium treatment. The blood was collected by heart puncture and serum was separated by centrifugation (3000 rpm at 4°C for 10 min). The liver was immediately removed.

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Estimation of SGPT, SGOT and ALP levels

Tissue and serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities were measured according to the method of Kind and King (1980) [16], alkaline phosphatase (ALP) activity was measured according to the procedure of Reitman and Frankel (1957) [17].

Measurement of SOD

Superoxide dismutase (SOD) was estimated by the method of Mishra and Fridovich (1972) [18] and Roy *et al.*, (2007) [15]. Brain tissue samples were homogenised with 5 ml of ice--cold 0.1 M phosphate buffer (pH--7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. Then, 0.1 ml of sample was mixed with 0.8 ml of TDB. Reaction was started by the addition of 4 μl of nicotinamide adenine dinucleotide phosphate (NADPH). Then, 25 μl of ethylenediaminetetraacetic acid- manganese chloride (EDTA-- MnCl_2) mixture was added to it. Thereafter, spectrophotometric readings were recorded at 340 nm. After recording of the spectrophotometric readings, 0.1 ml of mercaptoethanol was added to this mixture and again spectrophotometric readings were recorded at 340 nm.

Measurement of LPO

Lipid peroxidation was measured according to the method of Bhattacharya *et al* (2001) [19] and Roy *et al.*, (2007) [15]. Brain tissue samples were homogenised with 5 ml of ice--cold 0.1 M phosphate buffer (pH--7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. Then, 0.5 ml of sample was mixed with 1 ml of TDB and then the mixture was incubated at 37°C for 1 hour. To this, 0.5 ml of trichloroacetic acid (TCA) was added, vortexed and the absorbance was read at 350 nm. After recording of the spectrophotometric reading, 1 ml sample was mixed with 500 μl mercaptoethanol and again the absorbance was read at 350 nm.

Measurement of CAT

Catalase activity was estimated by the method of Cohen *et al* (1970) [20] and Roy *et al.*, (2007) [15]. Brain tissue samples were homogenised with 5 ml of ice--cold 0.1 M phosphate buffer (pH--7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. The precipitate was then stirred with the addition of 15 ml of ice--cold 0.1 M phosphate buffer and allowed to stand in cold condition with occasional shaking. The shaking procedure was repeated for thrice. 1 ml of sample was added to 9 ml of H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from the changes in absorbance at 350 nm. The activity of CAT was expressed as % inhibition unit.

Measurement of GSH

Reduced glutathione was measured according to the method of Ellman (1959) [21]. Equal quantity of homogenate was mixed with 10% TCA and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH--8.4), 0.5 ml of 5, - 5--dithiobis- (2--nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of GSH was expressed as µg/g of tissue.

Statistical Analysis

The data were expressed as MEAN ± SEM and were analysed statistically using one-way analysis of variance (one-way ANOVA), followed by multiple comparison 't'- test, which was used for statistical evaluation of the data. In addition to this, two-

tailed Student's 't'- test was performed to determine the level of significance between the means. Difference below the probability level 0.05 was considered statistically significant.

RESULTS

Twenty-eight days after diclofenac sodium treatment, the SGOT, SGPT, ALP, SOD and CAT activities, GSH and LPO levels were estimated. There was a sharp rise (P<0.001) in SGOT, SGPT and ALP activities in the diclofenac sodium treated experimental group as compared to the control group. The SGOT, SGPT and ALP activities were significantly (P<0.001) decreased in BH treated control group compared to the control group. BH significantly (P<0.001) decreased SGOT, SGPT and ALP activities in BH pretreated diclofenac sodium treated experimental group compared to the diclofenac sodium treated experimental group. There was a sharp decline (P<0.001) in SGOT, SGPT and ALP activities in the Hepamerz treated control group compared to the control group. The SGOT, SGPT and ALP activities were significantly (P<0.001) decreased in Hepamerz treated control group in comparison to BH treated control group. The SGOT, SGPT and ALP activities were significantly (P<0.001) decreased in Hepamerz pretreated diclofenac sodium treated experimental group when compared to diclofenac sodium treated experimental group. The results are shown in Table-1.

Table 1: Effect of BH on SGPT, SGOT and ALP activity in diclofenac sodium- induced hepatotoxic rat model

Group (n)	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)
Control	52.02 ± 0.03	56.45 ± 0.04	58.91 ± 0.06
Diclofenac treated	133.46 ± 1.02 ***	129.73 ± 0.32 ***	132.25 ± 0.24 ***
BH + Control	44.98 ± 0.02 *	45.87 ± 0.02 *	42.68 ± 0.02 *
BH + Diclofenac	89.96 ± 0.32 **	91.82 ± 0.06 **	90.89 ± 0.05 **
Hepamerz + Control	42.02 ± 0.02 *	41.87 ± 0.02 *	38.41 ± 0.03 *
Hepamerz + Diclofenac	85.91 ± 0.14 **	85.85 ± 0.04 **	86.92 ± 0.10 **

Values are mean ± SEM, n = 6. Data were analyzed statistically using one-way ANOVA test followed by multiple comparison t-test. * P < 0.001 when compared with control group; ** P < 0.001 when compared with diclofenac sodium treated group; *** P < 0.001 when compared with other mentioned groups.

There was a sharp decline ($P < 0.001$) in SOD activity both in serum and liver in the diclofenac sodium treated experimental group as compared to the control group. The SOD activity was significantly ($P < 0.001$) increased in BH treated control group compared to the control group, both in serum and liver. BH significantly ($P < 0.001$) increased SOD activity in BH pretreated diclofenac sodium treated experimental group in comparison to diclofenac sodium treated experimental group, both in serum and liver. There was a sharp increase ($P < 0.001$) in

SOD activity both in serum and liver in the Hepamerz treated control group compared to the control group. The SOD activity was significantly ($P < 0.001$) increased in Hepamerz treated control group in comparison to BH treated control group both in serum and liver. The SOD activity was significantly ($P < 0.001$) increased in Hepamerz pretreated diclofenac sodium treated experimental group compared to the diclofenac sodium treated experimental group both in serum and liver. The results are shown in Tables-2 and 3.

Table 2: Effect of BH on serum antioxidant enzymatic changes in diclofenac sodium - induced hepatotoxic rat model

Group (n)	SOD (% inhibition unit)	LPO (nmol of [TBARS /-g mol of tissue)	CAT (% inhibition unit)	GSH ($\mu\text{g/g}$ of tissue)
Control	13.42 \pm 0.02	4.97 \pm 0.02	14.71 \pm 0.02	31.96 \pm 0.07
Diclofenac treated	23.88 \pm 0.03 ^{***}	13.02 \pm 0.02 ^{***}	27.05 \pm 0.03 ^{***}	3.69 \pm 0.05 ^{***}
BH + Control	10.31 \pm 0.02 [*]	2.39 \pm 0.02 [*]	10.23 \pm 0.04 [*]	35.57 \pm 0.06 [*]
BH + Diclofenac	18.34 \pm 0.05 ^{**}	8.57 \pm 0.02 ^{**}	21.05 \pm 0.05 ^{**}	12.11 \pm 0.02 ^{**}
Hepamerz + Control	16.53 \pm 0.03 [*]	1.71 \pm 0.02 [*]	8.78 \pm 0.02 [*]	37.36 \pm 0.05 [*]
Hepamerz + Diclofenac	17.23 \pm 0.02 ^{**}	7.22 \pm 0.02 ^{**}	18.41 \pm 0.02 ^{**}	15.12 \pm 0.02 ^{**}

Values are mean \pm SEM, n = 6. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison *t*-test. ^{*} P < 0.001 when compared with control group; ^{**} P < 0.001 when compared with diclofenac sodium treated group; ^{***} P < 0.001 when compared with other mentioned groups. TBARS- Thio-barbituric acid reactive substances.

Table 3: Effect of BH on tissue antioxidant enzymatic changes in diclofenac sodium- induced hepatotoxic rat model

Group (n)	SOD (% inhibition unit)	LPO (nmol of [TBARS /-g mol of tissue)	CAT (% inhibition unit)	GSH ($\mu\text{g/g}$ of tissue)
Control	12.35 \pm 0.02	6.19 \pm 0.01	14.98 \pm 0.03	32.57 \pm 0.03
Diclofenac treated	24.98 \pm 0.02 ^{***}	13.41 \pm 0.02 ^{***}	27.43 \pm 0.03 ^{***}	3.66 \pm 0.02 ^{***}
BH + Control	10.41 \pm 0.01 [*]	2.78 \pm 0.02 [*]	10.22 \pm 0.02 [*]	37.34 \pm 0.02 [*]
BH + Diclofenac	17.42 \pm 0.01 ^{**}	8.67 \pm 0.04 ^{**}	19.35 \pm 0.03 ^{**}	11.96 \pm 0.04 ^{**}
Hepamerz + Control	15.53 \pm 0.02 [*]	1.67 \pm 0.01 [*]	8.92 \pm 0.03 [*]	38.67 \pm 0.02 [*]
Hepamerz + Diclofenac	16.51 \pm 0.02 ^{**}	6.72 \pm 0.02 ^{**}	17.41 \pm 0.04 ^{**}	14.89 \pm 0.03 ^{**}

Values are mean \pm SEM, n = 6. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison *t*-test. ^{*} p < 0.001 when compared with control group; ^{**} p < 0.001 when compared with diclofenac sodium treated group; ^{***} p < 0.001 when compared with other mentioned groups. TBARS- Thio-barbituric acid reactive substances.

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There was a sharp rise ($P<0.001$) in LPO level both in serum and liver in the diclofenac sodium treated experimental group as compared to the control group. The LPO level was significantly ($P<0.001$) decreased in BH treated control group compared to the control group, both in serum and liver. BH significantly ($P<0.001$) decreased LPO level in BH pretreated diclofenac sodium treated experimental group in comparison to diclofenac sodium treated experimental group, both in serum and liver. There was a sharp decline ($P<0.001$) in LPO level both in serum and liver in the Hepamerz treated control group when compared to the control group. The LPO level was significantly ($P<0.001$) decreased in Hepamerz treated control group compared to the BH treated control group, both in serum and liver. The LPO level was significantly ($P<0.001$) decreased in Hepamerz pretreated diclofenac sodium treated experimental group when compared to the diclofenac sodium treated experimental group, both in serum and liver. The results are shown in Tables-2 and 3.

There was a sharp decline ($P<0.001$) in CAT activity both in serum and liver in the diclofenac sodium treated experimental group as compared to the control group. The CAT activity was significantly ($P<0.001$) increased in BH treated control group when compared to the control group, both in serum and liver. BH significantly ($P<0.001$) increased CAT activity in BH pretreated diclofenac sodium treated experimental group in comparison to diclofenac sodium treated experimental group, both in serum and liver. There was a sharp increase ($P<0.001$) in CAT activity, both in serum and liver in the Hepamerz treated control group compared to the control group. The CAT activity was significantly ($P<0.001$) increased in Hepamerz treated control group in comparison to BH treated control group, both in serum and liver. The CAT activity was significantly ($P<0.001$) increased in Hepamerz pretreated diclofenac sodium treated experimental group when compared to the diclofenac sodium treated experimental group, both in serum and liver. The results are shown in Tables-2 and 3.

There was a sharp decline ($P<0.001$) in GSH level both in serum and liver in the diclofenac sodium

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treated experimental group as compared to the control group. The GSH level was significantly ($P<0.001$) increased in BH treated control group compared to the control group, both in serum and liver. BH significantly ($P<0.001$) increased GSH level in BH pretreated diclofenac sodium treated experimental group in comparison to diclofenac sodium treated experimental group, both in serum and liver. There was a sharp increase ($P<0.001$) in GSH level, both in serum and liver in the Hepamerz treated control group compared to the control group. The GSH level was significantly ($P<0.001$) increased in Hepamerz treated control group when compared to the BH treated control group, both in serum and liver. The GSH level was significantly ($P<0.001$) increased in Hepamerz pretreated diclofenac sodium treated experimental group compared to diclofenac sodium treated experimental group both in serum and liver. The results are shown in Tables-2 and 3.

DISCUSSION

The present study evaluates the protective role of aqueous pulp extract of BH on diclofenac sodium - induced hepatotoxicity in albino rat model with the possible involvement of the antioxidants. SGPT, SGOT and ALP are the most sensitive tests employed in the diagnosis of hepatic disease. It is evident from the results of the present investigation that treatment with aqueous pulp extract of BH significantly decreased the SGPT, SGOT and ALP activities at a dose of 400 mg/kg body weight. These findings can be explained by alterations of the LPO level and antioxidant activities such as that of SOD, CAT and GSH, both in serum and liver tissue.

Free radicals play an important role in the pathogenesis of liver damage. LPO can be used as an index for measuring the damage that occurs in membranes of tissue as a result of free radical generation [22, 23]. In our present study, oral administration of diclofenac sodium, significantly increased the LPO level. Significant elevation of LPO level observed in diclofenac sodium induced experimental- hepatotoxic group is possibly due to the generation of free radicals via auto-oxidation

or through metal ion or superoxide catalysed oxidation process. In our present study, BH significantly decreased LPO level at a dose of 400 mg/kg body weight compared to other groups. The aqueous pulp extract of BH was found to have excellent scavenging effect on LPO, which was well comparable with the standard drug Hepamerz. So, from the results obtained on LPO levels, it may be concluded that the protection by BH may be due to vitamin E, beta-carotene, flavonols, and flavonoids, which are present in BH pulp extract.

Endogenous antioxidant status in diclofenac sodium- induced experimental rat group was evaluated here by noting the activities of CAT, SOD and GSH as these are the important biomarkers for scavenging free radicals [24]. This result also suggest that diclofenac produces hepatic injury [25].

The primary role of CAT is to scavenge H₂O₂ that has been generated by free radicals or by SOD in its - removal of superoxide anions, and convert it to water [26]. From our experimental results of the aforesaid antioxidant enzyme activities in serum and liver tissues, it is clear that diclofenac sodium significantly decreased SOD, CAT, GSH activities in diclofenac sodium- treated experimental group compared to – control group, BH treated control group, Hepamerz treated control group, Hepamerz pretreated diclofenac sodium- treated experimental group and BH pretreated diclofenac sodium- treated experimental group. So, it may be concluded that the protection by BH may be due to vitamin E, beta-carotene, flavonols, and flavonoids, which are present in BH pulp extract.

The decreased level of GSH in diclofenac sodium-treated experimental group seen in our study indicates that there was an increased generation of free radicals and the GSH was depleted during the process of combating oxidative stress [27, 28]. This has probably been possible either from the low level of reactive oxygen species (ROS) production or through a rapid dissolution of ROS that has further been strengthened by the elevated activities of important antioxidant defense enzymes CAT and SOD, studied in this experiment. BH showed a strong antioxidant protection on colchicine- induced experimental rat model of

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Alzheimer's disease [15]. Phenolics are capable of scavenging peroxy radicals [29]. The preliminary phytochemical analysis of the extract showed the presence of flavonoids. Since the anti-oxidant and hepatoprotective activities of certain flavonoids from plant origin have already been established [30], we can conclude that these constituents may be responsible for the observed protective effects.

It has been reported that the BH fruit contains high level of vitamin E and beta-carotene which help in protecting the rat neurons against oxidative stress because both vitamin E (alpha tocopherol and other tocopherol) and beta-carotene are most potent chain breaking antioxidant. It may be inferred from the present results that BH protected rat liver against oxidative stress as is evidenced from our results of SGPT, SGOT, ALP, LPO, CAT, SOD and GSH activities, possibly by vitamin E, beta-carotene, flavonols and flavonoids, which are present in BH pulp.

The salient findings of our present study suggest that phenolic compounds of the BH pulp extract provide a good source of antioxidants that could offer potential protective effects against LPO and which could be exploited to make a hepatoprotective formulation.

ACKNOWLEDGMENTS

We are highly indebted to B.Sc final year students, Krishnath College, University of Kalyani, and B.Sc final year students, Katwa College, University of Burdwan, for their valuable co-operation. The authors have no conflicts of interest and there is no funding agency to support of this study and its publication.

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