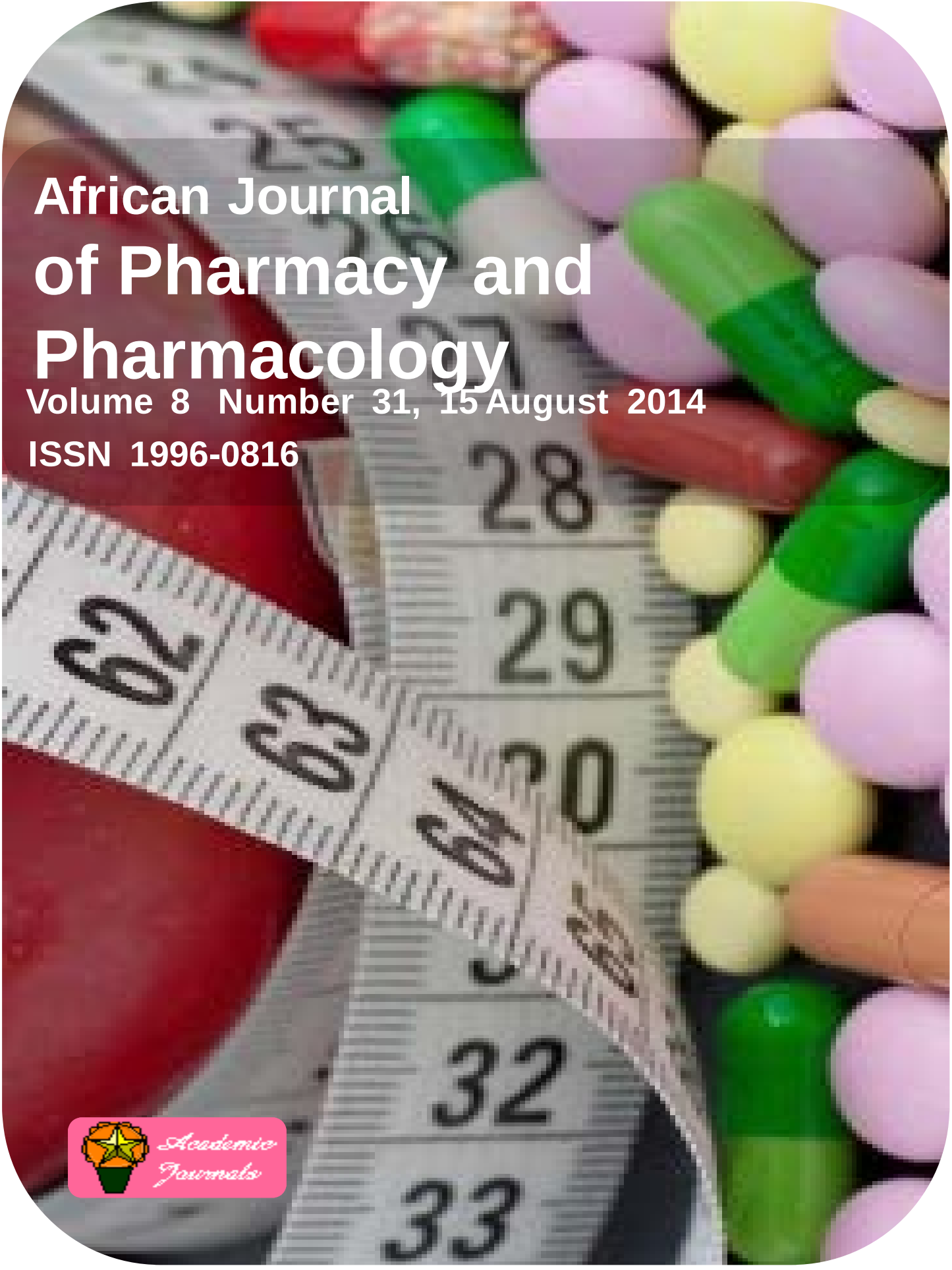

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Full length research paper

Protective action of *Taraxacum officinale* on CCl_4 induced hepatotoxicity in rats

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Plants are important source of medicines, especially in developing countries, where people use plant based traditional medicines for their health care. Leaves of *Taraxacum officinale* L. are being used against various human disorders in folk medicine since past. Current study was conducted to explore the hepatoprotective activity of methanolic leaves extract of *T. officinale* L. against carbontetrachloride (CCl_4) induced toxicity in rats. Various concentrations of plant extracts like 150 and 300 mg/kg of body weight as well as silymarin (100 mg/kg), a standard drug, was given to experimental animals. The results of this study indicates that the methanolic leave extracts has significantly reduced ($P < 0.05$) the level of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which was increased due to induction of CCl_4. It was also observed that due to CCl_4 induction, the level of bilirubin, lipid profile and antioxidants enzymes was also increased, that was significantly lowered after the treatment with the leave extracts. These results revealed that the leave extracts of *T. officinale* L. has protective effects against CCl_4 induced liver toxicity and damage. Furthermore, histopahtological results obtained during this study also supported this claim and was probably due to presence of valuable phytochemicals in the leave extracts.

Key words: Medicinal plants, *Taraxacum officinale*, leaves extract, animals, liver enzymes.

INTRODUCTION

Liver is a vital organ which plays a major role in different metabolisms and excretion of xenobiotics from the body. Liver dysfunction is a major health problem which is a challenge not only for health care professionals but also for the pharmaceutical industry and drug regulatory agencies. Liver cell injuries are caused by various toxic chemicals such as antibiotics, chemotherapeutic agents, carbon tetrachloride etc (Allies, 1990; Sing and Rao, 2008). Liver cells are also damaged due to excessive alcohol consumption and microbial action (Ahmed et al., 1987). The available synthetic drugs to treat liver disorders are also causing further damage to the liver (Singh and Rao, 2008). Furthermore, in the recent past, it was observed that the dosage of drugs might be adversely affecting the liver tissues. Many plasma proteins are synthesized in the liver and their plasma

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levels therefore depend on the balance between synthesis and catabolism and/or loss from the body (Edet et al., 2011; Ismail et al., 2010).

The liver enzymes and proteins are important biomarkers in the body utilized in the diagnosis and assessment of normal function of liver. Major or minor changes in the integrity of cellular membranes in tissues or organs have culminated changes in enzyme activities. For instance alanine amino transferase (ALT) and aspartate aminotransferase (AST) are useful in detecting alterations in liver disease while ALT and some other parameters are implicated in extra hepatic or intra-hepatic obstruction (Chatterjea and Shinde, 2002; Sing and Rao, 2008; Edet et al., 2011). In recent years, due to inadequacy of liver protective agents, researchers and traditional medicine practitioners concentrated on herbal based remedies for various liver disorders (Chaterrjee, 2000). Many folk remedies from plant sources are available for the protection of hepatic damages starting from ancient period. Medicinal plants possess valuable bioactive compounds that protects human from various complications. World Health Organization estimated that about 80% population in Africa and majority of population in Asia and Latin America still use traditional medicines for their primary health care. Phytotheraphy play vital role in detoxification of oxidative stress and other degenerative disorder with minimum or no side effects comparatively to other types of drugs (Reilly and Bulkley, 1990).

T. officinalis (Asteraceae) locally known as Dandelion has been commonly used by rural people against many human disorders including liver and kidney diseases (Dirlesi et al., 2012; You et al., 2010; Agarwal, 2001). The present work was conducted to prove scientifically hepatoprotective activity of *T. officinalis* in rats.

MATERIALS AND METHODS

Plant and chemicals

Carbontetrachloride (CCl_4) was purchased from Aldrich Chemical Co. All other chemicals used in this study were of analytical grade and products of May and Baker, England; BDH, England and Merck, Darmstadt, Germany. Reagents used for all the assays were commercial kits and products of Randox, USA. Fresh leaves of *T. officinalis* L. were collected in April, 2010 from different locations of Rawalpindi areas. The samples were identified and authenticated by a taxonomist and registered as a specimen (voucher specimen numbers 217).

Preparation of plant samples

The leaves were air-dried for 2 weeks and then ground into fine powder using an electric dry mill. A total of 200 g of the ground powder was soaked in 1 L of distilled water for 48 h at room temperature. The mixture was filtered into 500 ml conical flask with Whatman filter paper (No.1). The filtrate was dried at a temperature of 30°C for 10 h to produce a extract, which weighed 20.0 g. Appropriate concentration of the extract was then subsequently made by dilution with methanol into 150 and 300 mg/kg /body weight and administered to the animals.

Phytochemical analysis

Preliminary phytochemical tests were carried out on the methanol extract of the leaves using standard methods (Harbone, 1973; Trease and Evans, 1983).

Animals

Twenty five (25) male albino rats of Wistar strain were used in this study. The rats having weight of 100 to 150 g were maintained under standard animal house conditions and were fed with commercial rat chow (Feed Mills, Islamabad) and allowed water *ad libitum*. Animals were divided into 5 groups having 5 animals in each group and maintained in standard lab conditions with 12-hours cycle of light and dark. Room temperature was kept at 22 ± 2°C and humidity was maintained at 50 ± 5%. The protocol was approved by animal ethics committee of the University.

Acute toxicity tests

The acute toxicity tests were carried out by following a method reported by Lorke (1983). The extract was found to be relatively safe. Doses of 150 and 300 mg/kg of extracts were then chosen and administered to the rats.

Hepatoprotective activity

A total of 25 animals were divided into 5 groups of 5 animals in each group (n = 5). Group I (control) received olive oil orally for 14 days. Group II (hepatotoxin control) received a single dose of 5 ml/kg of CCl_4 diluted in olive oil, 1:1 ratio for 14 days alternatively. Group III (Test group 1) were administered with single dose of 5 ml/kg of CCl_4 along with vehicle alternatively for 14 days and it was followed by the treatment with 150 mg/kg of *T. officinalis* leaves extract orally for 21 days. Group IV (Test group 2) animals were administrated with single dose of 5 ml/kg of CCl_4 for 14 days, followed by treatment with 300 mg/kg of *T. officinalis* leaves extract for 21 days. Group IV (hepatoprotective agent control) animals were administered with CCl_4 for 14 days and followed by the treatment with 100 mg/kg of known hepatoprotective agent (silymarin). On 22nd day, the animals were anaesthetized using chloroform and blood was collected by cardiac puncture.

Preparation of serum

Blood was obtained from the rats by heart puncture technique into centrifuge tubes. Serum was prepared by centrifugation for 10 min at 3000 rev/h in a bench centrifuge. The clear supernatant was used for the biochemical tests.

Biochemical analysis

Liver enzymes such as AST, ALT, ALP and lipid profile - total cholesterol, very low density lipoprotein (VLDL), high density lipoprotein (HDL), triglycerides were estimated by using commercially available kits based on the methods reported by Reitman and Frankel (1957), King and Kind (1954). Whereas, bilirubin was determined by using method reported by Jendrassic and Grof (1938). The activities of anti oxidants enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase

Table 1. Phytochemical analysis of leave extracts of *T. officinalis*.

Constituent	Relative abundance
Polyphenols	++
Flavonoids	++
Alkaloids	++
Glycosides	+
Reducing sugar	++
Saponins	+
Tannins	+

++ = Highly present, + = Present

(GPx) were assayed in the hepatic tissue of control and experimental group of animals by using methods reported by Kakkar et al. (1984), Sinha (1972) and Rotruck et al. (1973), respectively. Whereas, the level of lipid peroxide-malondialdehyde (MDA) was also determined by using serum, as well as liver tissue of control and experimental groups of animals (Berton et al., 1998).

Histopathological studies

The liver tissues were subjected to normal routine histological procedures, stained with hematoxylin-eosin and examined using the light microscope for any morphological changes (Kleiner et al., 2005).

Statistical analysis

Data obtained was analyzed by one way analysis of variance (ANOVA), followed by Bonferoni test for comparison by using SPSS software version 16.0, and the $P < 0.05$ was considered as statistically significant.

RESULTS

The preliminary screening of phytochemicals from leaf samples of *T. officinalis* revealed the presence of polyphenols, alkaloids, flavonoids, glycosides, reducing sugar, saponins and tannins. Results indicate that proportion of polyphenols, flavonoids and alkaloids found in the extract was relatively higher than the other phytochemicals (Table 1). Methanolic leave extracts of *T. officinalis* (150 and 300 mg/kg bw) when given orally for 21 days showed hepatoprotective activity in CCl_4 induced hepatic damage in rats. Results show increases in the liver enzymes like ALT, AST, ALP and bilirubin in CCl_4 intoxicated animals when compared with that of the control group of rats (Table 2). Treatment of CCl_4 induced animals with different concentrations of plant extracts (150 and 300 mg/kg) significantly reduced ($P < 0.05$) CCl_4 induced elevations in enzymes on dose dependent manner as compared to control. The recovery of hepatic injury was observed in animals treated with plant extracts as well as with silymarin. Hepatic injury caused by CCl_4 administration at a dose of 5 ml/kg body weight showed significant increase in the lipid profile (total cholesterol, triglycerides, LDL and VLDL) levels in liver tissues. Whereas HDL level was decreased as compared to that of control group of rats ($p < 0.05$). However, the treatment of CCl_4 induced group of rats with extracts of *T. officinalis* at a dose of 150 and 300 mg/kg and also, a known hepatoprotective agent silymarin (100 mg/kg), showed significant reduction in level of liver cholesterol, triglyceride, VLDL and LDL, whereas HDL level was increased as compared to CCl_4 treated group (Table 3).

The effects of methanolic leaves extracts of *T. officinalis* on the antioxidants enzymes like catalase, GPx and SOD in the serum of control and CCl_4 treated group showed significant reduction ($p < 0.05$). The methanolic leaves extracts of *T. officinalis* and silymarin increased the revised activities of these antioxidants in the liver of CCl_4 induced group on dose dependent manner as compared to control and the change was significant ($p < 0.05$). These results suggested that the free radicals released in the liver were effectively scavenged in the animals treated with *T. officinalis*. Malondialdehyde (MDA) content in liver of CCl_4 treated group was significantly higher than that of the control group. However, MDA levels were significantly lowered in CCl4 treated group followed by treatment with methanolic leaves extracts of *T. officinalis* and silymarin ($p < 0.05$) (Table 4).

The results of histopathological study of the liver tissues of the control and CCl_4 treated rats are given in Figures 1 to 4, respectively. The liver section of the animal in control group showed normal hepatic cells, well defined cytoplasm prominent nucleus, nucleolus and a central vein with prominent small-sized (Figure 1). While liver section of CCl_4 induced animal showed total loss of hepatic architecture with centrilobur hepatic necrosis, fatty changes vacuolization and congestion of sinusoids (Figure 2). However, treatment of animals with 300 mg/kg of methanolic leaves extracts of *T. officinalis* (Figure 3) and 100 mg/g of silymarin (Figure 4) represent normal condition of liver tissues and it is assumed that treatment returned that injury towards normal side. Therefore, results of histopathological study also provided support for biochemical analysis carried out during this work.

DISSCUSION

Carbon tetrachloride (CCl_4) is assumed to initiate the biochemical processes leading to oxidative stress, which is the direct cause of many pathological changes in liver, kidney, testes, lungs, nervous system and blood tissues by producing free radicals (Abraham et al., 1999; ATSDR, 2003). These free radicals frequently damage different cell organelles through lipid peroxidation (Ahmed et al., 1987). For example, the acute exposure of CCl_4 to the liver tissues can cause damage of liver and lead to elevated levels of its enzymes (Sing and Rao, 2008; Okonkwo et al., 2004). These liver enzymes can then be

Table 2. Effect of methanolic extracts of *T. officinalis* leaves on liver enzyme and bilirubin.

Group	AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Control	72.4±0.24	38.1±0.21	114.21±0.42	0.15±0.07	1.73±0.07
CCl_4	217.43±0.23[a]	115.17±0.93[a]	224.93±0.54[a]	0.39±0.01[a]	2.17±0.05[a]
CCl_4+150 mg/kg extract	155±0.27	88±0.61	173.38±0.19	0.31±0.03	1.94±0.04
CCl_4+300 mg/kg extract	72.6±0.24[b]	35.15±0.27[b]	123.73±1.71[b]	0.24±0.06[b]	1.79±0.04[b]
CCl_4+ Silymarin (100 mg/kg)	149.81±1.53[b]	88±0.47[b]	138.83±0.61[b]	0.25±0.07[b]	1.83±0.05[b]

Results were expressed as Mean± SEM (n= 5); a P<0.05 compared with control group of rats; b P<0.05 compared with CCl_4 induced group of rats.

Table 3. Effect of methanolic extracts of *T. officinalis* leaves on liver lipid profiles.

Groups	Cholesterol (mg/dl)/dl)	Triglyceride (mg)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	88.76±0.32	77.35±0.23	29.38±0.35	54.7±0.19	19.41±0.33
CCl4	132.54±0.19[a]	135.48±1.18[a]	23.83±0.34[a]	98.37±0.23[a]	27.46±0.16[a]
CCl_4+150 mg/kg extract	112.29±0.14	97.11±0.24	20.78±0.27	58.57±0.14	16.9±0.43
CCl_4+300 mg/kg extract	84.59±0.26[b]	75.9±0.25[b]	21.11±0.17[b]	51.38±0.31[b]	13.39±0.18[b]
CCl_4+ Silymarin	99.41±0.18[b]	81.57±0.23[b]	22.51±0.17[b]	62.31±0.24[b]	14.13±0.16[b]

Results were expressed as Mean± S.E.M (n= 5). [a]P<0.05 compared with control group of rats. [b]P<0.05 compared with CCl4 induced group of rats.

Table 4. Effect of Methanolic extracts of *T. Officinalis* leaves on antioxidants enzyme.

Groups	Catalase U/mg of protein	GPX U/ mg of protein	SOD U/mg of protein	MDA nm/mg of protein
Control	16.32±0.06	2.65±0.05	46.45±0.04	2.87±0.75
CCl_4	9.74±0.04[a]	1.64±0.04[a]	29.5±0.07[a]	8.83±0.13[a]
CCl_4+150mg/kg extract	14.15±0.04	1.98±0.03	42.19±0.03	3.88±0.73
CCl_4+300mg/kg extract	15.67±0.09[b]	2.78±0.06[b]	45.34±0.08[b]	2.81±0.48[b]
CCl_4+ Silymarin	12.43±0.08[b]	1.65±0.08[b]	47.83±0.08[b]	4.51±0.81[b]

Catalase (U/mg of protein), glutathione peroxidase (U/mg of protein) superoxide dismutase (U/mg of protein). MDA-nm/mg of protein. Results were expressed as Mean ± SEM (n=5). [a]P<0.05 compared with control group of rats and [b]P<0.05 compared with CCl4 induced group of rats.

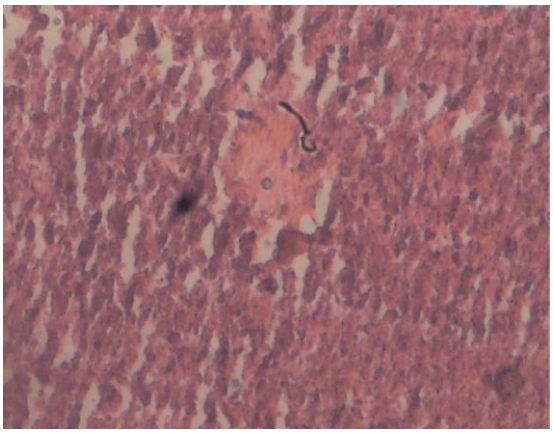

Figure 1. Liver tissues of normal rat.

released into blood stream and cause cellular necrosis, which is used as a diagnostic measure of liver damage (Alexander and Griffiths, 1993).

This study clearly indicates that a significant reduction in CCl_4 elevated liver enzymes was occurred after treatment with *T. officinalis* leaves extract in a dose dependent manner (Friday et al., 2010), which represents a protective effect of the extract on the damaged liver tissues (Chioma et al., 2008; Sumitha and Thirunalasundari, 2011). It was further investigated that the elevated level of serum marker enzymes (AST, ALT and ALP) produced by CCl_4 treatment was returned towards the normal level in the sample group of animals treated with plant extract compared with control (Friday et al., 2010). Furthermore, the biochemical parameters like

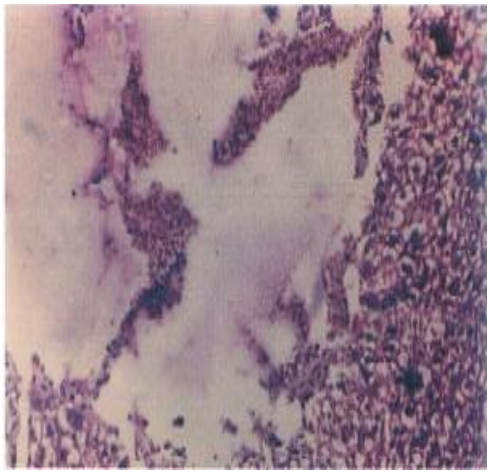

Figure 2. Liver tissue of rat treated with CCl4.

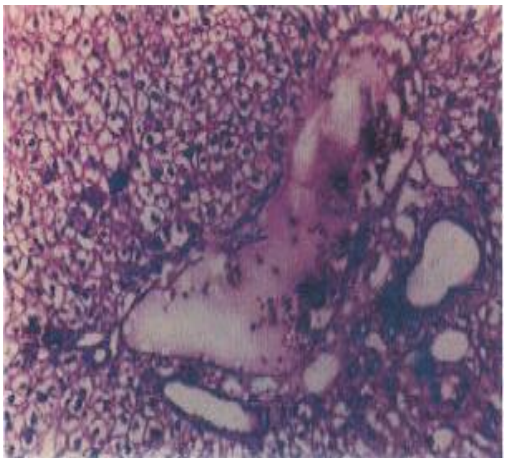

Figure 3. Liver tissue of rat treated with 300 mg/kg of methanolic leaves extract of *T. officinalis.*

bilirubin, total cholesterol LDL, VLDL and triglycerides were also restored towards their normal levels by the treatment of *T. officinalis* leaves extract. The reason was that the bioactive compounds (phytochemicals) in the extract minimized the adverse affects of CCl_4 by chelating with by-products produced from CCl_4 metabolites. This demonstrates the hepatoprotective role of plant extract which not only involved in considerably decrease in the effect of CCl_4 induced damage but also resulted in the recovery of damaged liver at a significant level (Chungma et al., 2007; You et al., 2010; Dirleise et al., 2012; Sing and Rao, 2008).

Moreover, the previous studies have reported that the oxidative damage to tissues and their cellular components can be prevented by certain antioxidant metabolites present in the plants (Khan and Ahmed, 2009). Therefore, the results obtained from this study clearly

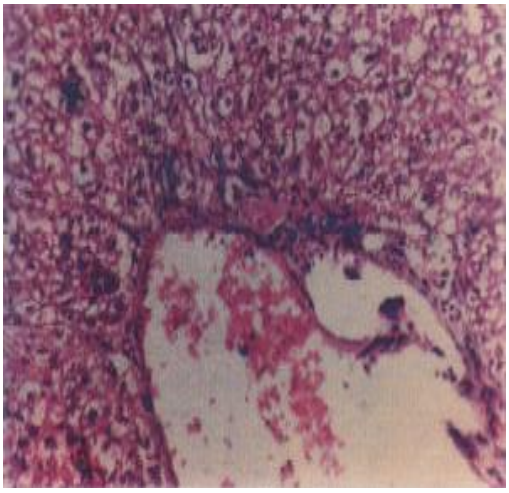

Figure 4. Liver tissue of rat treatments with 100 mg/kg of silymarin.

indicate that the antioxidant effect of *T. officinalis* extract resulted in the protection of liver against CCl_4 induced injury (Allis et al., 1990). It was found that the antioxidant activities of enzymes like SOD, GSH-Px and catalase were considerably decreased in the liver in response to CCl_4 administration compared with control group of animals, which indicates the CCl_4 induced oxidative damage of liver (Guven et al., 2003).The level of antioxidant enzymes was significantly improved by administration of 300 mg/kg of leaves extract to CCl_4 intoxicated rats. This further proves that in addition to hepatoprotective affect, *T. officinalis* has the ability to restore the antioxidant enzyme activities in CCl_4 damaged liver (Kakkar et al., 1984; Sinha, 1972; Rotruck et al., 1973).

From the whole discussion, it is concluded that the current study provides an important platform for the cure of liver damage caused by CCl_4 intoxication in rats. However, more studies are required to prove the availability of lead compounds from *T. officinalis* leaves extract having hepatoprotective nature.

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Full Length Research Paper

Study of visceral antinociceptive potential of bee *Apis mellifera* venom

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Pain is one of the most common reasons for patients to seek medical care. Bee *Apis mellifera* venom (AMV) has traditionally been used to treat inflammatory diseases and the alleviation of pain. Herein, we aimed to investigate the visceral antinociceptive potential of *A. mellifera* bee venom and its possible mechanism of action. Acetic acid-induced writhing assay was used in mice to determine the degree of visceral antinociception. Visceral antinociceptive activity was expressed as the reduction in the number of abdominal constrictions. Mice received an intraperitoneal injection of acetic acid after administration of AMV (0.08 or 0.8 mg/kg; intraperitoneally (i.p.)). In mechanistic studies, separate experiments were realized to examine the role of α2-receptors, nitric oxide, calcium channels, K^+_{ATP} channel activation, TRPV1 and opioid receptors on the visceral antinociceptive effect of AMV (0.8 mg/kg), using appropriate antagonists, yohimbine (2 mg/kg), L-NG-Nitroarginine methyl ester (L-NAME, 10 mg/kg), verapamil (5 mg/kg), glibenclamide (5 mg/kg), ruthenium red (3 mg/kg) or naloxone (2 mg/kg). AMV presented visceral antinociceptive activity in both doses tested (0.08 and 0.8 mg/Kg). Visceral antinociceptive effect of AMV was resistant to all the antagonists used. Mice showed no significant alterations in locomotion frequency, indicating that the observed antinociception is not a consequence of motor abnormality. Although AMV efficient diminished the acetic acid-evoked pain-related behavior, its mechanism is unclear from this study and future studies are needed to verify how the venom exerts its antinociceptive action.

Key words: *Appis mellifera venom*, antinociceptive, visceral pain.

INTRODUCTION

Pain is one of the most common reasons for patients to seek medical care. Current analgesics fall into two major classes: non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, both of which have critical liabilities and limitations. Opioids are tightly controlled because of their addictive effects and other serious side effects (McQuay,

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1999). Gastrointestinal side effects and effectiveness only in cases of mild to moderate pain limit NSAIDs use (Frölich,1997; Kingery, 1997). Pain control is an important medical problem. Much research has gone towards the identification of agents that can relieve chronic pain with out unwanted side effects.

Bee *Apis mellifera* venom (AMV) has traditionally been used to treat inflammatory diseases and the alleviation of pain (Lee et al., 2005; Son et al., 2007). Various components of AMV have been identified, but there is not a consensus about their concentration. The predominant component of the dried AMV is melittin (40 to 50%), a peptide of 26 amino acid residues. Moreover, many components with much lower concentration have been identified including hyaluronidase, acid phosphatase, apamin, mast cell degranulating peptide, adolapin, secapin, minimine, phospholipase A2 (PLA2) histamine, glycosidase, tertiapin, dopamine and carbohydrates (Gauldie et al., 1976; Habermann, 1972; Nelson and O'Connor, 1968; Vetter and Visscher, 1998; Vetter et al., 1999). These AMV components were reported to have a wide variety of pharmacological properties (Lariviere and Melzack, 1996).

An animal study suggested that melittin, the main component of whole AMV, is a likely candidate for the anti-inflammatory and antinociceptive effects observed in AMV treatment (Lee et al., 2004). According to Li et al. (2010), the mechanism of the antinociceptive effect of melittin is unknown, but several studies revealed that this effect may be partially explained by the following findings: the repeated application of capsaicin is followed by a prolonged period of hypoalgesia (Nolano et al., 1999); the initial nociceptive effect of melittin may function similarly to capsaicin by increasing the pain thresholds and desensitizing the nociceptor; the α2-adrenoceptor is involved in the anti-nociceptive effect of whole bacterial vaginosis (BV) (Kwon et al., 2001a,b,c); and the antinociceptive effect is dependent on the site-specific acupoint (Oliver et al., 2006).

It was demonstrated that subcutaneous bee venom injection produces a robust antinociceptive effect in several different rodent models of both somatic and visceral pain (Known et al., 2001a,b,c). These preliminary data imply that bee venom is useful for the management of both somatic and visceral pain, but it is not clear which constituent is responsible for its antinociceptive effect.

The effects induced by AMV and its components in experimental models of nociceptive and inflammatory pains have been reported (Merlo et al., 2011). These studies demonstrated that AMV antinociception involves the action of different components and does not result from non-specific activation of endogenous antinociceptive mechanisms activated by exposure to noxious stimuli. In the present study, we aimed to investigate the visceral antinociceptive potential of *A. mellifera* bee venom and its possible mechanism of action.

MATERIALS AND METHODS

Animals

Swiss mice (20 to 25 g) were used. Experimental groups consisted of 6 animals per group. They were housed at 22 ± 2°C under a 12 h light/12 h dark cycle and had free access to standard pellet diet (Purina chow) and tap water. Each animal was used only once for experimentation. The experimental protocols were in accordance with the ethical guidelines of the Brazilian Council for the Control of Animal Experiments (CONCEA) and were approved by the Animal Research Ethics Committee of the Federal University of Ceará, under entry #90/2011.

Venom

The bee AMV was donated by Professor Marcos Hiraki Toyama from Univerdidade Estadual do Litoral Paulista (UNESP). For the tests, it was prepared a stock solution (1 mg/ml) of the venom in phosphate buffered saline (PBS), pH 7.4, sterile.

Acetic acid-induced visceral nociception

Abdominal constrictions were induced by intraperitoneal injection of acetic acid (0.6%). The animals were pretreated with AMV (0.08 or 0.8 mg/kg, intraperitoneally (i.p.)), indomethacin (10 mg/kg, i.p.) or vehicle (PBS 10 ml/kg, i.p.) 30-min prior to acetic acid injection. After the challenge, each mouse was placed in a separate glass funnel and the number of contractions of the abdominal muscles, together with stretching, was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions, comparing the control animals with the mice pretreated with AMV.

In order to verify the possible involvement of noradrenergic, nitrergic, calcium, K^{+}_{ATP}, $TRPV_1$ and opioid mechanisms in the effects of AMV, the animals were treated with yohimbine (2 mg/kg, i.p), L-NAME (10 mg/kg, i.p), verapamil (5 mg/kg i.p.), rutehnium red (3 mg/kg subcutaneously (s.c.)), glibenclamide (5 mg/kg i.p.) or/and naloxone (2 mg/kg i.p.) 30 min before the administration of the AMV (0.8 mg/kg).

Evaluation of the motor activity

The motor coordination and performance of each mouse was evaluated in a rota-rod apparatus, 30 min after the intraperitoneal treatment with AMV (0.08 or 0.8 mg/kg), vehicle (PBS, 10 ml/kg) or Diazepam (1 mg/kg). This apparatus has a 2.5 cm diameter bar, divided into six parts, and it is placed at a height of 25 cm, rotating at 7 rpm. Latency to fall from the rotating bar during a 1 min period was registered.

Statistical analysis

The results are presented as the mean ± standard error of mean (SEM) of 8 animals per group. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey *post hoc* test for multiple comparisons. *P*-values less than 0.05 ($p < 0.05$) were considered as indicative of statistical significance.

RESULTS

In acetic acid-induced writhing test, AMV suppressed the

mean number of writhes, when compared with vehicle-treated control group (Table 1). These were in the order of 52.17 ± 5.05, 24.67 ± 4.59 and 1.17 ± 0.83, respectively, for the control and AMV at the tested doses of 0.08 and 0.8 mg/kg. The positive control group treated with indomethacin (10 mg/kg, i.p.) also manifested significantly diminished number of stretches (26.00 ± 3.90).

The acetic acid-induced visceral nociception was not significantly blocked by yohimbine, L-NG-Nitroarginine methyl ester (L-NAME), verapamil, rutehnium red, glibenclamide or naloxone. Their combinations with AMV failed to modify AMV antinociception (Table 2).

At the doses tested (0.08 and 0.8 mg/kg), AMV failed to produce any significant effect on motor coordination on rota-rod in mice. In contrast, diazepam (1 mg/kg, i.p.) significantly lowered the motor coordination (data not shown).

DISCUSSION

In this present study, it wad observed that the acetic acid-evoked visceral nociceptive behavior was significantly attenuated in mice pretreated with AMV. The acetic acid-induced writhing is a standard test for visceral pain, sensitive to opiates as well non-opiates analgesics (Steranka et al., 1987).

The α_2-adrenoceptor agonist has been shown to induce antinociceptive effect in the experimental model of formalin-induced colitis in rats and reduce visceral hypersensitivity in clinical settings (Lima-Júnior et al., 2006; Miampamba et al., 1992). Therefore, a possible involvement of α_2-adrenoceptors in the antinociceptive effect of AMV, using the antagonist yohimbine was investigated. Yohimbine could not reverse the antinociception produced by AMV.

Know et al. (2005) reported that a water soluble fraction (BVAF3, <10 kDa) from bee venom selectively activates the descending adrenergic system through α_2-adrenergic receptors and that activation is associated with the antinociceptive effect observed in the abdominal visceral pain model. BVAF3 produced a significant antinociceptive effect similar to that observed following injection of AMV. This discrepancy may be due to differences in experimental conditions and the route of AMV delivery.

This study also examined the possible participation of NO/cGMP/K^+_{ATP} pathway. Pretreatments with a K^+_{ATP} blocker, glibenclamide or the non-specific NOS inhibitor, L-NAME, could not reverse the antinociceptive effect of AMV, suggesting that AMV antinociception do not result from the modulation of K^+_{ATP} currents. Besides this, verapamil, blocker of Ca^{2+} channels, could not reverse the antinociception produced by AMV.

It is currently accepted that an endogenous opioid analgesic system is present at peripheral level (Smith, 2008; Alves et al., 2012), and most of opioid antinociceptive effects are mediated via activation of opioid receptors (Stein and Lang, 2009) and opioid receptors have been identified on peripheral terminals of afferent nerves, which can be the sites of the intrinsic modulation of nociception (Vadivelu et al., 2009). Attempts to mimic or augment such peripheral analgesia may potentially lead to analgesic effects in the absence of the central adverse effects caused by opioids.

The antinociceptive effect of AMV was not modified by a non-selective $TRPV_1$ antagonist ruthenium red. Roh et al. (2010) found that the destruction of capsaicin-sensitive primary afferents by resiniferatoxin (RTX) pre-treatment selectively decreased AMV-induced spinal Fos expression, but did not affect AMV-induced antinociception in the formalin test. They suggested that subcutaneous AMV stimulation of the Zusanli point activates central catecholaminergic neurons via capsaicin-insensitive afferent fibers without induction of nociceptive behaviour or by naloxone, a non-selective μ-opioid receptor antagonist, suggesting that there is no participation of an opioid mechanism. Kwon et al. (2001a,b,c) also showed that AMV acupoint stimulation can produce visceral antinociception that is not associated with naloxone-sensitive opioid receptors

The demonstration of the antinociceptive activity of AMV is in line with the demonstrations that AMV inhibits the nociceptive response induced by acetic acid in mice (Kim et al., 2007). In this study, AMV was injected into specific points of acupuncture. As the doses (0.06 to 6 mg/kg) used by these authors are in the range of those used in the present study, it is suggested that the antinociceptive effect induced by AMV is not related to injection into a specific point of acupuncture, but results from a systemic action.

Motor deficits may create confounds in studies in which antinociception is measured. To clarify if the analgesic effect is not a result of motor deficits, the effects of AMV on rota-rod test was assessed, that is, a classical model for screening central nervous system actions providing information on myorelaxant activity. AMV did not present myorelaxant activity as demonstrated in the rota-rod test that measures grip strength, suggesting that the AMV antinociception observed in this investigation is not exerted through induction of sedation. According to Heneine et al. (2007), AMV lacks analgesic action in test of hot-plate suggesting that its analgesic effect is only peripheral but not central.

It seems that antiinflammatory property of AMV may contribute to its antinociceptive effect. Recent studies have shown that bee venom treatment can induce a significant antiinflammatory response mediated by inhibition of inflammatory mediators, similar to what is achieved with the administration of non-steroidal anti-inflammatory drugs (Miampamba et al., 1992). In experimental rheumatoid arthritis, bee venom treatment significantly decreased the expression of inflammation-related

Table 1. Visceral antinociceptive activity of *Apis mellifera* venom.

Group	Dose (mg/kg)	Number of animal constrictions/20
Control	-	52.16 ± 5.05
Indomethacin	10	26.00 ± 3.90*
AMV	0.08	24.67 ± 4.59*
-	0.8	1.16 ± 0.83***

*p < 0.05 and ***p < 0.001 compared to the vehicle-administered control group (Control).

Table 2. Effect of yohimbine and L-NAME on visceral antinociception induced by of *Apis mellifera* venom.

Group	Dose	Number of animal constrictions/20
Control	-	40.166 ± 3.19
AMV	0.8	1.54 ± 0.63***
Yohimbine	2	44.00 ± 4.58
L-NAME	10	46.66 ± 3.74
Verapamil	5	46.16 ± 3.10
Glibenclamide	5	45.66 ± 3.57
Ruthenium red	3	28.83 ± 8.60
Naloxone	2	19.40 ± 2.13
Yohimbine+AMV	2 + 0.8	8.00 ± 3.15***
L-NAME+AMV	10 + 0.8	4.16 ± 2.22***
Verapamil+AMV	5 + 0.8	17.56 ± 7.17**
Glibenclamide+AMV	5 + 0.8	11.33 ± 9.94*
Ruthenium red+AMV	3 + 0.8	1.83 ± 0.65***
Naloxone+AMV	2 + 0.8	8.00 ± 3.74**

*p < 0.05 compared to the vehicle-administered control group (Control).

cytokines such as cyclooxygenase-2 (COX-2), phospholipase A2 (PLA2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, nitric oxide (NO) and reactive oxygen species (ROS) (Son et al., 2007) via NF-κB (Kim et al., 2013). It is known that after intraperitoneal administration of acetic acid, inflammatory reactions develop in the peritoneum (Clementi et al., 1999).

According to Know et al. (2005), the soluble fraction (BVF3) contains several small peptides including melittin, apamin, mast cell degranulating (MCD) peptide and minimine. The authors believed that BVAF3-induced antinociception may be produced by the interaction of several constituents of BVAF3 rather than by one specific antinociceptive component.

Conclusion

In conclusion, although AMV efficient diminished the acetic acid-evoke pain-related behaviour, its mechanism is unclear from this study and future studies are needed to verify how the venom exerts its antinociceptive action. Since AMV contains a wide number of constituents, attempts to investigate them individually are slightly challenging, because synergistic interaction among the AMV components may occur.

However, in order to gain better insight into the mechanisms of action of AMV, further efforts, including molecular biology methods, are necessary in the future.

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