Hepatoprotective Activity of Carvacrol in Paracetamol Induced Hepatotoxicity

[1]Umesh Kumar Singh Sikarwar,[2]Manoj Singh Bhadouria, [3]Navesh Veer

[1]Lecturer, [2]Associate Professor, [3]Assistant Professor
[1]Sarvodhya Adarsh Pharmacy College, Agra, UP, 282007, India
[2]Nagaji Institute of Pharmaceutical Sciences, GwaliorMP,474001,India
[3]F.S. University, Shikohabad, Firozabad, UP 283135, India

ABSTRACT:
AIM-The main aim of the present work is to study the hepatoprotective role of Carvacrol in Paracetamol induced hepatotoxicity. MATERIAL & METHODS-Carvacrol was evaluated for their possible hepatoprotective activity by paracetamol induced hepatic damage. Standard drug Silymarin was used as reference compound. For the paracetamol induced hepatotoxicity studies, paracetamol (500mg/kg po) suspension was prepared using 1% gum acacia and was administered to all animals except the animals of the normal control group. Silymarin (100 mg/kg po) was used as standard. The biochemical parameters like SGOT, SGPT, ALP and bilirubin were estimated using respective assay kits according to the methods described. RESULTS- Effect of carvacroland paracetamol on SGPT level of rats is shown in table. A single dose of paracetamol (500 mg/kg) caused significant elevation in the serum SGPT level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed. Paracetamol (500 mg/kg) caused significant elevation in the serum SGOT level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed. A single dose of paracetamol (500 mg/kg) caused significant elevation in the serum ALP level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed. CONCLUSION- The selected carvacrol are widely employed in various liver disorders in traditional systems of medicine. The present pharmacological evaluation will provide scientific data about the rationale behind their use.

KEYWORDS- Hepatoprotective Activity, Carvacrol, Paracetamol, SGOT, SGPT, ALP

INTRODUCTION:

When the drug or one of its metabolites acts as a hapten and induces hypersensitivity in the host. In many instances, drugs hepatotoxicity is associated with the appearances of auto-antibodies to liver-kidney microsomes (i.e. anti-LKM2) directed against cytochrome P450 enzyme [Sheth et al., 1970].Occasionally the drugs employed for management of different disorders turns fatal to the hepatic system because the metabolism of almost every compound ingested, is performed by the liver. By nature a majority of drugs used are lipophilic and they are converted chemically to hydrophilic substances during their metabolism. This process is termed as biotransformation. The hydrophilic substances are then excreted in urine or bile to get rid of toxic substances. The transformation process usually involves two reactions namely phase 1 and phase 2 reactions. For phase 1 reaction cytochrome P450 works as mediator and the reaction consist of metabolism of compound by oxidation or demethylation process. Paracetamol is a well known drug for relief of fever and management of pain. It is an over-the-counter remedy for almost all types of pain ranging from mild headache & body pain to more severe post-operative pain. Generally paracetamol is used in combination with other opioid analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) for the relief of severe pain and is widely used as an ingredient in numerous cold and flu remedies [Daly et al., 2008; Khashab et al., 2007; Hawkins et al., 2007; Larson et al., 2005]. The main aim of the present work is to study the hepatoprotective role of Carvacrol in Paracetamol induced hepatotoxicity.

MATERIALS AND METHODS:

SELECTION AND COLLECTION OF ACTIVE COMPOUND:

The active compound i.e. Carvacrol was identified as hepatoprotective agent and procured from Himedia Pvt. Ltd., Mumbai.

ANALYTICAL TECHNIQUES EMPLOYED:

Varioustechniques wereemployedandseveralinstruments were used for characterization and identification of the active compound for the determination of percentage purity.

Melting point determination:

This technique was used to determine the melting point of the active compound.

HEPATOPROTECTIVE ACTIVITY:

Carvacrol was evaluated for their possible hepatoprotective activity by paracetamol induced hepatic damage. Standard drug Silymarin [Saller et al., 2001] was used as reference compound.

Animals:
For the evaluation of hepatoprotective activity, wistar albino male rats weighing between 120–170g were used. The animals were accommodated in a group of four in clean polypropylene cages and maintained at 22 ± 2˚C under 12 h light/dark cycle and were fed *ad libitum* with standard pellet diet (Golden feed, New Delhi, India) and had free access to water. The animals were acclimatized to laboratory condition for seven days before commencement of experiments. The study protocol was approved by the Institutional Animal Ethical Committee as per the requirements of Committee for the Purpose of Control and Supervision on Animals (CPCSEA), New Delhi.

Acute toxicity studies:
The testing of acute oral toxicity was performed by using OECD, Guidelines 423 (2001). The acute toxic class method set out in this Guideline is a stepwise procedure with the utilization of 3 animals of a single sex per step. Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be required to permit judgment on the acute toxicity of the test substance. This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods (Test Guidelines 420 and 425).

Paracetamol Induced Hepatotoxicity:
For the paracetamol induced hepatotoxicity studies, paracetamol (500mg/kg po) suspension was prepared using 1% gum acacia and was administered to all animals except the animals of the normal control group. Silymarin (100 mg/kg po) was used as standard. Animals were divided into five groups of six animals each as stated under:
Group I :*Vehicle Control*: received 1% gum acacia suspension only for 5 days
Group II :*Toxic Control*: received paracetamol (500mg/kg po) suspension once only
Group III :*Standard*: received standard drug silymarin (100 mg/kg po) for five consecutive days
Group IV : Received test drug (Carvacrol, 25 mg/kg) for five consecutive days
Group V : Received test drug (Carvacrol, 50 mg/kg) for five consecutive days

Group I served as vehicle control and received 1% gum acacia suspension only. All other Groups received paracetamol once (500 mg/ kg, p.o., aqueous solution) with Group II serving as paracetamol treated control. 48 h after paracetamol administration, Groups III, IV & V received silymarin 100 mg/kg, Carvacrol 25 mg/kg & 50 mg/kg b.w., p.o. respectively, once daily for 5 consecutive days.

Blood samples of the experimental animals were collected by retro orbital artery bleeding after 16 hr of the last dose. Blood samples were centrifuged for 10 minutes at 2000 rpm to separate the serum. The rats were sacrificed by ether anesthesia on day 6 and liver was excised, rinsed in 0.25 M sucrose solution and 10% w/v homogenate was prepared in 0.15M KCl, centrifuged at 1000 rpm for 10 min followed by centrifugation of the supernatant at 12000 rpm for 15 min. The supernatant obtained was used for estimation of various oxidative enzymes.

Determination of Biochemical Parameters:
The biochemical parameters like SGOT, SGPT, ALP and bilirubin were estimated using respective assay kits according to the methods described.

Estimation of Serum Glutamic Pyruvic Transaminase (SGPT):
SGPT is a transaminase enzyme and is also called as Alanine transaminase (ALT) or Alanine Aminotransferase (ALAT). The enzyme is found in high concentration in the liver but is also present in a number of tissues, including the heart and skeletal muscles [Pratt et al., 2010].

Procedure:
The method proposed by International Federation of Clinical Chemistry (IFCC) utilizing the LDH-NADH coupled assay, was used for ALT estimation. The reaction mixture (a mixture of 13mM α-Ketoglutaric Acid, 400 mM D-Alanine, 0.2 mM NADH, 1200 U/L LDH and Tris Buffer, pH 7.5) was reconstituted by adding distilled water to it. One ml of compound under investigation was taken out into suitable tube and warmed at 37˚C for five minutes. About 0.10ml of sample was transferred to reagent to make total volume to 1.10 ml, mixed and incubated at 37˚C for one minute. After one minute absorbance was recorded and readings were repeated for next two minutes after regular interval of one minute. Average difference in absorbance per minute (Δabs./min.) was calculated. Results were expressed as IU/L (amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

Estimation of Serum Glutamic Oxaloacetic *Transaminase* (SGOT):
Serum Glutamic Oxaloacetic Transaminase (SGOT), also called Aspartate transaminase (AST) or aspartate aminotransferase (AspAT/ASAT/AAT) is a pyridoxal phosphate (PLP)-dependent transaminase enzyme [Reitman et al., 1957].

Procedure:
The method proposed by International Federation of Clinical Chemistry (IFCC) was used for AST estimation. The

reaction mixture (a mixture of 12mM α-Ketoglutarate, L-Aspartic Acid >150mM, NADH >0.2mM, LDH >800 U/L, MDH >600 U/L, Tris Buffer, pH 7.8) was reconstituted by adding distilled water to it. 1.0ml of compound under investigation was taken out into appropriate tube and warmed at 37˚C for five minutes. 0.10ml of sample was transferred to reagent to make total volume to 1.10 ml, mixed and incubated at 37˚C for one minute. After one minute absorbance was recorded and readings were repeated for next two minutes after regular interval of one minute. Average difference in absorbance per minute (Δabs./min.) was calculated.

Estimation of Alkaline Phosphatase (ALP):
The method described by King (1965) was used to estimate the serum alkaline phosphatase. *p*-Nitrophenyl phosphate (pNPP) is employed in the modified technique which produces a yellow-colored product upon hydrolysis by ALP. The maximum absorbance of the assay is achieved at 405 nm. The enzyme activity is said to be higher if reaction proceeds faster and vice versa.For each 96-well assay, 2 mL pNPP liquid substrate (10 mM), 5 mL Mg Acetate (5 mM) and 200 mL assay buffer. 200 mL distilled water (H_2O) and 200 mL Tartrazine standard were mixed to form working solution and transferred into wells of a clear bottom 96-well plate. 5 mL samples were transferred into other wells and 195 mL working solution was also transferred to sample wells. This made a total volume of 200 mL into the sample wells. The plate was tapped to mix the solutions. Optical density was read at 405 nm immediately after mixing and again after 4 mins on a plate reader. Kinetics of changes in OD was measured.

Estimation of TotalBilirubin:
Bilirubin is a reddish-yellow pigment found in bile. The production of the pigment usually takes place during the fate of heme. As the pigment liberated from the origin, it is in unconjugated or indirect state [Annino, 1960].

Procedure:
The procedure illustrated by Malloy et al., (1937) was exercised for total bilirubin estimation. Total bilirubin, both conjugated and free, were estimated by the technique. The process describes the use of stabilized diazonium salt of 3,5-dichloroaniline and bilirubin resulting in the formation of azobilirubin which shows its maximum absorbance at 540 nm. The concentration of bilirubin present is directly comparative to the absorbance of the azobilirubin measured spectrophotometrically at 540nm.1.0 mL of total Bilirubin Reagent (3, 5-dichlorophenyldiazoniumtetrafluoroborate, 0.36 mmol/L) was dispensed in test tube and 0.05 mL sample was added to it. The solutions were properly mixed before their incubation for 5 min. at room temperature. The absorbance was measured at 540nm.

BiostatisticalInterpretation:
Results were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test. For antioxidant activity results were expressed as mean±SEM of three observations and the outcomes of hepatoprotective studies were expressed as the mean±SEM of six animals in each group.

RESULTS AND DISCUSSION:
HEPATOPROTECTIVEACTIVITY:
Acute toxicitystudies:
Different doses of carvacrol solution (250 mg/kg) were given orally. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily then after, for a total of 14 days. Since no mortality was observed upto 250 mg/kg of carvacrol, $1/10^{th}$i.e.25mg/kg & $1/5^{th}$ i.e. 50 mg/kg of the dose was selected as treatment dose for further studies of pharmacological activities.

Determination of Biochemical Parameters:
The biochemical parameters like SGOT, SGPT, ALP and bilirubin were estimated using respective assay kits according to the methods described.

Estimation on Serum Glutamate Pyruvate Transaminase (SGPT) :
Effect of carvacroland paracetamol on SGPT level of rats is shown in table. A single dose of paracetamol (500 mg/kg) caused significant elevation in the serum SGPT level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed.

Table No.1:Effect of carvacrol on SGPT level

Group	Treatment	Dose	No. of animals	SGPT (IU/L)
I	**Normal control**	1 ml	6	34.22±2.28
II	**Paracetamol control**	500mg/kg	6	99.44±3.70
III	**Silymarin**	100mg/kg	6	48.44±1.88

IV	Carvacrol	25mg/kg	6	65.71±2.68
V	Carvacrol	50mg/kg	6	53.56±2.55

[a]p<0.001; [b]p<0.01; [c]p<0.05; [ns]non significant

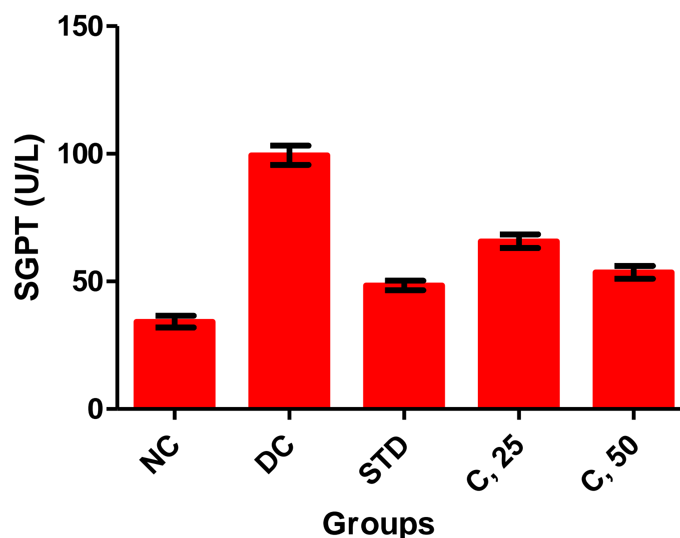

Figure No.1: Effect of Carvacrol on SGPT level

Estimation on Serum Glutamate Oxaloacetate Transaminase (SGOT):
Paracetamol (500 mg/kg) caused significant elevation in the serum SGOT level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed. A graph of comparative SGOT level of different groups is shown in figure.

Table No.2 Effect of carvacrol on SGOT level

Group	Treatment	Dose	No. of animals	SGOT (IU/L)
I	Normal control	1 ml	6	44.31±2.17
II	Paracetamol control	500mg/kg	6	107.73±2.38
III	Silymarin	100mg/kg	6	53.11±1.46
IV	Carvacrol	25mg/kg	6	76.355±1.14
V	Carvacrol	50mg/kg	6	57.33±2.52

[a]p<0.001; [b]p<0.01; [c]p<0.05; [ns]nonsignificant

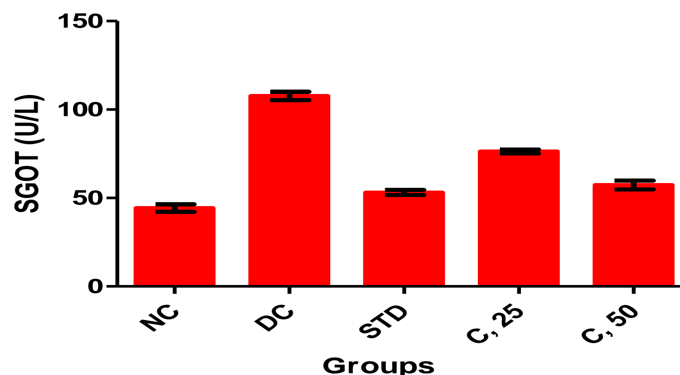

Figure No. 2: Effect of Carvacrol on SGOT level

Estimation of Alkaline phosphatase (ALP) :

Effect of carvacroland paracetamol on ALP level of rats is shown in table. A single dose of paracetamol (500 mg/kg) caused significant elevation in the serum ALP level when compared to normal group, while on treatment with carvacrol, a significant dose dependent reduction was observed. A graph of comparative ALP level of different groups is shown in figure.

Table No 3. Effect of carvacrol on ALP level

Group	Treatment	Dose	No. of animals	ALP (IU/L)
I	**Normal control**	1 ml	6	41.72±1.95
II	**Paracetamol control**	500mg/kg	6	114.83±2.73
III	**Silymarin**	100mg/kg	6	57.77±4.81
IV	**Carvacrol**	25mg/kg	6	78.56±3.46
V	**Carvacrol**	50mg/kg	6	62.44±3.62

[a]p<0.001; [b]p<0.01; [c]p<0.05; [ns]non significant

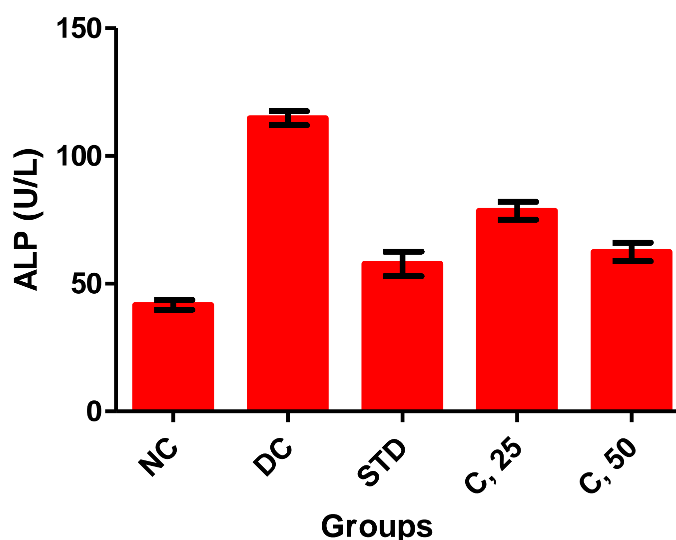

Figure No. 3: Effect of Carvacrol on ALP Level

Serum ALP is related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure. The elevated level of these entire marker enzymes observed in paracetamol treated rats corresponded to the extensive liver damage induced by toxin. The reduced concentration of ALP as a result of carvacrol administration observed during the present study might probably be due in part to the presence of polyphenolics compounds.

Estimation on serum bilirubin of carvacrol .

Comparative effect carvacrol and paracetamol on serum bilirubin level is presented in graph. There was a considerable increase in serum bilirubin level after paracetamol administration which was then reversed by carvacrol.

Table No 4. Effect of carvacrol on bilirubin level

Group	Treatment	Dose	No. of animals	Total bilirubin (mg/dl)
I	**Normal control**	1 ml	6	1.23±0.01
II	**Paracetamol control**	500mg/kg	6	5.91±0.49
III	**Silymarin**	100mg/kg	6	2.37±0.18

IV	**Carvacrol**	25mg/kg	6	4.17±0.39
V	**Carvacrol**	50mg/kg	6	2.57±0.03

[a]p<0.001; [b]p<0.01; [c]p<0.05; [ns]non significant

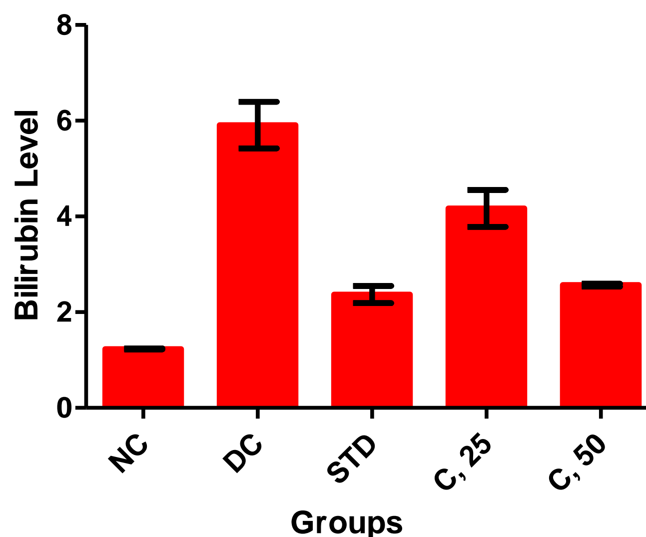

Figure No. 4: Effect of Carvacrol on Bilirubin Level

Bilirubin is one of the most useful clinical clues to the severity of necrosis and its accumulation is a measure of binding, conjugation and excretory capacity of hypatocyte. Decrease in serum bilirubin after treatment with the carvacrol in liver damage induced by paracetamol, indicated the effectiveness of the extracts in normal functional status of the liver.

CONCLUSION:

The selected carvacrol is widely employed in various liver disorders in traditional systems of medicine. The present pharmacological evaluation will provide scientific data about the rationale behind their use. Moreover, the safety of the phytoconstituents will be identified to use them for regular therapy. The scientific evidence obtained in this way might be sufficient for the preparation of the herbal medicine can be developed as drug in the sense of modern medicine.

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