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Full Length Research Paper

## Effect of the methanolic extract of *Brachylaena discolor* in a streptozotocin-induced diabetic rat model

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Traditionally, leaf infusions of *Brachylaena discolor* are used for the treatment of diabetes and renal conditions. Therefore, the aim of this study was to evaluate the effect of a methanolic leaf extract of *B. discolor* in a streptozotocin (STZ)-induced diabetic rat model. Diabetes was induced in Wistar rats by an intraperitoneal injection of STZ; blood glucose levels greater than 20 mmol/L measured after 7 days confirmed a stable diabetic mellitus state. Two doses of the test extract (50 and 150 mg/ml) were administered daily via oral dosing to both STZ-induced and control rats. Blood was obtained from the tail vein and used to measure the effects of the extract on the biochemical profile of the rats over 28 days. The methanolic leaf extract of *B. discolor* at both doses (50 and 150 mg/ml) caused a significant reduction in the blood glucose levels at 7, 14, 21 and 28 days in STZ-induced diabetic rats when compared with the normal rats (negative control). Significant differences were also observed in alkaline phosphatase, creatinine, total bilirubin and body weights of extract treated diabetic rats when compared with untreated diabetic rats, normal rats and metformin treated rats. Findings suggest that *B. discolor* exhibits significant anti-hyperglycaemic activity in STZ-induced diabetic rats.

**Key words:** Diabetes, streptozotocin, *Brachylaena discolor*, Wistar rats.

### INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease which occurs as a result of insulin deficiency and/or insulin resistance and is a major cause of disability and hospitalization (Kamgang et al., 2008). Diabetes mellitus has become a major health concern in the world, with an expectation that diabetes and obesity will reach epidemic proportions, affecting third world developing countries to a far greater extent than the developed world (Rheeder, 2006). This may be attributed to the high number of African countries, which are undergoing a demographic transition and coming under increased western influence.

This has resulted in an increase in the consumption of fat, salt and sugar, coupled with a rapid increase in urbanization in African countries and increase in nutritional deficiency, thereby providing an elevated risk factor for the prevalence of diabetes (Colagiuri et al., 2006). Estimates from the World Health Organization (WHO) have projected an increase from 171 million in the year 2000 to 366 million in 2030, with approximately 70% occurring in developing countries (WHO, 2002; Wild et al., 2004). Currently, type 2 diabetes is the most prevalent form of diabetes mellitus; however, oral

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hypoglycemic drugs which are the main form of treatment, have been shown to have undesirable side-effects and high secondary failure rates (Moller, 2001). In addition to the lack of efficacy by pharmacological agents for the treatment of type 2 diabetes, there is also limited access to these drugs for people living in many rural areas as well as cost implications (Holt, 2004; Tonye Mahop and Mayet, 2007). These limitations have prompted research into investigating traditional medicines as potential replacements for conventional pharmacological agents used in the treatment of diabetes (Grover et al., 2002; Odhav et al., 2010). There are already an estimated 122 drugs from 94 plant species, which have been discovered through ethnobotanical leads such as indigenous knowledge. Plants such as *Syzygium cordatum*, *Allium sativum* and *Ficus thonningi* prevail as the treatment for diabetes in poor socio-economic conditions (Musabayane et al., 2005). These plants have been found to control diabetes mellitus by exhibiting hypoglycaemic and antioxidant effects. These ethnobotanical studies are promoted by the WHO for the discovery of novel mechanisms for diabetes treatment (WHO, 1999). Currently, in South Africa, there are no regulations with regard to the prescription and use of traditional medicine, exposing people to misadministration, especially of toxicity of plant compounds. The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also cause for alarm (Fennell et al., 2004). Therefore, it becomes necessary to identify the amylase inhibitors from natural sources having lesser side-effects. The traditional African herbal medicinal system practiced for thousands of years have reports of anti-diabetic plants with no known side effects. Such plants and their products have been widely pre-scribed for diabetic treatment all around the world with less known mechanistic basis of their functioning. Therefore, these natural products need to be evaluated scientifically in order to confirm claims for their anti-diabetic properties (Iwueke and Nwodo, 2008). The aim of this study was to investigate *Brachylaena discolor* var. *discolor* as a possible dietary adjunct or therapeutic for diabetes therapy. *B. discolor* is a species of tree belonging to Asteraceae family. There are three known varieties: var. *discolor* which is commonly known as the Coast Silver Oak or Coastal Silver Oak, var. *transvaalensis* commonly known as the Forest Silver Oak or Natal Silver Oak and var. *rotundata*. *B. discolor* var. *discolor* is an evergreen shrub or small tree usually 4 to 10 m in height which is often multi-stemmed. It is confined mainly to the coastal bush and associated bushveld (van Wyk and van Wyk, 2007). These trees are distributed from the Eastern Cape in South Africa and into Southern Mozambique, but are most common in the coastal vegetation of KwaZulu-Natal. The leaf infusions of *B. discolor* var. *discolor* are used for diabetes and renal conditions by different ethnic groups in Southern Africa and are reported to act as a

tonic (Hutchings, 1996). The roots are used to treat roundworms and chest pains.

## MATERIALS AND METHODS

### Plant

Leaves of *B. discolor* var. *discolor* was collected in Durban, province of Kwazulu Natal, South Africa, and identified by using available floral keys. The leaves were carefully examined for insect-damage or fungus-infection and these were discarded. Healthy leaves were dried at 40°C for 48 h in a convection oven. Once completely dry, plant was ground to a fine powder using a Waring blender. The dried plant was then stored in Amber Schott bottles at room temperature until required. The methanolic extract of the dried plant material was prepared according to the procedure outlined by Jeremy and Whiteman (2003) with minor modifications. 20 g of the dried plant material was stirred for 24 h in 200 ml of 80% methanol. The slurry was filtered using Whatman No. 1 filter paper and the supernatant was collected. The supernatant was concentrated using a Buchi RE Rotoevaporator including a Buchi 461 water bath set at a temperature of 45°C. The concentrate was placed in a biofreezer and freeze-dried using a Virtis Benchtop Freeze Dryer. The freeze-dried material was used as a stock and working solutions were prepared for appropriate applications.

### Animals

Male Wistar rats of 250 to 300 g body weight (BW) were obtained from the Biomedical Resource Centre (BRC) at the University of KwaZulu-Natal and divided into non-diabetic and diabetic groups of n=12 to be used for acute and chronic studies. These animals were maintained under laboratory conditions of temperature (22 to 24°C), humidity (40 to 60%) and 12 h light/12 h dark regime at the Biomedical Resource Unit (University of Kwazulu Natal, Westville Campus) where animals were exposed to both food and water *ad libitum* for the entire duration of the study. All animals used for this study were maintained according to the rules and regulations outlined by the Ethics Committee of the University of KwaZulu-Natal, South Africa (Ethics approval was obtained from the University of KwaZulu Natal, Ethics reference 089/11/Animal).

### Experimental design

The two groups of animals were sub-divided into a control group, metformin group (500 mg/kg BW) and treated group, each with 12 rats per group. These animals were administered two different concentrations of the crude plant extract (200 and 600 mg/kg BW) reconstituted in 1% Tween 80 and standard treatments through feeding 1 ml of the plant extract (gavage) and standard treatment to alleviate any strain on the animal during dosage. The crude extract and standard treatment were administered to the animals for 28 days after identification of streptozotocin (STZ)-induced rats. All blood samples were obtained from the tail vein every 7 days. The study also involved a Humane Endpoint for the experiment and was conducted by veterinary support staff on a daily basis. This was used to determine the severity of the animals' well-being for the duration of the study through visual and intrusive tests (blood glucose levels, weight loss, dehydration etc).

### Induction of diabetes

Diabetes was induced in male Wistar rats of 250 to 300 g BW by

intraperitoneal injection of STZ (60 mg/kg BW) in citrate buffer, at a pH of 4.5 (Musabayane et al., 2005). The maximum quantity for the STZ injection used was 1.0 ml/250 g body mass. Blood was collected via a tail prick after 48 h and tested using a Glucocheck blood glucose monitoring system. Animals that exhibited blood glucose levels greater than 20 mmol/L after 48 h were considered to be diabetic. Plasma glucose levels of 20 mmol/L measured after 7 days confirmed a stable diabetic mellitus state.

### Serum biochemical analysis

For serum biochemical tests rats were warmed in a warming cabinet for 10 to 15 min in order to dilate blood vessels prior to taking the sample. During the warming process, rats were monitored for signs of hyperthermia and dehydration. After warming, the tail of the rat was washed with diluted Hibitane in order to see the blood vessel. Rats were restrained for the minimum time while the blood was being drawn from the lateral tail vein at the base of the tail. Approximately 1 ml of blood was collected from 3 rats in each of the respective group every 7 days. After tail bleeding, blood flow was stopped by applying finger pressure to the blood sampling site for approximately 30 s before the rat was returned to its cage. Blood was centrifuged and the serum was collected and analyzed immediately using a Chemvet analyzer for the evaluation of serum biochemical parameters. The parameters measured were albumin (ALB), alkaline phosphatase (ALKP), alanine amino transferase (ALT), amylase, blood urea nitrogen (Urea), calcium (Ca), cholesterol (CHOL), creatinine (CREA), globulin (GLOB), phosphorous (P), total bilirubin (TBIL), total protein (TP) and blood glucose (GLU).

### Measurement of BW

All experimental animals were weighed every 7 days for the entire 28 day treatment period, to monitor any weight losses or gains as a result of extract dose.

### Statistical analysis

Analysis was carried out using one-way analysis of variance (ANOVA; GraphPad Prism), followed by Tukey test for multiple comparisons. Values are expressed as a mean  $\pm$  standard deviation ( $n = 3$ ). A P-value less than 0.05 was considered to be statistically significant.

## RESULTS

The result of the effect of *B. discolor* methanolic extract on the blood glucose levels of STZ-induced diabetic rats are as shown in Figure 2. These results show that non-diabetic rats (Groups 5 to 8) had blood glucose levels that fell within the normal range. The methanolic leaf extract of *B. discolor* in both doses (200 and 600 mg/kg BW), the reference drug used (metformin) caused a time dependant ( $P < 0.001$ ) reduction in the blood glucose levels of the STZ-induced diabetic rats (Groups 1 to 3) when compared with the negative control (Group 4). There was also a variation between the different doses tested on the blood glucose levels with the higher dose (600 mg/kg BW) giving the highest activity followed by the reference drug metformin.

The activity of ALKP in diabetic treated rats is as shown in Figure 1. Rats treated with *B. discolor* extracts and the reference drug, decreased the ALKP levels in comparison to the untreated diabetic rats. However, the lower dose extract (200 mg/kg BW) decreased the ALKP levels within the acceptable range when compared with the higher dose and reference drug.

Creatinine levels as shown in Figure 1 shows that the diabetic rats (Group 4) exhibited a CREA level below the acceptable limits; however, animals that received both doses of *B. discolor* (Groups 1 and 2) extract and the reference drug (Group 3) increased the CREA levels to an acceptable limit. Urea levels also varied with the STZ-induced control group (Group 4) and the group receiving the reference drug (Group 3) having elevated urea levels, which exceeded the reference range at the end of the 28 days treatment period.

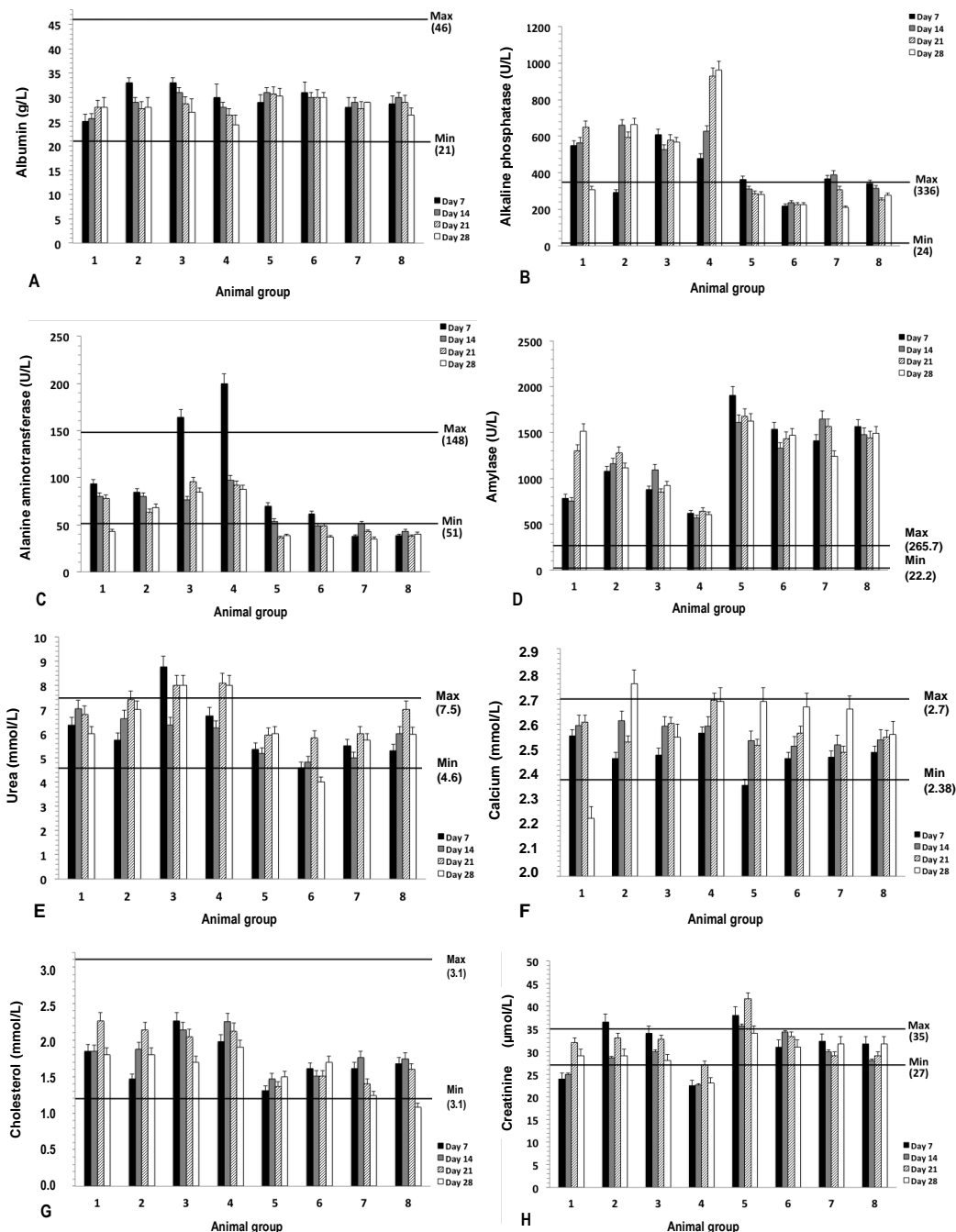
There was a significant difference in TBIL levels between the different groups as reflected in Figure 2. Diabetic rats which received the *B. discolor* extracts (Groups 1 and 2), showed TBIL levels within the normal range when compared with the diabetic negative control (Group 4) and reference drug (Group 3).

The levels between the different treatment groups for ALB, ALT, Ca, CHOL, GLOB, P, TBIL and TP were not significant, with only small variations in levels.

The data for BW change is represented in Figure 3. Although, there was no significant difference in the initial BW (Day 0) between the different groups, the BW gains for the diabetic negative control (Group 4) and the STZ-induced rats which received the low *B. discolor* dose (200 mg/kg BW, Group 1) were significantly lower when compared with the other groups. It is important to note that the STZ-induced rats which received the higher *B. discolor* dose (Group 3) had a better BW gain in comparison to those STZ-induced rats which received the reference drug (Group 4).

## DISCUSSION

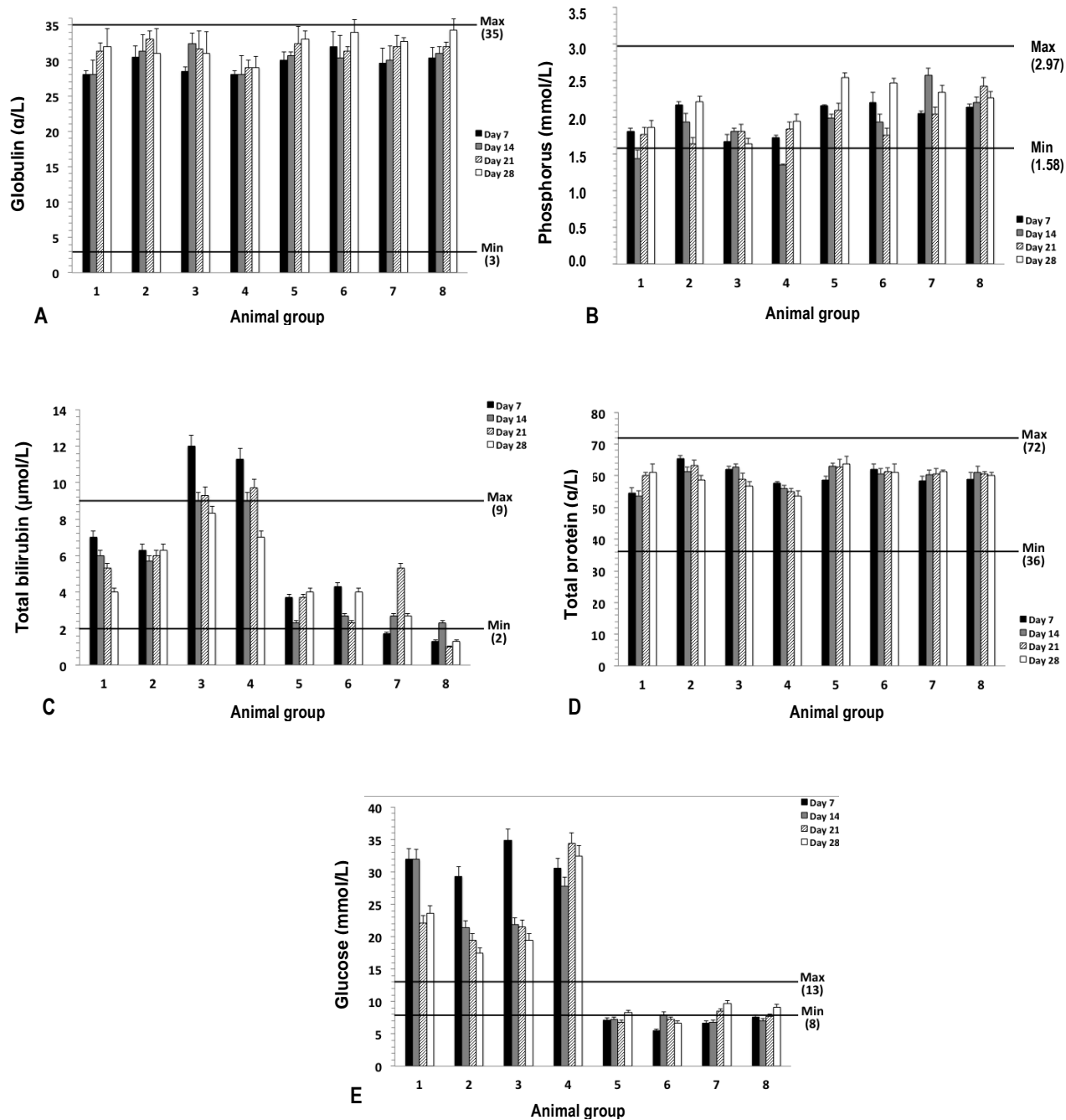
There are currently no regulations controlling the prescription and use of traditional medicine in South Africa resulting in possible misadministration, especially of toxic plants. The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also cause for alarm (Fennell et al., 2004). Therefore, it becomes necessary to identify the amylase inhibitors from natural sources having lesser side-effects. The purpose of this study was to investigate the anti-diabetic activity of a methanolic extract of *B. discolor* in a STZ-induced diabetic rat model. This was achieved by monitoring the blood glucose levels over a 28 day period using a Chemvet analyzer. Results from the study show that the methanolic extract of *B. discolor* at the test doses and the reference drug (metformin) exhibited a significant time dependant decrease in blood glucose levels of the STZ-induced diabetic rats with different levels of



**Figure 1.** Effect of oral administration of *B. discolor* crude methanolic extract at doses of 200 (Ext 1) and 150 mg/kg BW (Ext 2) BW on albumin (A), alkaline phosphatase (B), alanine amino transferase (C), amylase (D), blood urea nitrogen (E), calcium (F), cholesterol (G) and creatinine (H) levels in normal and diabetic male Wistar rats. Each column represents mean  $\pm$  SD (n = 3). Group 1: Diabetic + Ext 1; Group 2: diabetic + Ext 2; Group 3: diabetic control; Group 4: diabetic + metformin; Group 5: normal + Ext 1; Group 6: normal + Ext 2; Group 7: normal control; Group 8: normal + 1% Tween 80.

reduction on day 28 when compared with the negative control rats. In this experiment, the extract dose of 600 mg/kg BW produced the highest antidiabetic effect and suggests that this dose may be an effective antidiabetic dose for the crude *B. discolor* extract. The variation

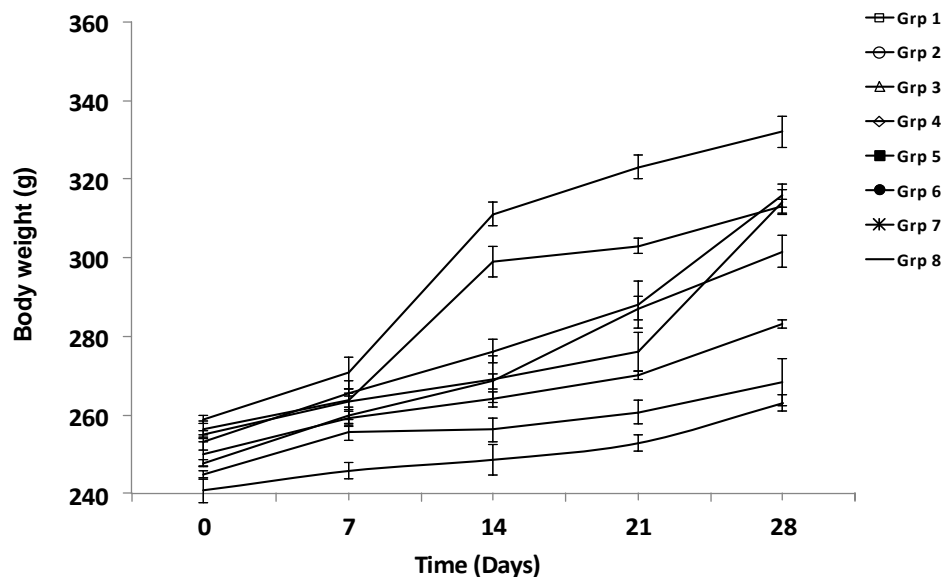
between the 200 and 600 mg/kg BW doses may be due to the concentrations of phytochemicals and active compounds within the extract. The exact mechanism by which *B. discolor* reduces blood glucose levels are unclear, but some attribute the anti-hyperglycaemic



**Figure 2.** Effect of oral administration of *B. discolor* crude methanolic extract at doses of 50 (Ext 1) and 150 mg/kg (Ext 2) BW on globulin (A), blood glucose (B), phosphorus (C), total bilirubin (D) and total protein levels in normal and diabetic male Wistar rats. Each column represents mean  $\pm$  SD ( $n = 3$ ). Group 1: diabetic + Ext 1; Group 2: diabetic + Ext 2; Group 3: diabetic + metformin; Group 4: diabetic control; Group 5: normal + Ext 1; Group 6 normal + Ext 2; Group 7: normal control; Group 8: normal + 1% Tween 80.

activity of other studied medicinal plants to their ability to restore the function of pancreatic tissue, by causing an increase in the insulin output or by a decrease in the intestinal absorption of glucose (Ibeh and Ezeaja, 2011; Neelesh et al., 2010). Biomarker enzymes such as alkaline phosphatase and alanine aminotransferase may

be used for the evaluation of hepatic disorders (Ghandi et al., 2012). This may be substantiated by the ALKP results in Figure 1 depicting elevated ALKP levels found in the STZ-induced diabetic groups (Groups 1 to 4). A significant reduction in the activity of ALKP in *B. discolor* treated diabetic rats indicates a potential hepatoprotective



**Figure 3.** Body weight gains in the different animal groups during the 28 day experimental period. Data is expressed as a mean of  $n = 4$ . Group 1: Diabetic + Ext 1; Group 2: diabetic + Ext 2; Group 3: diabetic + metformin; Group 4: diabetic control; Group 5: normal + Ext 1; Group 6: normal + Ext 2; Group 7: normal control; Group 8: normal + 1% Tween 80.

role in preventing possible diabetic complications. The elevation of urea, creatinine and total proteins may be considered as a significant marker for renal dysfunction (Carlo et al., 1996). Results in Figure 1 show a decrease in the urea levels of animals treated with *B. discolor* extract. The increase in urea levels for the STZ-induced control rats (Group 4) and the STZ-induced rats receiving the reference drug (Group 3) may be due to the high protein present in *B. discolor* (Haschick and Kerley, 1997). Blood urea nitrogen is a metabolite produced from dietary protein (Al Faris et al., 2011). Hyperglycaemia results in the generation of free radicals which may exhaust antioxidant defenses, thereby leading to the disruption of cellular functions and oxidative damage to membranes (Dangi and Mishra, 2011). Decreased protein levels may be attributed to the antioxidant activity of organic compounds within the *B. discolor* extract which may play a role in reducing the protein levels as can be seen in Figure 2 (Al Faris et al., 2011). Creatinine levels for STZ-induced rats receiving the *B. discolor* extract and those receiving the reference drug (metformin) all had creatinine levels which were within the normal range when compared with the STZ-induced control group whose levels remained below the acceptable minimum range. By the end of the 28 days treatment period, the serum levels for ALB, Amyl, ALT, Ca, CHOL, GLOB, P, TBIL and TP were not significantly different in comparison to the control group. The induction of diabetes by STZ has been associated with a loss in BW due to increased muscle wasting and loss of tissue protein (Chatterjee and Shinde, 2002; Gupta et al., 2012). The use of *B. discolor* at the 600 mg/kg BW dose improved the

BW in diabetic rats over the 28 days study period. This increase in the BW of diabetic rats may be attributed to an improvement in insulin secretion and glycemic control (Eliza et al., 2009; Genet et al., 1999). It has also been reported by Rajkumar et al. (1997) that increased anabolic reactions may be responsible for weight gain in diabetic rats.

Conclusively, the results of this study have demonstrated that *B. discolor* has significant antidiabetic potential and may be acting through the restoration of pancreatic tissue, stimulation of  $\beta$ -cells or by decreasing intestinal glucose absorption. Further studies are required to isolate and characterize antidiabetic bioactive compounds, and establish the mechanism(s) of action.

## ACKNOWLEDGEMENTS

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*Full Length Research Paper*

## The effect of hydroxy safflower yellow A on inflammatory reaction in myocardium of the rats after acute myocardial infarction

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This study was designed to investigate the effect of hydroxy safflower yellow A (HSYA) on inflammatory reaction in rat myocardium after acute myocardial infarction (AMI). 138 male Wistar rats were randomly divided into six groups: normal group, sham group, control group, injecting SY positive control group (SY group, 90 mg/kg), HSYA high-dose group (HSYA-H group, 40 mg/kg), and HSYA low-dose group (HSYA-L group, 20 mg/kg), n = 23. The AMI injury of rats was induced by ligating the anterior descending coronary artery. After the treatment of drugs, the concentrations of IL-1 $\beta$  and IL-6 in serum of the rats on HSYA-H, HSYA-L and SY groups significantly decreased when compared with those of the rats in the control group (P<0.05). The percentage of cells with the positive expressions of NF- $\kappa$ B and CRP in the drug treatment groups significantly decreased when compared with those of cells in the control group (P<0.05). Moreover, when compared with those of NF- $\kappa$ B in the control group, mRNA and protein expressions of NF- $\kappa$ B in myocardium in SY, HSY-L and HSY-H groups significantly decreased (P<0.05). In addition, the mRNA and protein expressions of NF- $\kappa$ B in myocardium in HSY-H group significantly decreased (P<0.05). HSY-A and SY can reduce the levels of hs-CRP, IL-1 $\beta$  and IL-6 in serum of the AMI rats. The inhibitory effect of HSY-A on inflammation is the main mechanism to improve AMI rats.

**Key words:** hydroxy safflower yellow A; acute myocardial infarction; inflammation

### INTRODUCTION

Acute myocardial infarction (AMI) is the leading cause of morbidity and mortality among all the cardiovascular pathologies, including embolic vascular occlusions, angina pectoris, peripheral vascular insufficiency, cardiac surgery, and cardiogenic shock (Mann and Nolan, 2006). This is despite controlling some risk factors such as arteriosclerosis and treatments via surgical intervention. AMI is a circumstance characterized by two events: ischemia and reperfusion of myocardium, leading to myocardium injury and loss of its function. Furthermore,

an acute loss of myocardium following MI results in increased loading conditions that induces ventricular remodeling of infarcted border zone and remote non-infarcted myocardium (Wu et al., 2011). Currently, AMI and unstable angina have progressively become a major concern, because of their high prevalence and mortality as well as their related high treatment costs (Robert et al., 2005).

Inflammatory process plays an important role in the myocardial healing process after an acute ischaemic

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event (Luigi et al., 2007). Important cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 and IL-6 are the starting promoters of the humoral post-MI healing process. They directly interfere with the myocardial contractility, the vascular endothelial function, and the recruitment of other inflammatory cells.

One of the major therapeutic goals for AMI is to alleviate myocardial necrosis and optimize cardiac repair following myocardial infarction. Hydroxy safflower yellow pigment A (HSYA) is the active ingredient of the safflower plant which has been demonstrated to antagonize platelet-activating factor receptor binding, and thus is used to treat several ischemic diseases, including myocardial ischemia, cerebral ischemia, coronary heart disease, and cerebral thrombosis (Liu et al., 2008; Zhu et al., 2003, 2005). According to recent studies, HSYA is a hydrophilic drug with low oral bioavailability, belonging to the biopharmaceutics classification system (BCS) III class of drugs (Wang et al., 2008). Recently, HSYA has been found to alleviate carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis in rats (Zhang et al., 2011). Also, HSYA was demonstrated to prevent cerebral ischemia-reperfusion injury by inhibition of thrombin generation (Sun et al., 2010). Yan et al. (2011) found that HSYA could significantly alleviate bleomycin-induced early pulmonary inflammation by suppressing the activation of nuclear factor-kappa B (NF- $\kappa$ B), phosphorylation of p38 mitogen-activated protein kinase (MAPK) and inhibiting the augmentation of pro-inflammatory and pro-fibrogenic cytokines expression (Yan et al., 2012). But it is still unknown whether HSYA can prevent AMI by anti-inflammatory effect. In this study, we designed to investigate the effect of HSYA on inflammatory factors and nuclear transcription factors during the prevention AMI.

## MATERIALS AND METHODS

### Animals and reagents

Male Wistar rats (138; aged 5 to 7 weeks, 200  $\pm$  20 g) were purchased from Chinese Academy of Medical Sciences, Institute of Experimental Animals. IL-1 $\beta$ , IL-6 and IL-10 kits were purchased from American Rapid Bio Lab Company; high sensitivity C-reactive protein (hs-CRP) kit was purchased from American Adlitteram Diagnostic Laboratories (ADL). Monoclonal antibody rabbit anti-CRP antibody, concentrated polyclonal antibody of NF- $\kappa$ B p65 antibody, versatility secondary antibody, and diaminobenzidine (DAB) staining kit were purchased from Beijing Boaosen Biotechnology Co., Ltd. Reverse transcriptase polymerase chain reaction (RT-PCR) kit was purchased from Sigma, USA; NF- $\kappa$ B p65 primers were provided by Invitrogen Corporation, USA.

### Experimental drug

Injection safflower yellow pigment, (SY; 150 mg/branch, contains flavonoids (80 mg) and hydroxy safflower yellow pigment A (HSYA; 67 mg)) was provided by Shanxi Huahui Kai Tak Pharmaceutical Co., Ltd. 80302003; HSYA (98.1135%) from Taiyuan Hua Wei Pharmaceutical Co., Ltd., 20070910.

### The preparation of rat AMI model

Chloral hydrate (0.8 ml/100 g) of 3.5% was intraperitoneally injected to anesthetize rats. After fixing on the back and after disinfection, the skin of the rat was cut open in 4,5 intercostal skin, and the thoracic cavity was open and the heart exposed. The pericardium of rat was cut and the heart was extruded, the root of left anterior descending coronary of the rat was ligated between pulmonary cone and the left atrial appendage, threaded and ligated on the bottom 2 to 3 mm of the left atrial appendage root. Then, the thoracic cavity of the rat was sutured. Electrocardiogram was recorded immediately before and after thoracotomy. The remaining operation of the rats on sham group was the same as rats on AMI group except for ligation of coronary artery.

### Animal grouping and treatment

Male Wistar rats (138) were randomly divided into six groups: normal group, sham group, control group, injecting SY positive control group (SY group, 90 mg/kg), HSYA high-dose group (HSYA-H group, 40 mg/kg), and HSYA low-dose group (HSYA-L group, 20 mg/kg),  $n = 23$ . 360 min after the ligation, two rats were dead in the control, HSYA-H, and HSYA-L groups and one rat was dead in SY group.

### Administration of dose

According to the results of pharmacodynamics study before injecting SY in clinic, the rats on the normal, model and sham groups were injected with saline at the same volume. The aforementioned drugs were injected just after and 120 min after induction of AMI.

### Experimental animal

Rats (138) were treated with euthanasia 360 min after ligation, and about 5 ml blood was collected from abdominal aorta of rats. After centrifugation, the serum was collected and frozen on -80°C. Six hearts from each group were removed, and fixed with formalin. Then 6 hearts were routinely embedded with paraffin for pathological examination and immunohistochemistry. The other 5 hearts from each group were removed to store for molecular biology.

### Preparation of pathological specimens of hearts

After anesthesia, the rat hearts were removed and rinsed cleanly with normal saline. According to surgical marker of infarction location, myocardial tissue was excised below the ligation site. Then, the myocardial tissue was fixed in 4% paraformaldehyde for 24 h, and was embedded in paraffin. 4- $\mu$ m slices were cut along the long axis of the left ventricular at interval of 1 mm cross-section, and stained with hematoxylin-eosin (HE) staining. The pathological change of myocardial tissues was observed in optical microscope.

### Immunohistochemistry method and measurement

Serial 4- $\mu$ m paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. After blocking sections with 20% (v/v) goat serum in phosphate-buffered saline, sections were incubated overnight at 4°C with NF- $\kappa$ B antibody (Scant Cruz, 1:100) and CRP (1:100, Scant Cruz). Sections were then incubated with the

appropriate secondary antibodies. Positive areas were counted and expressed as a percentage of the myocardial tissue. A negative control, where the primary antibody was replaced with either mouse or rat IgG at the same dilution, was always included. Blinded analysis of positive immunostained sections was performed with the image-analysis program (Image Pro Plus, Media Cybernetics).

### Western-blotting

Total protein was isolated from rat myocardial tissues. Tissues were collected and homogenized in protein extraction buffer [50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% protease inhibitor cocktail]. The lysate was centrifuged at 12,000 ×g for 10 min, and the supernatant was collected. The supernatant (30 ×g of protein) was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk, the membranes were probed with the primary NF-κB antibody (1: 5000) for 1 h, followed by a secondary antibody (goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge, Beijing, China), or probed with the primary antibodies anti-actin (1:500; Sigma) and anti-β-actin (1:3000; Proteintech Group, Inc., Chicago, IL), followed by a secondary antibody (goat anti-mouse IgG horseradish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge). Protein expression was detected with an enhanced chemiluminescence detection system (Vigorous, Beijing, China).

### RT-PCR

Total RNA was isolated from each specimen of frozen rat myocardial tissue using Trizol reagent according to the manufacturer's instructions. RNA concentration and purity were determined by the Thermo Scientific NanoDrop 2000 (Wilmington, U.S.A.). First-strand cDNA synthesis was performed with 2 μg of the total RNA in a reaction volume of 20 μl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. One microliter of cDNA was amplified in 20 μl reactions using SYBR® Premix Ex Taq™ on an iCycler iQ Real-time Detection System. The following gene-specific primers were used. β-actin primer: Sense, 5'-AACACCCAGCCATGTACG-3'; Antisense, 5'-CG CT CAGGAGGAGCAATGA-3'; NF-κB primer: Sense, 5'-AAGATCAATGCTA CACAGG-3'; Antisense, 5'-CCTCAATGTCTTCTTTCTGC-3'; PPAR-γ primer: Sense, 5'-GACCACTCCCCTCCTTTGA-3'; Antisense 5'-CGAC ATCAATTGCC ATGAG-3'. 30 μl Reaction system: RT-PCR enzyme mix, 2 μl; 20X buffer (Mg<sup>2+</sup> free), 1.5 μl; MgCl<sub>2</sub> (25 mM), 1.5 μl; deoxyribonucleotide triphosphate (dNTP; 10 mM), 1.0 μl; CX1 sense primer, 1.0 μl; CX1 anti-sense primer, 1.0 μl; RNA, 2.0 μl; dH<sub>2</sub>O, 20 μl. PCR amplification was carried out as follows: initial denaturation at 95°C for 15 s, 35 cycles with denaturation at 95°C for 5 s, annealing at 61°C for 15 s. Relative quantification was determined using the 2<sup>-ΔCt</sup> method with data normalized to β-actin house keeping gene.

## RESULTS

### Effect of HSYA on pathological myocardial tissue of AMI rats

The results of HE staining from the rats in the normal group in light microscope showed that normal myocardial

cells were arranged regularly and cellular nucleus was well-stacked, cardiac muscle fibers were arranged uniformly and myocardial structure was normal. While the results from AMI rats on the control group showed that myocardial cells were arranged in scattered manner and cardiac muscle fibers were broken. The intercellular space of myocardial cells in infarction zone was widened and infiltrated with some inflammatory cells including neutrophil granulocyte and monocytes, and red blood cells (Figure 1).

### Effect of HSY-A on the concentrations of hs-CRP, IL-1β, IL-6, and IL-10 in serum of AMI rats

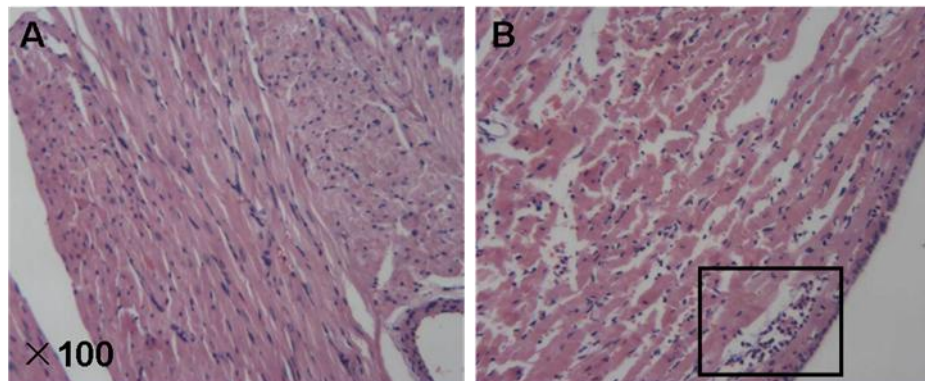
As shown in Figure 2, 360 min after AMI induction, the concentrations of IL-1β and IL-6 in serum of the rats in the control group significantly increased when compared with those of rats on the sham group (P<0.05), while the concentration of IL-10 in serum of the rats in the control group significantly decreased when compared with those of rats in the sham group (P<0.05). It indicates that inflammatory reaction obviously happened on acute ischemic stage during AMI. After the treatment of drugs, the concentrations of IL-1β and IL-6 in serum of the rats in HSY-high dose group, HSY-low dose group and SY group significantly decreased when compared with those of rats in the control group (P<0.05), while there was no significant difference in the concentration of IL-10 in serum of the rats between all the drug-treatment and control groups (P > 0.05).

### The effect of HSY-A on the NF-κB and CRP protein expression in myocardium of AMI rats

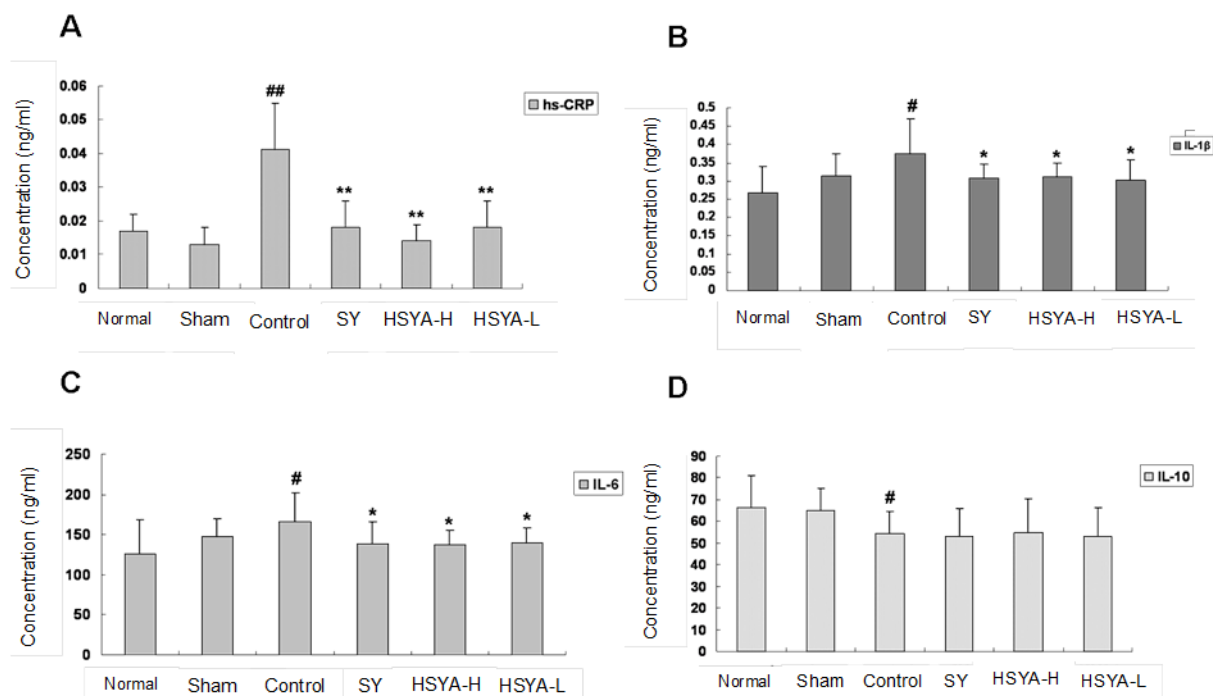
As shown in Figure 3, the immunochemical results showed that small amounts of the cells with the positive expressions of NF-κB and CRP were detected in myocardium of the rats in the normal group and sham group. There were small amounts of yellow brown granulation in intracytoplasm. The percentages of the cells with the positive expressions of NF-κB and CRP in the control group were significantly increased when compared with that of the cells on the sham group (P<0.05), while the percentage of the cells with the positive expressions of NF-κB and CRP in the drug treatment groups significantly decreased when compared with that of cells in the control group (P<0.05). Compared with the drug-treatment group, there were no significant difference on the percentage of the cells with the positive expressions of NF-κB and CRP (P >0.05).

### Effect of HSY-A on the NF-κB expression in myocardium of AMI rats

As shown in Figure 4, the results detected by RT-PCR



**Figure 1.** The pathological change of the infarction myocardium of the rats after ligation treatment. A. normal group; B. control group. Black rectangle indicated the infiltration of inflammatory cells.

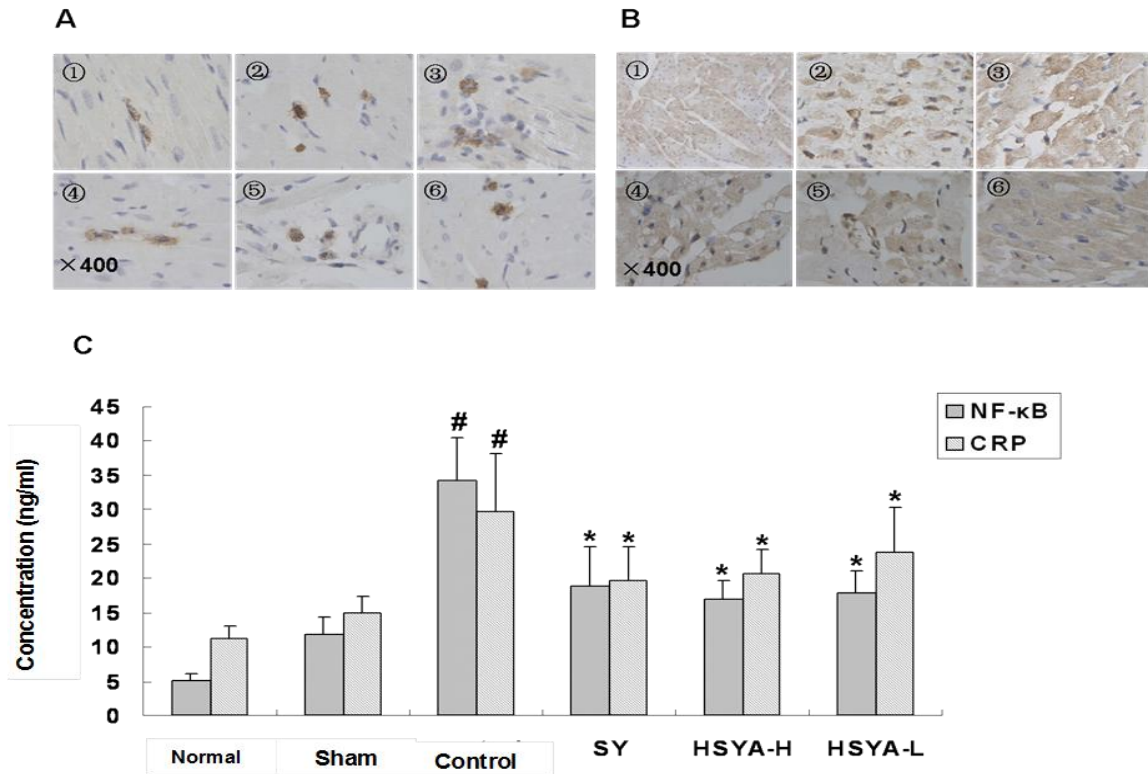


**Figure 2.** The effect of HSY-A on the concentrations of hs-CRP, IL-1 $\beta$ , IL-6, and IL-10 in serum of AMI rats. A. hs-CRP; B. IL-1 $\beta$ ; C. IL-6; D. IL-10 (n=12).

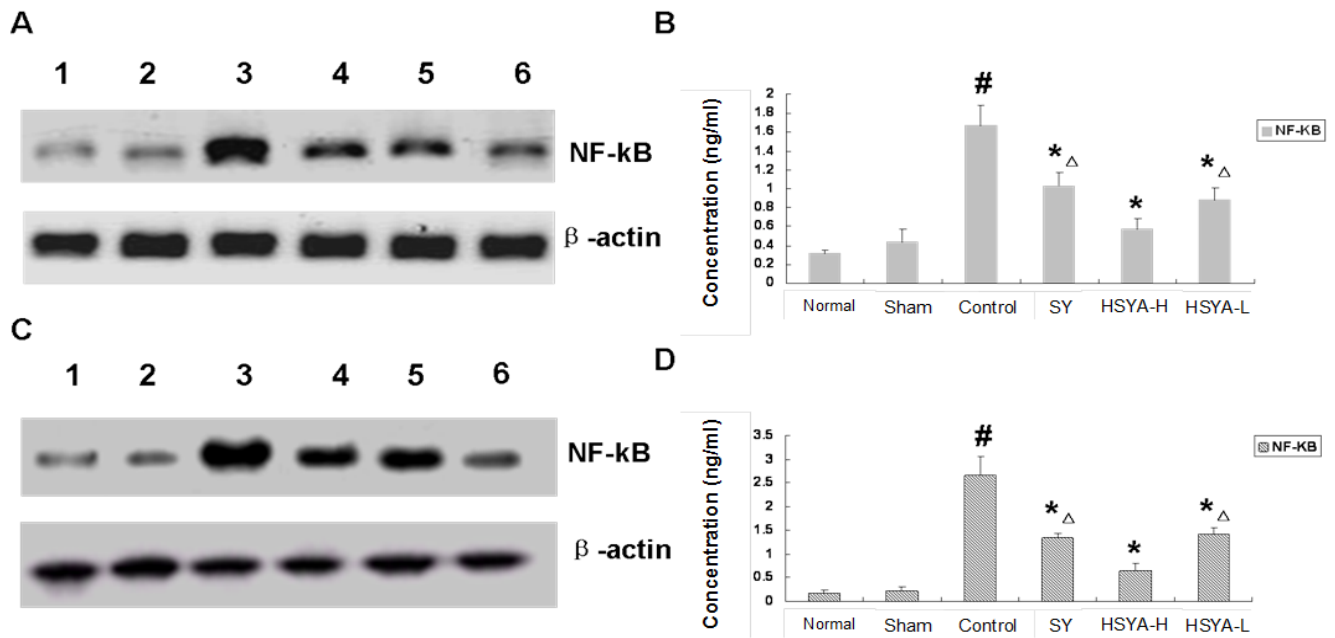
and Western-blot showed that when compared with that of NF- $\kappa$ B on sham group, the mRNA and protein expression of NF- $\kappa$ B in myocardium on the control group significantly increased ( $P < 0.05$ ). Compared with that of NF- $\kappa$ B in the control group, the mRNA and protein expressions of NF- $\kappa$ B in myocardium of SY, HSY-L and HSY-H groups significantly decreased ( $P < 0.05$ ). In addition, compared with the other drug treatment group, the mRNA and protein expressions of NF- $\kappa$ B in myocardium in HSY-H group significantly decreased ( $P < 0.05$ ).

## DISCUSSION

HSYA was isolated from the dried flower of *Carthamus tinctorius* L, which was extensively used in traditional Chinese medicine (TCM) to treat cirrhosis. In our previous study, the data showed that HSYA can protect myocardium from ischemia and reduce the levels of cardiac troponin T (cTn-T) and creatine kinase-MB (CK-MB) in serum of AMI rats (Fu et al., 2011). In this study, based on the key role of inflammation in the process of AMI, our data showed that HSYA can inhibit significantly



**Figure 3.** The effect of HSY-A on the NF-κB and CRP expression in myocardium of AMI rats. A. The immunochemical results of NF-κB of the rats on each group. B. The immunochemical results of CRP of the rats on each group. C. The statistical results of NF-κB and CRP expression in myocardium of AMI rats on each group.



**Figure 4.** The effects of HSY-A on the expression of NF-κB in AMI cardiac of the rats on each group. A. The results of the expression of NF-κB in AMI cardiac of the rats on each group by RT-PCR. B. The histogram according to the results of the expression of NF-κB in AMI cardiac of the rats on each group by RT-PCR. C. The results of the expression of NF-κB in AMI cardiac of the rats on each group by Western-blotting. D. The histogram according to the results of the expression of NF-κB in AMI cardiac of the rats on each group by Western-blotting.

inflammatory reaction by reducing the level of hs-CRP, IL-6 and IL-1 $\beta$  in serum and possibly by regulating the activation of NF- $\kappa$ B.

Myocardial infarction is associated with inflammatory reaction in a complex interaction between a variety of pleiotropic inflammatory mediators, which is a prerequisite for healing and scar formation. Among them, the elevation of interleukin, as an indicator that reflects immune system, is significantly related to cardiovascular events (Chamorro, 2004). IL-1 $\beta$  can not only reflect the intensity of inflammatory reaction, but also indirectly predict the extent of myocardial injury. IL-6, also known as a pro-inflammatory cytokine, is a central regulatory factor of inflammation and plays a key role in acute myocardial ischemia and vascular injury in coronary heart disease. IL-6 can enhance the adhesion of white blood cells and myocardial cells, and aggravate damage of myocardial cells (Liu et al., 2007). Anti-inflammatory cytokine IL-10 may inhibit inflammatory reaction and immune reaction (Sheng et al., 2004). When myocardial ischemia occurs, the levels of IL-1 $\beta$  and IL-6 are increased rapidly, and are positively correlated to the increased degree of myocardium damage. A large number of studies have shown that anti-inflammatory factors and pro-inflammatory cytokines are involved simultaneously in the process of ischemic injury. In the process of myocardial ischemia-induced inflammatory response, the increase of anti-inflammatory cytokine IL-10 can inhibit inflammatory response and injury (Prasanna et al., 2010). Monocyte-derived macrophages and mast cells may produce cytokines and growth factors necessary for fibroblast proliferation and neovascularization, leading to effective repair and scar formation. At this stage expression of inhibitory cytokines such as IL-10 may play a role in suppressing the acute inflammatory response and in regulating extracellular matrix metabolism.

The results suggested that after the treatment of injection SY and HSYA, the concentrations of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in serum were reduced when compared with the control group, but there was no significant change in anti-inflammatory cytokine IL-10 after the treatment of SY and HSYA. It suggested that the inhibitory effect of HSYA on myocardial ischemic injury induced by ligating coronary artery of rats was related to the inhibitory effect of inflammatory response in AMI.

Previous study showed that hs-CRP was one of the independent risk factors for coronary heart disease, and it is an accurate and objective indicator to reflect inflammation activation (Lin and Li, 2007). In the case of AMI, serum CRP increases following cytokines activation and binds to the damaged myocardial cells. Further, it stimulates the complement cascade, which may finally increase the MI size, worsening the overall post-MI outcomes (Barrett et al., 2002; Paoletti et al., 2004).

In this study, 360 min after AMI, the concentration of hs-

CRP in the serum of rats in the control group significantly increased when compared with that of rats in the sham group, which suggested that inflammatory reaction is significant at acute phase of ischemia. After drug treatment, the levels of hs-CRP in the serum of rats in hydroxyl high-dose, low dose and safflower yellow pigment groups significantly reduced when compared with that of rats in the control group. It indicated that HSYA can inhibit inflammatory reaction during myocardial infarction. CRP is one of the most powerful predictors of myocardial infarction, stroke, and vascular death currently known. NF- $\kappa$ B activation may be responsible for the synergistic effect of IL-1 $\beta$  on IL-6-induced CRP expression. In this study, the immunohistochemical results indicated that 360 min after myocardial ischemia, inflammatory reaction is significant in ischemic myocardium and the protein expression of CRP in myocardium is significantly decreased, which corresponds with the results of serum determination. The percentage of the cells with the positive expressions of NF- $\kappa$ B and CRP after the treatment of HSYA and SY significantly decreased when compared with those of cells in the control group.

NF- $\kappa$ B is considered as one of the most important transcription factors, and is the central link of regulating immune response, stress response, apoptosis, and inflammation. It can be activated by a variety of different stimuli and it participates in expression and regulation of a variety of genes, especially genes involved in defense functions of the body. In recent years, studies have shown that myocardial ischemia at early stage can induce activation of NF- $\kappa$ B to regulate gene transcription (Xiyuan et al., 2009) and NF- $\kappa$ B plays an important role in myocardial ischemic preconditioning, ischemia, hypoxia, reperfusion injury and apoptosis, especially in the process of inducing myocardial inflammatory reaction, and increasing the release of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Lu et al, 2009; Yao et al., 2011), and aggravating myocardial injury. At present, a variety of NF- $\kappa$ B inhibitors have been used to relieve myocardial ischemia and reperfusion injury. NF- $\kappa$ B is activated by a vast number of agents, including cytokines such as TNF- $\alpha$  and IL-1 $\beta$  and free radicals. The genes regulated by the NF- $\kappa$ B family of transcription factors are diverse and include those involved in the inflammatory response, cell adhesion and growth control (Martine et al., 2011). NF- $\kappa$ B activation has been demonstrated in various models of experimental myocardial ischemia and reperfusion (Kupatt et al., 1999; Shimizu et al., 1998).

According to the results from RT-PCR and Western blotting, after AMI, compared with that of rats in normal and sham groups, the mRNA and protein expression of NF- $\kappa$ B on the AMI control group is significantly increased, while the expressions of NF- $\kappa$ B on HSYA-L and HSYA-H groups, especially on HSYA-L group, significantly reduced when compared with that of rats in the AMI control group. It suggested that HSYA can inhibit the

release of downstream inflammatory factors to protect ischemic myocardium by inhibiting the activation of NF- $\kappa$ B in the process of myocardium ischemia.

The immunohistochemical results indicated that 360 min after myocardial ischemia, inflammatory reaction is significant in ischemic myocardium and the protein expression of CRP in myocardium is significantly decreased, which corresponds with the results of serum determination.

Previous studies showed that treatment with HSYA also alleviated bleomycin-induced increase of mRNA level of TNF- $\alpha$ , IL-1 and transforming growth factor (TGF- $\beta$ 1) in lung homogenates. Moreover, HSYA inhibited the increased activation of NF- $\kappa$ B (Sun et al., 2010). HSYA decreased NF- $\kappa$ B p65 nuclear translocation, inhibited the mRNA expressions of pro-inflammatory cytokine TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in mice with acute lung injury (Sun et al., 2010). The results also were corresponding with our experimental results in this study.

In summary, it can be suggested that HSY-A and SY can reduce the levels of hs-CRP, IL-1 $\beta$  and IL-6 in serum of the AMI rats. HSYA is the main component of SY to reduce myocardial ischemia. The inhibitory effect of HSY-A on inflammation after AMI is the main mechanism to improve AMI of rats.

## ACKNOWLEDGEMENT

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Full Length Research Paper

# Quantitative determination of lappaconitine in plasma by liquid chromatography-tandem mass spectrometry and its application in the pharmacokinetic study in rabbits

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A high sensitive and rapid method was developed for the analysis of lappaconitine in rabbit plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Blood was taken from the ear vein after transdermal administration of lappaconitine gel on the back of male rabbits, followed by liquid-liquid extraction with n-hexane from plasma, the analyte was separated in an Inertsil ODS-3 column (2.1×50mm, 0.5µm) through gradient elution with acetonitrile-water-formic acid at a flow rate of 0.4 mL/min. Detection was performed by positive ion electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode, monitoring the transitions m/z 585.5 → m/z 535.5 and m/z 531.3 → m/z 489.4, for the quantification of lappaconitine and the internal standard (IS) ketonconazole, respectively. The method was linear over the concentration range of 13.125-1050.0ng/mL and the lower limit of quantification was 13.125 ng/mL. The intra- and inter-day precisions were less than 7.51%, and accuracy ranged from 92.3 to 102%. Under the transdermal concentrations of 13.125n g/mL, 65.625 ng/mL and 525.00 ng/mL, the absolute recoveries of lappaconitine were 77.8, 84.4 and 79.0%, respectively. The concentration versus time curve in 24 h was fitted using the bimodal model of DAS ver1.0 software and the results showed that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model.

**Key words:** Lappaconitine, LC-MS/MS, plasma drug concentration, pharmacokinetics.

## INTRODUCTION

Lappaconitine, a diterpenoid alkaloid extracted from the roots of *Aconitum Sinomontanum* Nakais (Chen et al., 1980; Wang et al., 1992; Ma et al., 1998), has strong central analgesic, local anesthesia, antifebric and anti-inflammatory activities so as to be the first developed non-narcotic analgesics in China (Gao and Qi, 2008). Its clinical application is primarily to treat cancer pain, postoperative analgesia, induced abortion and cesarean section analgesia, neuralgia caused by herpes zoster,

osteoarthritis of the knee and urinary tract syndrome and so on (Tang and Mo, 2007). Meanwhile, injection therapy is regularly used, especially epidural injection (Lu, 2012). However, lappaconitine has narrow safe range because of its certain toxicity (Li et al., 2012a), and its existing delivery methods of lappaconitine still have many drawbacks, which hinder the further application of the drug.

There are few studies of the pharmacokinetic proper-

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ties of lappaconitine in animals or human body. The plasma concentrations of lappaconitine hydrobromide and its similar alkaloids, such as aconitine, mesaconitine and hypaconitine, were determined by high performance liquid chromatography (HPLC) (Guan et al., 2006; Shi et al., 2008), LC-ultra violet (UV) (Yang et al., 1989; Wang et al., 1987; Li, 2010), LC-MS (Hikoto O et al., 1997; Wang et al., 2011) or LC-MS/MS (Sun et al., 1999; Zhang et al., 2008) methods. Because of the low sensitivity and poor specificity of UV detection, it only can be used to determine the plasma concentration of moderate level and the results have large errors and low credibility, which are in contrast with the high sensitivity and strong specificity of MS detection.

In addition, investigation indicates that lappaconitine provides rather appropriate oil-water partition coefficient and percutaneous permeability displayed by its chemical structure. Hence, it is fairly an ideal administration method to be made transdermal absorption preparation (Li et al., 2012a). There are reports of patch, micro-emulsion and gel of lappaconitine at present (Han et al., 2008; Li et al., 2012b; Wang et al., 2009), and their analgesic effects are quite evident (Zhou et al., 2005; Qiu et al., 2012). Based on the above reasons, to further improve the drug delivery and enhance the efficacy, we prepared lappaconitine gel and first developed a rapid and sensitive method to determine the concentration of lappaconitine monomer in rabbit plasma with utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS) in this study. Determination of drug concentration in plasma can not accurately reflect the efficiency of the transdermal delivery method, but provide scientific basis for optimizing dosage regimen and clinical medication.

## MATERIALS AND METHODS

### Apparatus, drugs and chemicals

HP1100 Series liquid chromatography system (Agilent, USA); API4000 mass spectrometer (Applied Biosystems, USA); SZ-1 Fast vortex mixer (Jincheng Guosheng Test Instrument Factory, Jintan City, Jiangsu Province); centrifuge (model LD-Z4, Beijing Medical Centrifuge Factory) were used for this study.

Lappaconitine gel was made in our laboratory (Wang et al., 2007). Lappaconitine standard (purity 98.8%) was provided by the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine (TCM). Ketoconazole (purity 99%), as the IS, was from Zhejiang East Asia Medicine Chemical Industry Ltd. Co., N-hexane, acetonitrile, and methanol were all of analytical grade purchased commercially. Double-distilled water was used.

### Animals

Male New Zealand rabbits, weighing (2.5±0.5) kg, were provided by the Xing-long Experimental Animal Center, Haidian District, Beijing (certification number SCXK2006-0001). The study adopted a single dose administration of 8.0 mg/kg.

### Sample collection

Six male rabbits were fasted 24 h prior to the drug administration but with free access to water. The skin hair on the back at an area of 2×10 (cm<sup>2</sup>) was shaved. Each rabbit was uniformly spread with 1.0 g lappaconitine gel that was weighed precisely (equivalent to 20 mg lappaconitine, 8.0 mg/kg). The gel was covered with 2×10 (cm<sup>2</sup>) double-deck gauze for 12 h. The gauze was removed with warm water after 12 h. Three ml blood samples were collected from the vein of the edge of rabbit ear into heparinized tubes before drug administration (0 h) and at 10, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 h after drug administration. The blood samples were centrifuged at 4000 rpm for 15 min, and the plasma was separated and kept frozen at -20°C until analysis.

### LC-MS/MS conditions

Chromatographic separation was performed using C18 Inertsil ODS-3 column (2.1×50 mm, 0.5 μm). The mobile phase A was 2.0 mM ammonium acetate and 0.1% formic acid aqueous solution. Mobile phase B was 0.1% formic acid acetonitrile solution. A gradient elution was carried out at the following schedules: 0 min, 10% B; 2.5 min, 55% B; 3.5 min, 55% B; 4.0 min, 10% B; 7.5 min, 10% B. The flow rate was 0.4 mL/min.

The mass spectrometer parameters were set at: voltage 5.4 kV, collision induced voltage 43 V for lappaconitine and 42 V for ketoconazole, ionization source temperature 650°C, curtain gas pressure 25 psi, sheath gas pressure 50 psi, and auxiliary gas pressure 50 psi. Detection was performed by positive ion electrospray ionization (ESI) in multiple reactions monitoring (MRM) mode. Scan time was 0.2 s.

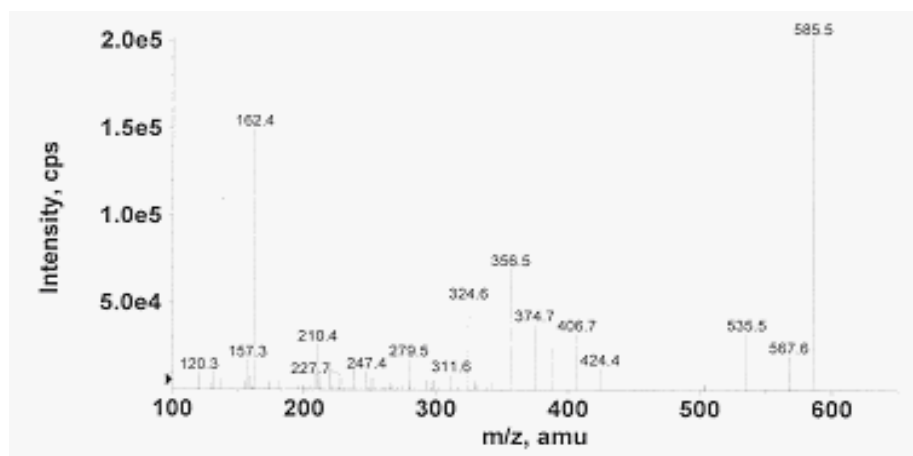
### Sample preparation

Lappaconitine stock solution was prepared by dissolving 1.05 mg lappaconitine standard, which was dried to constant weight in 105°C and weighed precisely, in 50 mL methanol and water (50:50, v/v) to obtain a concentration of 21.0 μg/mL. It was stored in a refrigerator until use. Similarly, 50.00 mg of ketoconazole weighed precisely was dissolved in 50 mL methanol and water (50:50, v/v) to obtain a concentration of 1 mg/mL. Twenty-five microliter (25 μL) of ketoconazole solution (1mg/mL) was diluted into 50 mL (500 ng/mL) methanol and water (50:50, v/v) and stored in a refrigerator until use.

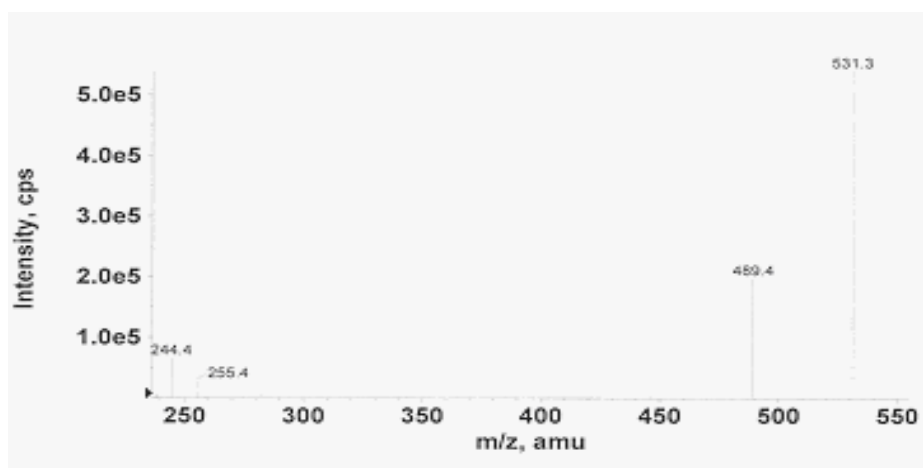
The plasma samples were thawed and 1.0 mL plasma was spiked with 100 μL IS solution (500 ng/mL ketoconazole) and 500 μL of 1 mol/L NaOH, and mixed well. Three milliliter (3 mL) of n-hexane was added for extraction. The mixture was vortexed for 2 min and centrifuged for 10 min. The organic phase was collected and evaporated to dryness at 40°C under a nitrogen stream. The residue was reconstituted in 200 μL methanol and water (50:50, v/v). 20 μL was injected into the LC-MS system.

### Validation of the analytical method

Specificity of the LC-MS/MS method was assessed by analyzing drug-free plasma samples. Rabbit plasma standard samples spiked with various concentrations of lappaconitine were prepared by adding 100 μL lappaconitine standard solution into 900 μL drug free plasma and mixed. The final lappaconitine, concentrations were 13.125, 26.250, 32.800, 65.625, 262.5, 525.0 and 1050.0 ng/mL. The samples were processed and analyzed to obtain the linear range of the calibration curve. The lowest concentration of the



A



B

**Figure 1.** The full scans mass spectrometry of lappaconitine (A) and IS (B).

linear curve was treated as the lower limit of quantification (LOQ). The precision was assessed by repeatedly running three standard samples on the same day and on different days. The differences (relative standard deviation, RSD) of the determined concentrations and the spiked concentrations were used to assess the accuracy of the method. The absolute recovery was also determined in three standard samples. The stability of lappaconitine in the plasma samples was determined from three different concentration levels with three replicates each under the following conditions: long-term stability at  $-20^{\circ}\text{C}$  for 60 days, general stability at  $25^{\circ}\text{C}$  for 24 h, and after three freeze-thaw cycles at  $-20^{\circ}\text{C}$ .

## RESULTS

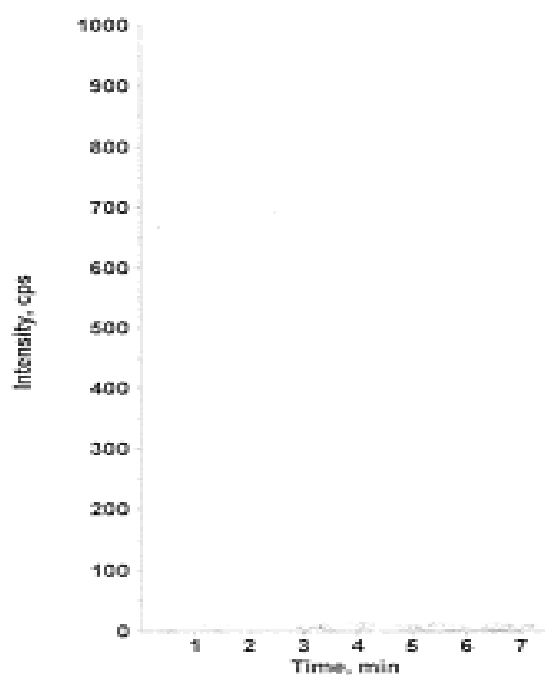
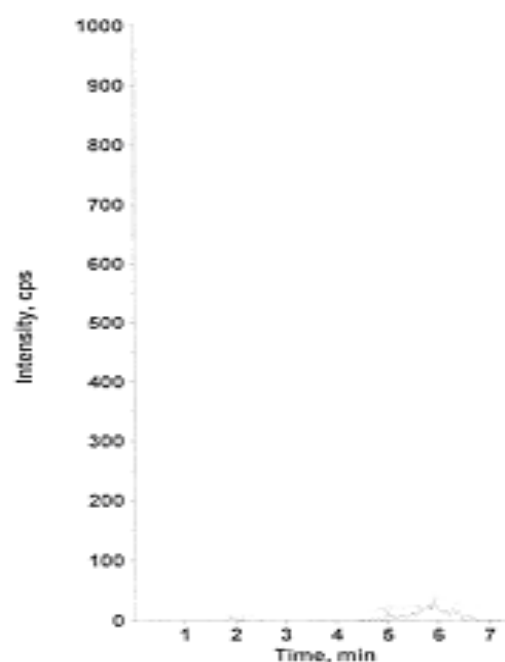
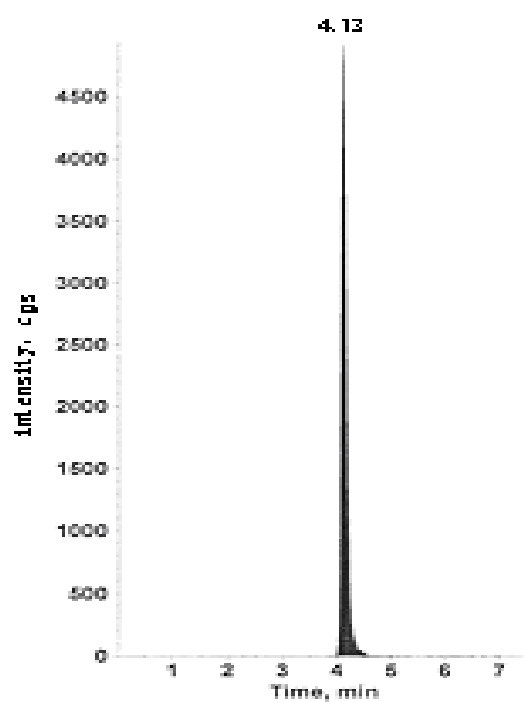
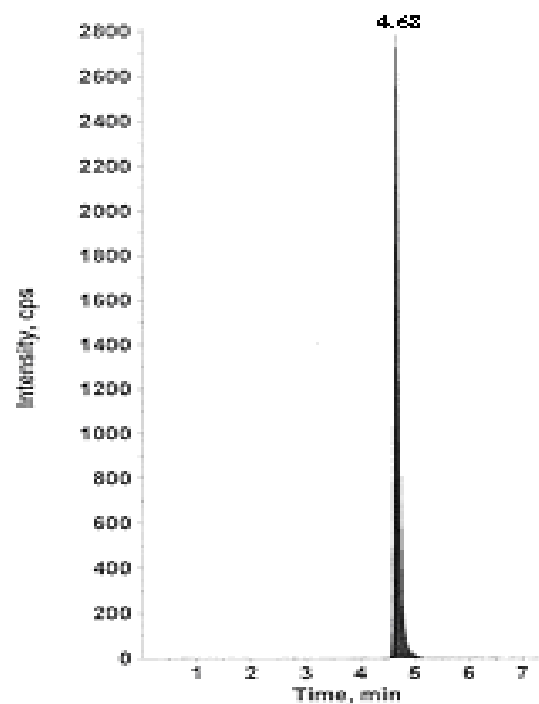
### Method development

Full scans were performed in the positive ion detection

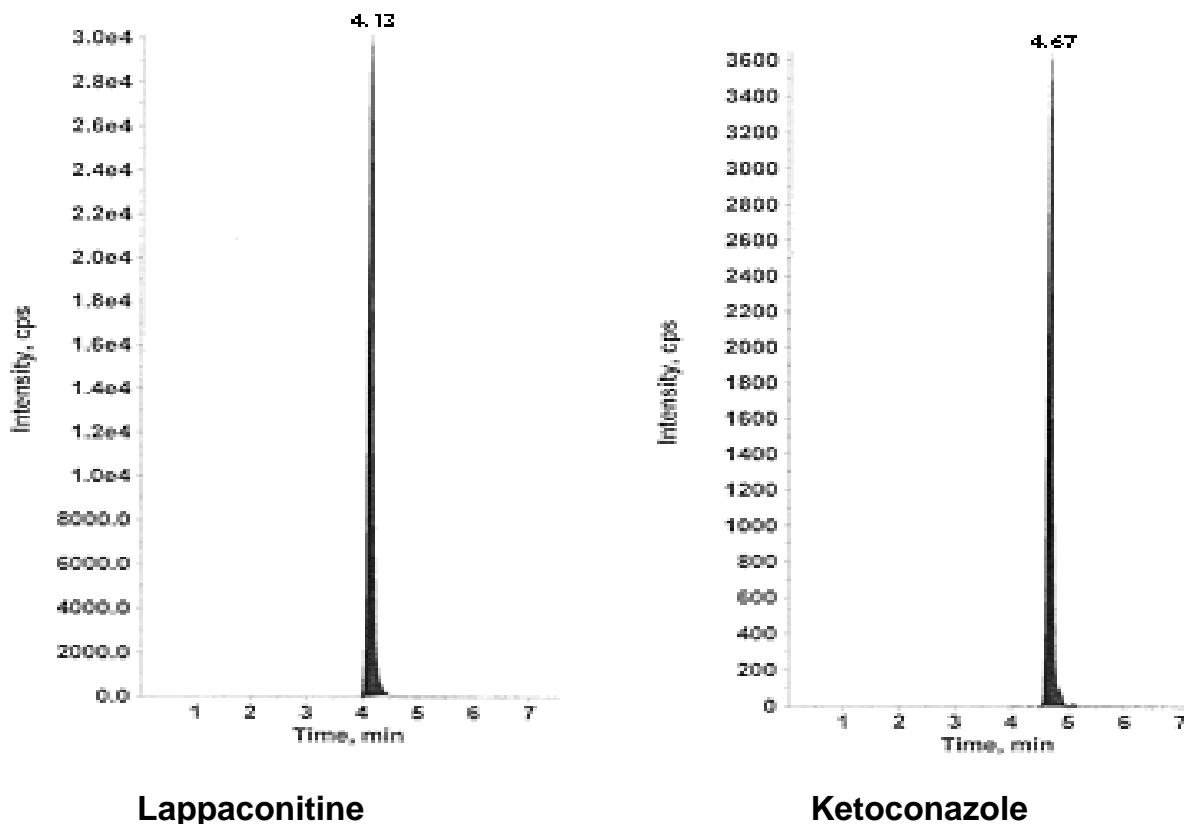
mode to develop the ESI conditions for lappaconitine and IS. Lappaconitine predominantly formed protonated molecular ions  $[\text{M}+\text{H}]^{+}$  and had the most abundant at  $m/z$  585.5 while IS at  $m/z$  531.3 (Figure 1). Multiple reaction monitoring (MRM) mode with parent/daughter mass transitions of 585.5/535.5 and 531.3/489.4 was used to quantify Lappaconitine and IS, respectively.

### Specificity

Drug-free plasma was processed and analyzed by the developed LC-MS/MS method. No interference peaks were observed (Figure 2A). The chromatograms of the plasma spiked with lappaconitine at the concentration of 13.1 ng/mL and IS are shown in Figure 2B. The retention

**A****Lappaconitine****Ketoconazole****B****Lappaconitine****Ketoconazole**

C



**Figure 2.** LC-MS/MS chromatograms of drug-free plasma (A), plasma spiked with lappaconitine and IS (B), and plasma of rabbit administered with lappaconitine gel spiked with IS (C).

times of lappaconitine and ketoconazole were 4.13 and 4.67 min, respectively. Chromatograms of the rabbit plasma administered with lappaconitine gel are shown in Figure 2C. The results show that under the chromatographic conditions the peaks of lappaconitine and ketoconazole were well separated with good symmetry, and no endogenous sources of interference were observed, indicating the method is specific for the analysis of lappaconitine.

#### Linear range

Rabbit plasma standard samples spiked with various concentrations of lappaconitine were prepared by adding 100  $\mu$ L lappaconitine standard solution into 900  $\mu$ L drug free plasma and mixed. The final lappaconitine, concentrations were 13.125, 26.250, 32.800, 65.625, 262.500, 525.000 and 1050.000 ng/mL. The samples were processed and analyzed. The ratios of the peak

area of lappaconitine to that of ketoconazole (Y axis) were plotted against the concentrations of lappaconitine (X axis). A linear regression analysis with the weighted least square method was performed. The resulting regression equation was  $Y=0.209X-1.26$  and the linear regression coefficient  $r=0.9959$ . The lappaconitine standard curve was linear in the range from 13.125 to 1050.0 ng/mL. Therefore, the lower limit of quantification was determined at 13.125 ng/mL.

#### Precision, accuracy, recovery

Three concentrations of lappaconitine (13.125, 65.625 and 525.00 ng/mL) were prepared and analyzed. Each concentration was analyzed five times in one day or in five consecutive days. Concentration of lappaconitine was calculated using the calibration curves prepared on the same day, and was compared with the nominal concentration to estimate the methodological recovery.

The precisions, accuracies, and recoveries were assessed, which are shown in Table 1. The data indicated that intra-day and inter-day precisions and accuracies were well within the requirement of relevant international practice (China Pharmacopeia, Part II, 2010).

### Limit of detection

A standard plasma sample containing 0.1 ng/mL lappaconitine was prepared and analyzed as described. A signal to noise ratio of 5 was obtained, indicating that 0.1 ng/mL lappaconitine was detectable. Therefore, the lower limit of detection of lappaconitine in rabbit plasma was 0.1 ng/mL, which is much lower than the lower limit of quantification.

### Matrix effect

Three concentrations of lappaconitine (13.125, 65.625 and 525.000 ng/mL) were prepared and each sample was analyzed five times and the peak area was recorded. Meanwhile solutions of lappaconitine standard (26.25, 131.25 and 1050.00 ng/mL) were prepared and directly analyzed. The matrix effects were assessed by comparing the peak area obtained from extracts of spiked plasma samples with the peak area obtained from the direct injection of known amounts of standard solutions of lappaconitine. They are 77.8, 84.4 and 79.0%, respectively, at three concentrations.

### Stability

Rabbit plasma samples containing lappaconitine at low, medium and high concentrations (13.125, 65.625 and 525.00 ng/mL) were prepared. The stability of lappaconitine in the plasma, under different conditions, was investigated. Lappaconitine was found to be stable after three freeze-thaw cycles, at 25°C for 24 h, and at -20°C for 60 days. The stability results are summarized in Table 2.

### Pharmacokinetic study of lappaconitine gel

The validated method was successfully applied to analyze the plasma concentration of lappaconitine at different times after external administration of lappaconitine gel. The maximum plasma concentration of lappaconitine was 189 ng/mL. The lower limit of quantification of this method was lower than 1/10 of  $C_{max}$ , which met the requirement of Chinese Pharmacopeia (version 2010). The averaged plasma concentration-time curve of the lappaconitine gel is shown in Figure 3. The concentration versus time curve was fitted using the

bimodal model of DAS ver1.0 software. The results show that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model, having a higher  $r^2$  (0.9994) compared with the two-compartment model (0.685). The calculated pharmacokinetic parameters are shown in Table 3.

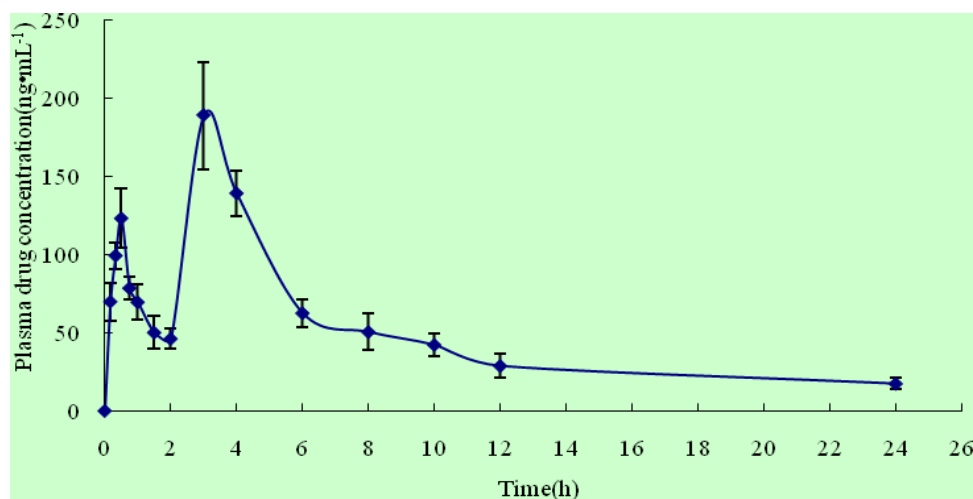
## DISCUSSION

The reports are less concerning the pharmacokinetic study of lappaconitine and mainly about *in vivo* metabolic analysis of its hydrobromide and similar alkaloids, which using intravenous injection or oral administration. It is the first study on pharmacokinetics of transdermal delivery of lappaconitine monomer that has never been reported at home and abroad.

Experiment indicated that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model after transdermal delivery, which was inconsistent with the literature reported that drug concentration-time data of lappaconitine hydrobromide in mouse plasma were fitted to a two-compartment open model by intravenous injection and oral administration (Guan et al., 2006; Shi et al., 2008). The reasons may be related to the drug form, route of administration and experimental animals. However, the specific reasons deserve further study.

Sample preparation played a key role in the development of this method. Several organic solvents were used to extract lappaconitine from the plasma, which included methanol, acetonitrile, ether, ethyl acetate, isopropyl alcohol, chloroform, dichloromethane, 1,2-dichloroethane, n-hexane, and mixed solvents like n-hexane-dichloromethane-isopropyl alcohol (300:150:15), ether-dichloromethane (2:1) and ethyl acetate-isopropyl alcohol (85:15). The results show chloroform, dichloromethane, 1, 2-dichloroethane and n-hexane-dichloromethane-isopropyl alcohol (300:150:15) extracted the drug efficiently, but they were prone to form emulsions, which led to varied recovery. When more hydrophobic solvent, like n-hexane, was used, the recovery is high and no emulsion was formed. We therefore chose n-hexane as the extraction solvent. We also found that basification of the plasma increased the extraction efficiency. The best result was obtained when the plasma was basified with 1 M NaOH solution.

The transdermal lappaconitine plasma concentration-time curve had shown double peaks, the first time to peak about 0.5 h, the second time about 3 h. There are three possible reasons. For one thing, Lappaconitine is an alkaloid with smaller solubility. It is possible that lappaconitine dissolves in the liquid of the surface of skin, some of them in the medicine are precipitated caused by pH change or solvent decrease. Over time, precipitated crystals re-dissolve and absorb again causing fluctua-



**Figure 3.** The averaged plasma concentration-time curve after administration of a single dose of lappaconitine gel.

**Table 1.** Intra-day and inter-day analysis of rabbit plasma samples containing different concentrations of lappaconitine (n=5).

Concentration (ng/mL)	Intra-day variation $\bar{X} \pm S$	Methodological recovery (%)	RSD (%)	Inter-day variation $\bar{X} \pm S$	Methodological recovery (%)	RSD (%)
13.125	13.4±0.563	102	5.42	14.3±1.21	109	7.51
65.625	63.8±2.21	97.2	3.16	63.2±3.37	96.4	5.03
525.00	484±26.7	92.3	4.53	472±32.2	90.0	5.87

**Table 2.** Stability of lappaconitine in the rabbit plasma samples.

Condition	Concentration (ng/mL)			RE (%)		
	Low	Medium	High	Low	Medium	High
Room temperature, 24 h	12.319	63.433	495.443	-6.14	-3.34	-5.63
	12.340	63.545	496.388	-5.98	-3.17	-5.45
	12.314	63.597	496.545	-6.18	-3.09	-5.42
Three freeze-thaw cycles	12.612	62.167	548.258	-3.91	-5.27	4.43
	12.579	62.160	546.263	-4.16	-5.28	4.05
	12.609	62.114	546.630	-3.93	-5.35	4.12
-20°C, 60 day	12.684	63.092	517.860	-3.36	-3.86	-1.36
	12.641	62.245	536.498	-3.69	-5.15	2.19
	13.200	62.718	538.913	0.57	-4.43	2.65

tions of blood concentration.

The difference of crystal size causing different dissolving and absorbing rate results in larger fluctuations of blood concentration. For another, the fat-soluble lappaconitine results in rapid distribution of drug into the

tissue after absorption. Second release of drug into the blood appears when it is metabolized. Therefore double peaks appear. Finally, the drug concentration increases because of blood volume in animals reducing caused by large number of blood losing.

**Table 3.** The pharmacokinetic parameters of lappaconitine gel (one-compartment model).

Parameter	$t_{1/2ka}$ (h)	$t_{1/2}$ (h)	Time of resorption (h)	CL (L/h/kg)	AUC <sub>0-12</sub> (ng/mL·h)	AUC <sub>0-∞</sub> (ng·mL <sup>-1</sup> ·h <sup>-1</sup> )	T <sub>max</sub> (h)		C <sub>max</sub> (ng/mL)		MRT <sub>0-∞</sub> (h)	VF (1/h)	Ka (1/h)	Ke (1/h)
							1	2	1	2				
$\bar{X}$	0.231	4.460	2.000	0.014	1208.602	1437.292	0.500	3.000	123.000	189.000	7.900	0.089	3.000	0.155

CL, Clearance; AUC, area under the curve; VF, ventricular fibrillation; MRT, mean residence time.

## CONCLUSIONS

In this paper, the elimination process of lappaconitine in the body of rabbit after transdermal administration of lappaconitine gel was researched. The results indicated the method we established was simple, selective, sensitive and could successfully used for the pharmacokinetic study of lappaconitine gel in rabbits.

To our knowledge, this is the first report on the drug plasma concentrations and pharmacokinetic parameters of lappaconitine monomer in rabbit treated with the therapeutic dose. Our method can be used for the in vivo study of transdermal absorption of other TCM and the results provide information for the development of lappaconitine and its analogs.

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Full Length Research Paper

## Development and pharmacokinetic evaluation of once-daily sustained-released matrix capsules of nifedipine using solid dispersion technique

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The purpose of this study was to develop once-daily sustained-release matrix capsules of nifedipine (F1) using the combination of solid dispersion and drug controlled release techniques. F1 were prepared by the wetting granules methods using hydroxypropyl methyl cellulose (HPMC) as hydrophilic retard drug release agent and ethylcellulose (EC) ethanol solution based on the polyvinyl pyrrolidone / stearic acid solid dispersion. *In vitro* drug release kinetic model of F1 was fitted well with the zero-order kinetic equation:  $Q=0.0736 t+0.0871$  ( $R=0.993$ ) in the range of 0-6 h, the first-order kinetic equation:  $\ln(1-Q)=-0.0934 t-0.1375$  ( $R=0.999$ ) in the range of 6-24 h, respectively. The relative bioavailability of F1 was studied in rabbits after oral administration using a commercial available controlled release tablet (F2) as a reference. The pharmacokinetic results showed no significant differences in  $C_{max}$ , MRT and AUC (0-24h). The relative oral bioavailability value from F1 in comparison with F2 was 97.12 %. The results of both *in vitro* and *in vivo* studies indicated that once-daily sustained-release matrix capsules of NFD prepared by the optimized formulation exhibited excellent sustained-release effects and high relative oral bioavailability.

**Key words:** Once-daily sustained-release matrix capsules of nifedipine, solid dispersion, pharmacokinetic behavior.

### INTRODUCTION

For the treatment of chronic diseases, such as hypertension and angina pectoris, the most suitable administration route is the oral route. Tablets and capsules are the most popular oral dosage forms available in the market and are preferred by physicians and patients. In long-term therapy of the treatment of hypertension, conventional formulations of the most antihypertensive drugs need to be administered twice or

three times a day, which result in marked blood pressure fluctuations. However, the formulations of drug sustained (controlled) release delivery systems are preferred for such therapy because of the advantages like reduced frequency of administration and fluctuation in plasma drug concentration, maintenance of stable blood pressure and improvement of patient compliance (Barakat and Almurshedi, 2011; Sousa de Silva et al., 2011).



Successful treatment of hypertension in clinical practice means maintenance of blood pressure at a normal physiological level. Therefore, the oral drug sustained (controlled) release delivery system for the treatment of hypertension is an ideal strategy.

Nifedipine (NFD), a well known dihydropyridine type Calcium channel blocker that inhibits entry of calcium ions through membrane into cardiac and vascular smooth muscle cells, is widely used in hypertension treatment (Iwai et al., 2011). Conventional oral formulations of NFD are required to be administered in multiple doses and therefore have several disadvantages because of its short elimination half-life (Lee et al., 1999; Li et al., 2009). For example, use of short-acting nifedipine was associated with increased risk of stroke occurrence in elderly hypertensive patients (Lee et al., 2011). To increase therapeutic index and reduce side effects, more attention has been focused on the development of NFD oral sustained (controlled) release delivery system. At present, the main oral dosage forms of NFD used in the treatment of hypertension include twice-daily sustained-release preparation and once-daily controlled-released tablets. Though once-daily controlled-release tablets of NFD as oral osmotic pump tablets produced in Bayer Schering Pharma AG have many advantages, such as zero-order drug delivery rate throughout the gastrointestinal tract, reducing risk of adverse reactions, a high degree of *in vitro* and *in vivo* correlation and improving patient compliance (Liu and Xu, 2008; Mehramizi et al., 2007), many patients in developing countries like China could not accept them due to its high cost- because of complex processing technology involved in their manufacturing. Therefore, the development of once-daily sustained-release dosage form of NFD is desirable.

According to the lower solubility of NFD, the combination of solid dispersion and drug controlled release techniques were employed to develop once-daily sustained-release matrix capsules of NFD for the first time. The formulation design and *in vitro* drug release evaluation were studied. *In vivo* study in rabbits was carried out to evaluate the oral NFD pharmacokinetic behavior from once-daily sustained-released matrix capsules (F1) developed by the optimized formulation in comparison with imported NFD once-daily controlled-release tablets (F2) used in clinic as the reference after oral administration.

## MATERIALS AND METHODS

### Materials

NFD was obtained from Chongqing Kerui Pharmaceuticals Com., Ltd. (Chongqing, P.R. China). Ethylcellulose (EC, 10cp), hydroxypropyl methyl cellulose (HPMC) were provided by Shanghai Coloron Coating Technology Com., Ltd. (Shanghai, P.R. China). Nifedipine controlled-released tablets (30mg) (batch: BJ06208) were purchased from the First Affiliated Hospital of Luzhou Medical

College (Sichuan, P.R. China). Polyvinyl pyrrolidone (PVP k-29/32) was a gift sample from ISP Technologies, Inc (USA). Microcrystalline cellulose (MCC, Avicel PH101) was donated by Japan Credit Rating Agency, Ltd. (Tokyo, Japan). Stearic acid was purchased from Sinopharm Chemical Reagent Co.Ltd. (Shanghai, P.R. China). Magnesium Stearate was purchased from Luzhou Juhe Chemical Reagent Co.Ltd. (Sichuan, P.R. China). Acetonitrile and methanol (HPLC grade), Ethanol and other reagents used throughout the study (analytical grade) were purchased from Luzhou Kelong Reagent Co.Ltd. (Sichuan, P.R. China).

### Preparation of sustained-release capsules

A solid dispersion of NFD was prepared by solution-evaporation method. NFD, PVP and Stearic acid with different mass ratio were dissolved in ethanol at 50°C. The organic solution was stirred using a half-moon paddle stirrer at 600 rpm to evaporate the solvent and the precipitate was dried at 40°C for 24 h to solid dispersion granules and stored in a sealed desiccators.

Sustained-release capsules of NFD were prepared by wet granulation technique. The solid dispersion granules of NFD obtained from the above procedure, HPMC and MCC were mixed well in a different mass ratio. EC ethanol solution (12% wt/vol) was chosen as bonding agent to prepare wet granulation using 24 mesh sieves. The granules were dried at 40°C for 3h, and then dry granules were mixed with magnesium stearate, which serves as lubricant, and passed through 20 mesh sieves. Based on the result of NFD content determination of semi-finished products, the sustained-release capsules of NFD (30 mg of NFD/capsule) containing granules of about 305 mg total weight were obtained by filling hard gelatin capsules (2 #).

### Evaluation of granules

#### Angle of repose

The angle of repose of granules was measured by the funnel method. In brief, the accurately weighed granules were placed in the funnel. The height of the funnel was adjusted in such a way that the tip of the funnel just touched the apex of the heap of the granules. The granules were allowed to flow through the funnel freely onto the surface. The diameter of the powder cone was determined and then the angle of repose was calculated according to equation (1).

$$\tan \theta = h/r \quad (1)$$

Where, h and r in (1) represent the height and radius of the powder cone.

#### Bulk density

The loose bulk density (LBD) and tapped bulk density (TBD) of granules were determined. A quantity of 3 g of powder was introduced into a 10 ml measuring cylinder followed by lightly shaking to prevent any agglomerates from forming. When the initial volume was observed, the cylinder was allowed to fall freely under its own weight onto a hard surface from the height of 2.5 cm at 2 s interval. The tapping was continued until no further change in volume was observed. LBD and TBD were calculated according to equation (2) and (3).

$$\text{LBD} = \text{weight of the powder} / \text{volume of the packing} \quad (2)$$

$$\text{TBD} = \text{weight of the powder} / \text{tapped volume of the packing} \quad (3)$$

**Table 1.** Factors and levels for orthogonal experiments.

Level	Experimental factor		
	The amount of NFD solid dispersions (g)	The amount of HPMC (g)	The concentration of EC (%)
	(A)	(B)	(C)
1	0.75	0.75	3.0
2	0.9	0.9	4.0
3	1.05	1.05	5.0

### X-ray powder diffraction

Solid dispersions and corresponding physical mixtures were analyzed using an X' D/MAX-2500/PC diffract meter (Rigaku Corporation, Tokyo, Japan) with a copper anode (Cu K $\alpha$  radiation,  $\lambda=0.15405$  nm, 40 kV, 40 mA). The diffraction behavior was measured between 10-90  $\theta$  at room temperature.

### Differential scanning calorimeter (DSC)

A differential scanning calorimeter (METTLER 1100LF RT-350, Switzerland) was used to investigate crystalline nature of the drug in the solid dispersions. About 3 mg of samples in an open aluminum standard pan was heated at a scanning rate of 10°C/min from a temperature to 350°C under an argon gas (99.999%) flow.

### Orthogonal experimental design

According to the results of the preliminary experiments, the L9 (3<sup>4</sup>) orthogonal array was used to optimize the formulation of sustained-release capsules of NFD. The orthogonal experiment with four factors and three levels are shown in Table 1. The *in vitro* drug release behavior was chosen as a main marker to investigate these formulations. For once-daily sustained-release dosage form of NFD, the desirable drug release percentages at 2, 12 and 24 h were 20, 70 and 90%, respectively. Q2, Q12 and Q24 were the cumulated release of sustained-release capsules of NFD developed in this study at 2, 12 and 24 h. Therefore, the equation of  $K=|20-Q2|+|70-Q12|+|90-Q24|$  was obtained. K represented comprehensive score. When smaller the value of K was, the percentages of the drug release from the experimental preparation were more similar with the desirable drug release values.

### *In vitro* release test

The basket method of the China Pharmacopoeia (2010 version) drug release test was used to study *in vitro* dissolution behavior of NFD. The release medium composed of 900 ml of 0.25% sodium dodecyl sulphate (SDS) with a stirring speed of 100 rpm was maintained at 37 $\pm$ 0.5°C. Five milliliter (5 ml) of samples was withdrawn at regular intervals and then the samples withdrawn were filtered through a 0.45  $\mu$ m hydrophilic Millipore membrane. The level of the drug release was determined by using a UV-visible spectrophotometer (UV-2102, Shanghai, P.R., China) at 238 nm. A 5 ml of blank release medium at 37 $\pm$ 0.5°C was added to the container after sampling to maintain a constant volume of the release medium.

### Pharmacokinetic study

Pharmacokinetics studies were performed using New Zealand albino rabbits weighing 2 $\pm$ 0.5 kg, which were purchased from the

Laboratory Animal Center of Luzhou Medical College. The use of rabbits in this study was approved by the Luzhou Medical College animal ethical experimentation committee (Sichuan, P.R. China). These rabbits were fasted for at least 12 h before the experiments, but had free access to water. Both once-daily sustained-released matrix capsules of NFD (F1) developed by the optimized formulation and imported NFD controlled-release tablets (F2) as the reference were orally administered to six rabbits at a dose of 30 mg/rabbit. Two milliliter (2 ml) of blood sample was withdrawn before and after oral administration. Each blood sample was centrifuged immediately at 3000 rpm for 5 min and plasma was separated and then stored at -20°C until analysis.

### HPLC analysis

The above biosamples were analyzed by HPLC method as previously described (Sawada et al., 2004). In brief, 50  $\mu$ L of nicardipine solution in methanol (10 $\mu$ g/mL) as internal standard were introduced in 10 ml tubes and dried at 45°C under a nitrogen steam. Then, the plasma samples (1 ml), 1ml of Na<sub>2</sub>HPO<sub>4</sub> (50  $\mu$ M) and 5 ml of the organic solvents composed of n-hexane and ethyl acetate (1:1, v/v) were successively added and vortex-mixed for 15 min. The mixture was centrifuged at 8000 rpm for 10 min. Then, the organic solvent was collected and evaporated to dryness at 45°C under a nitrogen steam. The residues were reconstituted in 200  $\mu$ L mobile phase and centrifuged at 10000 rpm for 10 min. Samples (20  $\mu$ L) injected into the HPLC system consisted of Dionex ultimate 3000 series including pump (LPG-3400SD), UV-vis detector (VWD-3100), auto injector (WPS-3000) and column oven (TCC-3000). Separation was performed on a reverse phase C18 column (Inertsil ODS-SP; 4.6 $\times$ 250 mm, 5 $\mu$ m particle size, made in Japan) with a guard column (Dionex C18, 4.3 mm $\times$ 10.0 mm) at 30°C and mobile phase consisted of acetonitrile - methanol - distilled water (15:55:30, v/v) at a flow rate of 1.0 ml/min. Chromatographic separation was monitored at 235 nm with UV detector. The following pharmacokinetic parameters were determined by using DAS 2.0 pharmacokinetics software: the area under the plasma drug concentration-time curve up to 24 h post-administration (AUC<sub>0-24 h</sub>), the time to reach the maximum plasma drug concentration (T<sub>max</sub>), the maximum plasma drug concentration (C<sub>max</sub>), the elimination half-life (T<sub>1/2</sub>) and the mean residence time (MRT).

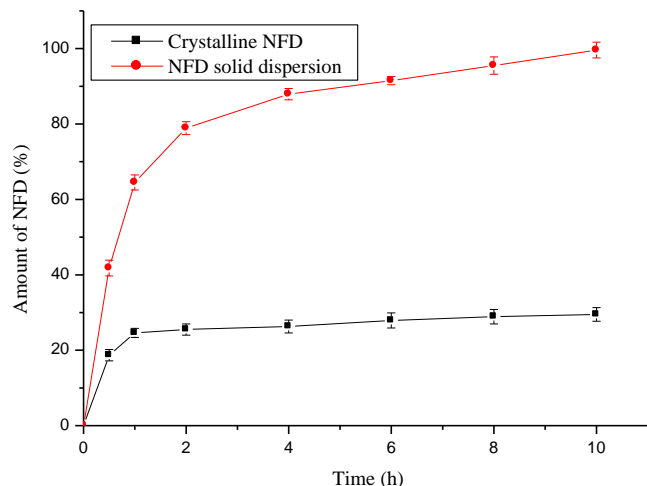
### Statistical analysis

Students't-tests were used to evaluate the significant differences between the pharmacokinetic data of F1 and F2. Values were reported as mean  $\pm$ SD and a significance level of less than 5% was considered as the significant difference.

## RESULTS AND DISCUSSION

### Preliminary experiments

Nowadays, NFD has been widely used as a



**Figure 1.** *In vitro* dissolution profiles of crystalline NFD and NFD-to-solid dispersion containing PVP and stearic acid (1:3 w/w) at a load of 20% in 0.25 % sodium dodecyl sulphate. (Mean±S.D., n=3).

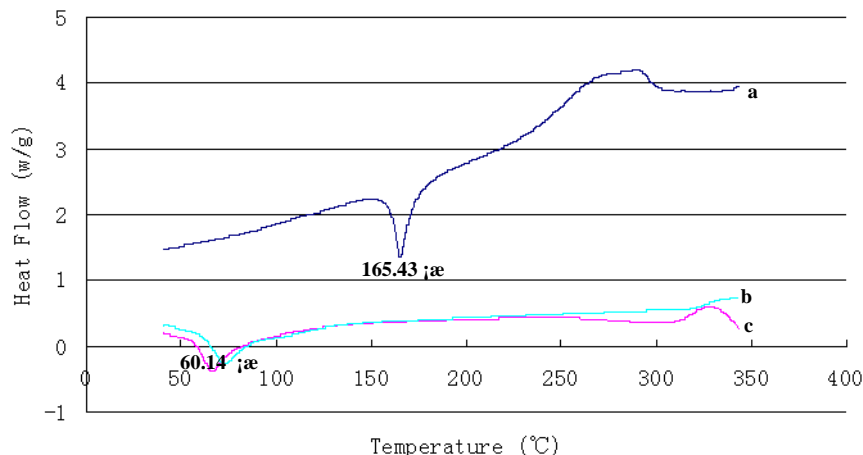
dihydropyridine type  $\text{Ca}^{2+}$  channel blocker for the treatment of cardiovascular diseases such as hypertension and angina pectoris. However, the highly crystalline and poorly soluble characterizations of NFD result in lower oral bioavailability (Sawada et al., 2004). Application of solid dispersions composed of hydrophilic carrier in which a lipophilic drug is incorporated is a proven technique to improve the poor water solubility of drug (Huang et al., 2011; Srinarong et al., 2012). Furthermore, many studies have been reported that the solid dispersion technique was used to enhance oral bioavailability of poor water soluble drugs (Boghra et al., 2011; Tran et al., 2011). Therefore, in order to solve the poor aqueous solubility problem NFD and improve the oral bioavailability and prolong the drug release, the combination of solid dispersion and drug controlled release techniques were used to develop once-daily sustained-release matrix capsules of NFD for the first time. In the preliminary experiments, the effect of solid dispersion components with PVP and stearic acid, the amount of HPMC and concentration of EC on the dissolution rate of NFD was investigated. On the basis of a series of preliminary experiments, it was found that, when the solid dispersions was composed of NFD (0.2 g), PVP (0.2 g) and stearic acid (0.6 g), the dissolution of NFD (20% w/w) in the solid dispersion was significantly greater than that of crystalline NFD (Figure 1).

Then further studies were conducted to confirm the physical state of the drug incorporated in solid dispersion by using differential scanning calorimetry (DSC) and X-ray powder diffraction (XRD). The results show that the thermograms of crystalline NFD, blank solid dispersions without NFD and solid dispersions at a drug load of 20% w/w are shown in Figure 2. The melting peak of NFD was about 165.43°C while the glass transition temperature of

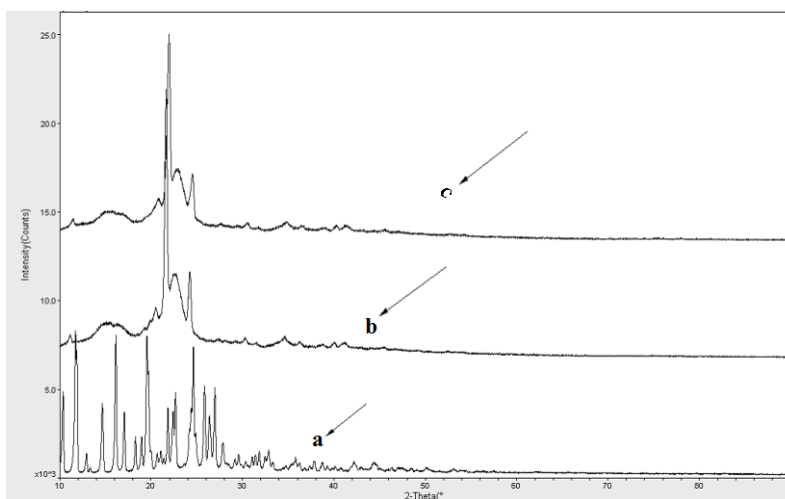
blank solid dispersions was 60.14°C. The thermograms of PVP and stearic acid-based solid dispersion with a drug load of 20% w/w did not show a melting peak of NFD, which indicated that the solid dispersions are most likely amorphous. The X-ray diffraction behaviors of crystalline NFD, blank solid dispersions without NFD and solid dispersion at a drug load of 20% w/w are shown in Figure 3. The results show that the characteristic diffraction peaks for crystalline NFD were not observed in the solid dispersions, indicating that the NFD in the solid dispersion was amorphous form. Therefore, the solid dispersions of NFD were used as the drug source for next experiments. HPMC is the most commonly and successfully employed drug release retarding agent for the preparation of oral controlled delivery systems (Colombo, 1993). The transport mechanism involved in the drug release from hydrophilic matrices is complicated since the macrostructure and microstructure of HPMC exposed to release medium is mainly time dependent. In general, the level of HPMC used as a rate-controlling polymer in capsules or tablets to retard the release of drug from a matrix ranged from 10 to 80% (Avachat and Kotwal, 2007). When NFD sustained release capsules were prepared by using mixture of the above solid dispersion and HPMC and ethanol as granulating agent, the effect of HPMC level on the release of NFD for capsules containing 10, 20 and 30% is shown in Figure 4. The results show that, when HPMC level is increased to 30%, prolonged release of NFD was achieved up to 22 h. Therefore, it was selected for further formulation development. In order to overcome initial burst release, the above formulation was modified by using different concentration of EC in ethanol (2, 4 and 6%) as granulating agents. In the case of EC concentration (2, 4 and 6%), NFD sustained release capsules released 23.5, 14.5 and 13.8% of the drug at the end of 0.5 h; 97.4, 97.8 and 78.9% of the drug at the end of 24 h, respectively (Figure 5). When the concentration of EC was increased to 4%, the preparations could control the drug release in a better manner, which could be contributed to the decreased penetration of the solvent molecules in the presence of hydrophobic polymer film, resulted in a lower diffusion rate of the drug from the matrix. However, when the concentration of EC was increased to 6%, the drug could not be completely released at the end of 24 h. Therefore, the concentration of EC (4%) as granulating agents was chosen to go for further study.

### Optimization of formulation

It was found that main pharmaceutical characterizations including angle of repose, bulk density of granules and weight variation and drug content of capsules except for *in vitro* release behavior were similar among all the preliminary experimental formulation. Therefore, the drug release behavior was chosen as the estimated marker to optimize the formulation of once-daily sustained-released



**Figure 2.** DSC thermograms of (a) crystalline NFD, (b) solid dispersion containing 20% w/w of NFD and (c) blank solid dispersion without NFD.



**Figure 3.** X-ray diffraction behaviors of crystalline NFD, blank solid dispersion without NFD and solid dispersion containing 20% w/w of NFD.

matrix capsules of NFD. The results of orthogonal experiments are shown in Table 2. According to the R-values, the order of the influence of above three main factors on *in vitro* release of NFD from the preparation was as follows: B>C>A. On the basis of the results of orthogonal experiments, the optimal formulation (F1) was composed of the amount of NFD solid dispersions (1.05 g) containing PVP / stearic acid weight ratio (1:3) at a drug load of 20%, HPMC (0.75 g) and the concentration of EC (4%).

In order to validate the optimal formulation and investigate reproduction, three batches (110506, 110507, and 110508) of once-daily sustained-release matrix capsules of NFD (30 mg) were prepared using the optimized formulation. The angle of repose of granules was  $23.24 \pm 0.02$ ,  $22.99 \pm 0.03$  and  $23.18 \pm 0.02$ , respec-

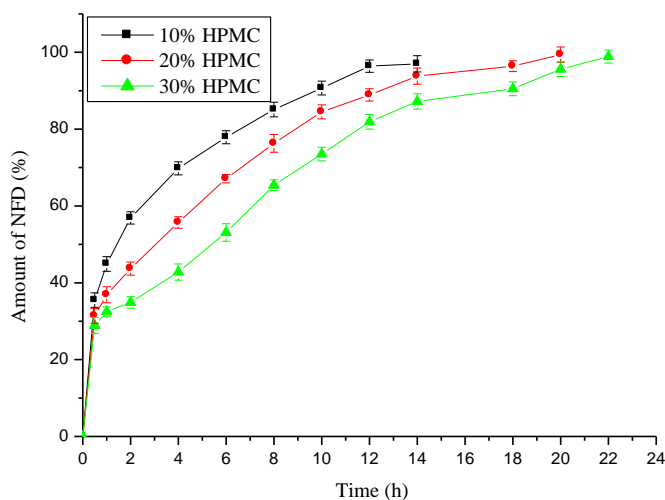
tively. The LBD and TBD values of granules were  $0.77 \pm 0.02$  and  $0.84 \pm 0.03$ ,  $0.75 \pm 0.02$  and  $0.83 \pm 0.02$ ,  $0.77 \pm 0.02$  and  $0.84 \pm 0.03$ , respectively. The content of these preparations was 10.03, 9.94 and 10.02%, respectively. Drug release profiles from the three batches developed in this study and NFD controlled release tablets (30mg) (batch: BJ06208) purchased from the First Affiliated Hospital of Luzhou Medical College are shown in Figure 6. The similar results of *in vitro* release rate of NFD from the three batches of once-daily sustained-release matrix capsules were observed. The above results showed excellent reproduction and validation for the optimized formulation. The mean release amount of NFD of the three batches at the end of 2, 12 and 24 h was 25.5, 72.2 and 90.5%, respectively. In the case of NFD controlled-release tablets, the release rate was

**Table 2.** The results of L9 (3<sup>4</sup>) orthogonal experiments.

S/N	The amount of NFD solid dispersions (g) (A)	The amount of HPMC(g) (B)	The concentration of EC (%) (C)	K
1	1 (0.75)	1 (0.75)	1 (3)	17.75
2	1 (0.75)	2 (0.9)	2 (4)	19.53
3	1 (0.75)	3 (1.05)	3 (5)	20.77
4	2 (0.9)	1 (0.75)	2 (4)	36.48
5	2 (0.9)	2 (0.9)	3 (5)	15.89
6	2 (0.9)	3 (1.05)	1 (3)	13.29
7	3 (1.05)	1 (0.75)	3 (5)	31.48
8	3 (1.05)	2 (0.9)	1 (3)	20.57
9	3 (1.05)	3 (1.05)	2 (4)	24.43
K1	19.35	29.16	17.20	
K2	21.89	18.67	26.81	
K3	25.49	19.50	22.71	
R	6.24	10.49	9.61	

**Table 3.** Pharmacokinetic parameters of NFD after oral administration of once-daily sustained-release matrix capsules of NFD (F1) developed in this study (closed squares) and once-daily NFD controlled release tablets (F2) as reference (closed circles) to rabbits at a dose of 30 mg/ rabbit. (n=6).

Parameter	Tmax (h)	Cmax µg/L	T1/2 (h)	MRT (h)	AUC(0-24h) µg/L*h
F1	4±0.8	38.7±3.2	13.3±1.7	9.7±0.8	572.6±57.8
F2	6±0.6	35.7±2.9	16.7±2.0	10.7±1.2	589.6±38.4

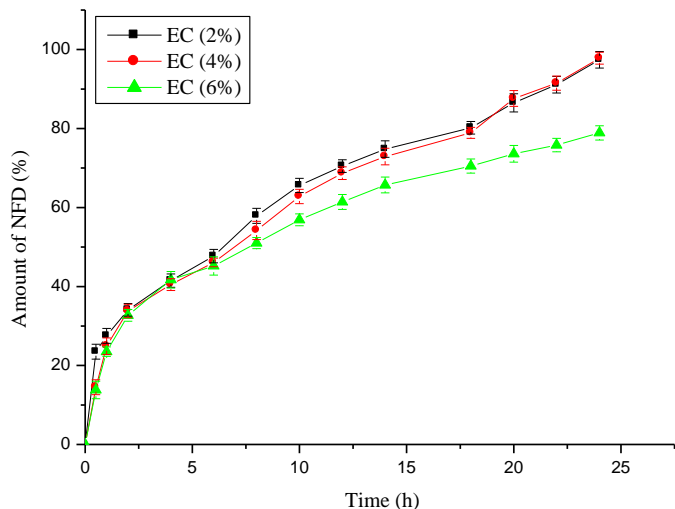
**Figure 4.** *In vitro* dissolution profiles of NFD sustained release capsules prepared by using mixture of above solid dispersion and HPMC (10%, 20% and 30% w/w) and ethanol as granulating agent in 0.25 % sodium dodecyl sulphate. (Mean±S.D., n=3).

observed in a steadier constant way without burst release behavior. On the other hand, it was found that *in vitro* drug release kinetic model of once-daily sustained-

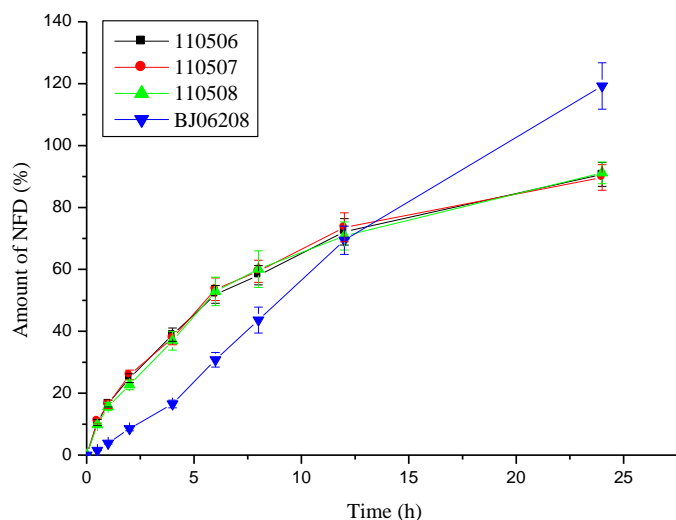
release matrix capsules of NFD was fitted well with the zero-order kinetic equation:  $Q=0.0736t+0.0871$  ( $R=0.993$ ) in the range of 0-6 h, the first-order kinetic equation:  $\ln(1-Q)=-0.0934t-0.1375$  ( $R=0.999$ ) in the range of 6-24 h, respectively. For NFD controlled-release tablets, the release rate during 24 h showed a zero-order kinetic model and could be expressed by the following equation:  $Q=5.1562t-0.3561$  ( $R=0.996$ ), indicating difference in the underlying drug release mechanism.

### *In vivo* pharmacokinetic characterization

The aim of *in vivo* study in rabbits was to evaluate the oral NFD pharmacokinetic behavior from once-daily sustained-release matrix capsules (F1) developed by the optimized formulation and once-daily NFD controlled-release tablets (F2) used in clinic as a reference after oral administration. The mean plasma drug concentration – time curve after a single oral dosage is shown in Figure 7. The pharmacokinetic parameters are summarized in Table 3. After oral administration of F1 and F2 to rabbits, no sharp peak of the plasma drug concentration was observed throughout the experiments, that is, the plasma drug concentration was maintained within a narrow range for a long period of time with the mean residence time

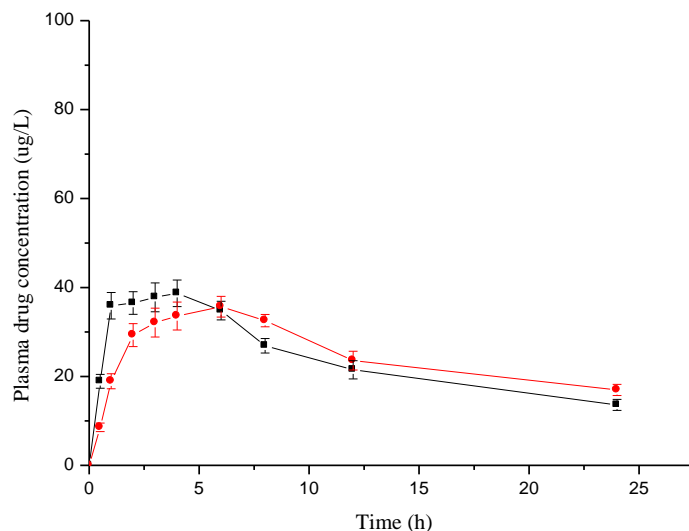


**Figure 5.** *In vitro* dissolution profiles of NFD sustained release capsules prepared by using mixture of above solid dispersion and HPMC (30% w/w) and EC ethanol solution (2%, 4% and 6% w/v) as granulating agent in 0.25 % sodium dodecyl sulphate. (Mean $\pm$ S.D., n=3).



**Figure 6.** Cumulative release of NFD from once-daily sustained-release matrix capsules (110506, 110507, 110508) developed in this study and once-daily controlled released tablets (BJ06208) as reference in 0.25 % sodium dodecyl sulphate (Mean $\pm$ S.D., n=3).

(MRT) values of  $9.7\pm 0.8$  and  $10.7\pm 1.2$  h, and the maximum plasma drug concentration ( $C_{max}$ ) values were  $38.7\pm 3.2$  and  $35.7\pm 2.9$  ng/L, respectively. The time to reach the maximum plasma drug concentration ( $T_{max}$ ) and elimination half-life ( $T_{1/2}$ ) of F1 and F2 was  $2, 4\pm 0.8$  h and  $6\pm 0.6$  h,  $13.3\pm 1.7$  and  $16.7\pm 2.0$  h, respectively. All the results confirmed the sustained-release characterization of NFD sustained release capsules developed in this study in the rabbit model. The AUC (0-24h) value for NFD formulations F1 and F2 was  $572.6\pm 57.8$  and



**Figure 7.** NFD plasma concentration-time curve after oral administration of once-daily sustained-release matrix capsules of NFD (F1) developed in this study (closed squares) and once-daily NFD controlled release tablets (F2) as reference (closed circles) to rabbits at a dose of 30 mg/ rabbit. Each point represents the mean $\pm$ S.D., n=6.

$589.6\pm 38.4\mu\text{g/L}\cdot\text{h}$ , respectively. When student's t-tests were used to analyze the two pharmacokinetic data, no significant differences in  $C_{max}$ , MRT and AUC (0-24h) were found ( $P>0.05$ ). The relative oral bioavailability value from F1 in comparison with F2 was 97.12%, indicating bioequivalence that means the rate and extent of absorption of F1 prepared in this study do not show a significant difference from the rate and extent of absorption of the reference drug F2 when administered at the same drug dose under similar experimental conditions (Toal et al., 2012).

## CONCLUSION

Once-daily sustained-release matrix capsules of NFD were successfully developed by the wetting granules methods using HPMC as hydrophilic retard drug release agent and EC ethanol solution as a bond agent based on the polyvinyl pyrrolidone / stearic acid solid dispersion. The results of both *in vitro* drug release test and *in vivo* pharmacokinetic investigation showed that once-daily sustained-release matrix capsules of NFD prepared by the optimized formulation exhibited excellent sustained-release effects and high relative oral bioavailability.

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## DECLARATION OF INTEREST

The authors participate in this study report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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Full Length Research Paper

## Synthesis of folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid for *in vivo* tumor cell labeling

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Iron (II, III) oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles modified with folic acid were synthesized and effectively mediated into the tumor cells through the binding of folate and folic acid receptor. This study made us know the method of synthesis of folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid and elucidate *in vivo* labeling effect of folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid on the hepatoma cells in tumor-bearing rats. Results showed that folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid could target and enter the hepatoma cells, enhancing the signal contrast of tumor tissue and surrounding normal tissue in magnetic resonance imaging (MRI). It was in favor of the tumor cells labeling, tracing and MRI target detection.

**Key words:** Nanoparticles, folic acid, tumor, synthesis, labeling.

### INTRODUCTION

Tumor cell membranes and Iron (II,III) oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles are both negatively charged, which affects tumor cell uptake of Fe<sub>3</sub>O<sub>4</sub> nanoparticles. The naked Fe<sub>3</sub>O<sub>4</sub> nanoparticles cannot effectively label tumor cells. To achieve an effective labeling of magnetic nanoparticles on tumor cells, the optimal method is to couple magnetic nanoparticles to specific targeting molecules which have high affinity with the target tumor cells (Weissleder et al., 1992; Veisheh et al., 2005; Huh et al., 2005). An increasing number of reports suggest many specific targeting molecular markers for the tumor cells, such as monoclonal antibodies (Cerdan et al., 1989; Bulte et al., 1992; Weissleder et al., 1991; Tiefenauer et al., 1993), proteins (Kresse et al., 1998) and peptides (Wunderbaldinger et al., 2002). However, these agents are expensive and antibody molecules are too big and hinder the crossing of the coupled magnetic nanoparticles through biological barriers (Jain et al., 1987; Mcneil et al., 2005).

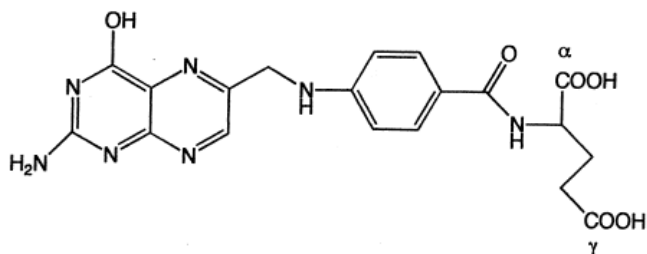
Folic acid is a small-molecule vitamin (Cezar et al.,

2007) that is essential for the human body, especially single carbon metabolism of eukaryotic cells and nucleoside synthesis. It is an important coenzyme in DNA synthesis and serves as a co-factor in the synthesis of several enzyme systems. Its molecular structure is shown in Figure 1. Compared with protein-targeting molecules such as antibodies, folic acid is stable, cost-effective and non-immunogenic. In addition, folic acid binds strongly to folic acid receptors through ligand-receptor binding. Therefore, folic acid can be efficiently mediated into tumor cells and may be useful in targeting these cells (Weitman et al., 1992; Ross et al., 1994).

The expression of the folate receptor is highly conserved in normal animal cells. This may be due to the lack of a key enzyme for folate biosynthesis in normal animal cells, so folate receptors on the cell surface actively uptake exogenous folate to maintain normal life activities. However, folate receptors are over-expressed in many malignant tumors, such as ovarian cancer, nasopharyngeal cancer, kidney cancer, breast cancer,

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**Figure 1.** Schematic diagram of folic acid structure.

endometrial cancer, testicular cancer, and liver cancer. Because folate receptors are over-expressed on the surface of malignant tumor cells, and folic acid has a high affinity and specificity with acid analogs, we can mediate folic acid endocytosis into tumor cells through specific binding with folic acid substances. Therefore, folic acid has high potential as a tumor-targeting molecule (Park et al., 2005; Sudimack et al., 2000; Zhang et al., 2002, 2004).

In this study, we coupled the 3-aminopropyltriethoxysilane (APTES)-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles to folic acid, and mediated the entrance of the folic acid-conjugated  $\text{Fe}_3\text{O}_4$  nanoparticles into hepatoma cells in tumor-bearing rats, in a broader attempt to elucidate the *in vivo* labeling effect of folic acid-modified  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid on the hematoma cells and MRI imaging.

## MATERIALS AND METHODS

### Chemicals

Ferric chloride hexahydrate (99.99%), ferrous sulfate heptahydrate (99.9%), sodium dodecyl benzene sulfonate (analytical reagent), folic acid (analytical reagent), dimethyl sulfoxide (analytical reagent) and toluene (analytical reagent) were provided by Beijing Chemical Reagent Company, China. APTES (analytical reagent), 1,3-dicyclohexylcarbodiimide (analytical reagent), N-hydroxysuccinimide (analytical reagent) and pentobarbital sodium imported packaging were all provided by Shanghai Chemical Reagent Company, China. RPMI1640 medium (Gibco, USA); human skin fibroblast cell line (from Dr. Yong Chen, Hunan Province Population and Family Planning Commission, China, as gifts); and male Wistar rats weighing 180 g were provided by the Experimental Animal Center, Central South University, China.

### Experimental

#### Synthesis of APTES-modified superparamagnetic $\text{Fe}_3\text{O}_4$ nanoparticles

Ultrapure water (25 ml) was fluxed with high-purity argon to remove oxygen, then stirred at high-speed with  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (2 mM) and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (4 mM). After this had dissolved completely, 15.8 ml dibutyl sebacate (DBS) solution (25 mM) and 75 ml xylene were added to the solution and stirred at high-speed for 10 min, then 1 M NaOH was added to adjust to pH 8 to 9 and the sample was stirred

for 30 min. The mixed solution was placed into the separatory funnel and rested for stratification. After the lower layer of water was removed, the upper layer of black liquor was transferred into three-necked flasks and fluxed with argon to remove oxygen. Then the water and part of the xylene in solution was streamed out through slow heating, refluxed for 30 min, and cooled to room temperature. An appropriate amount of APTES and acetone was added to the sample and it was subjected to ultrasound for 30 min, separated with external magnetic field and alternately rinsed with ultra-pure water and ethanol 4 to 5 times, then put through dialysis (the cutoff was 8000 Da) and dispersed in ultrapure water for a week to obtain APTES-modified  $\text{Fe}_3\text{O}_4$  nanoparticles.

#### Synthesis of folic acid-modified superparamagnetic $\text{Fe}_3\text{O}_4$ nanoparticles

Folic acid (0.3 mM) was dissolved in 10 ml dimethylsulphoxide (DMSO), subjected to ultrasound for 10 min, mixed with 0.3 mmol N-hydroxysuccinimide (NHS), 0.3 mmol 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), HCl and 5 ml APTES-modified  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid (containing 5 mg  $\text{Fe}_3\text{O}_4$  nanoparticles), subjected to ultrasound for 20 min, and stirred overnight. The mixed solution was separated with an external magnetic field, alternately rinsed with ultra-pure water and ethanol 4 to 5 times, then put through dialysis and dispersed in ultrapure water to obtain folic acid-modified  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid.

#### Characterization of folic acid-modified superparamagnetic $\text{Fe}_3\text{O}_4$ nanoparticles

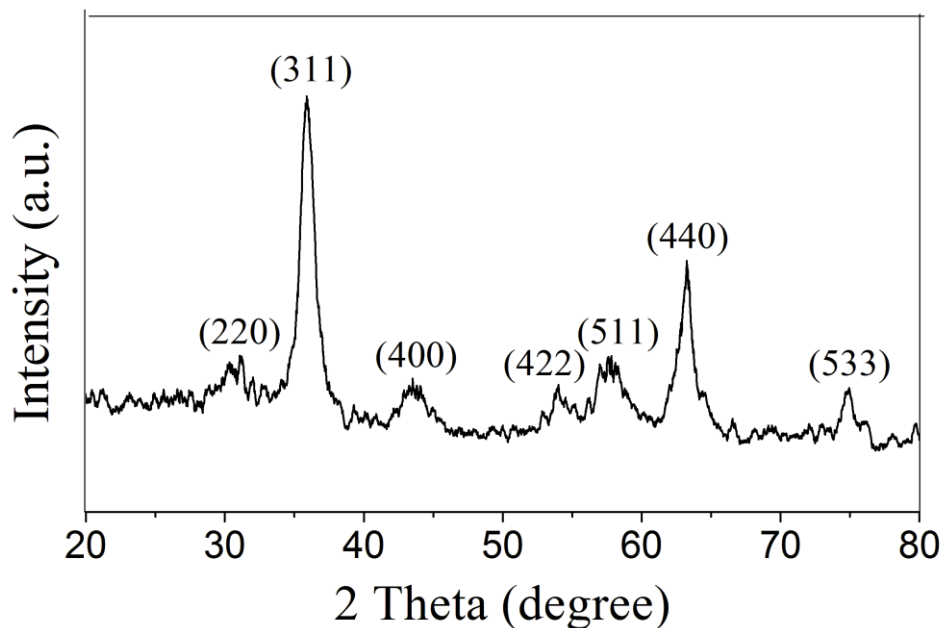
The structure of the sample was determined with a Bruker D8 X-ray diffraction meter. Scanning voltage was 40 kV. Scanning current was 40 mA, at 0.25°/min with sampling interval of 0.02°. Transmission electron microscopy (TEM) was done using JEM 3010 microscopes. Fourier transform infrared spectroscopy (FTIR) spectra was obtained using a WQF 410 FTIR spectrometer with a resolution of 4  $\text{cm}^{-1}$  and scans of 32. A small amount of nano-composite powder was milled with KBr, and the mixture was pressed into a disc for analysis. The magnetic measurement was measured using a Lake Shore 7310 VSM at 27°C. The dynamic light scattering characterization was measured with dynamic light scattering instrument HPPS-ET 5002 (Malvern Instruments, UK) using the He-Ne laser ( $\lambda = 632.8 \text{ nm}$ ). The data were collected using back-scattering mode at  $25 \pm 0.1^\circ\text{C}$ .

#### The preliminary safety assessment

Folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid was injected via the tail vein, Wistar rats were observed for 3 consecutive days for activities, death and damage to major organs after death. The acute side effects of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid were also observed to make a preliminary evaluation of its safety.

#### Sulforhodamine B (SRB) assay

Human skin fibroblast cell line at logarithmic growth phase were cultured with RPMI1640 complete culture medium containing 150 ml/L inactivated newborn calf serum to adjust cell density as  $1 \times 10^9/\text{L}$ , then added to the first line of wells in a 24-well plate, with 0.2 ml in each well. Cells were respectively incubated with folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid and saline. Each group contained 12 parallel wells and was revaccinated for 4



**Figure 2.** XRD patterns of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles

plates. After cells were adhered to 24-well plates and culture fluid was disposed of, 50  $\mu\text{l}$  of 500 ml/L trichloroacetic acid pre-cooled 4°C was gently added to each well to a final concentration of 100 ml/L, and the plate was kept at 4°C for 1 h. Cells were washed five times to remove water, trichloroacetic acid, culture medium, low molecular weight metabolites and serum protein. After air drying, cells were stained with 4 g/L SRB for 20 to 30 min, washed three times with acetic acid and dried, then dissolved in 200  $\mu\text{l}$  of 10 mmol/L Tris solution. The absorbance at 515 nm was determined with enzyme-linked immunosorbent assay.

#### ***In vivo* tumor cell labeling**

A human hepatocellular carcinoma subcutaneous tumor xenograft model was established in tumor-bearing rats. All procedures involving animal use conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the animal study protocol was approved by Institutional Animal Care Committee at the Beth Israel Deaconess Medical Center, Harvard Medical School. In brief, a human hepatoma Bel 7402 cell line (Experimental Animal Center of Central South University, China) was prepared into  $5 \times 10^7/\text{ml}$  cell suspension, and 0.2 ml of the suspension containing approximately  $1 \times 10^7$  living cells was collected using a syringe with a No. 7 gauge needle, and inoculated subcutaneously into the left foreleg of Wistar rats to establish a tumor-bearing mouse model. Tumor-bearing rats were housed in a standard super-clean environment for 1 to 2 weeks and used for hepatoma carcinoma cell *in vivo* labeling experiments when the subcutaneous tumor grew to 5  $\text{mm}^3$ . After tumor-bearing rats were anesthetized with 3% sodium pentobarbital, an MRI scan of their legs was performed to obtain plain scan unenhanced T2WI images. Then, the same tumor-bearing rat was injected with folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid via the tail vein, with an injection volume of 0.4 mg Fe/kg body weight. Forty minutes later, the same position was scanned with the same parameters to obtain an enhanced T2WI image and to compare the inspection of tumor.

#### **Statistical analysis**

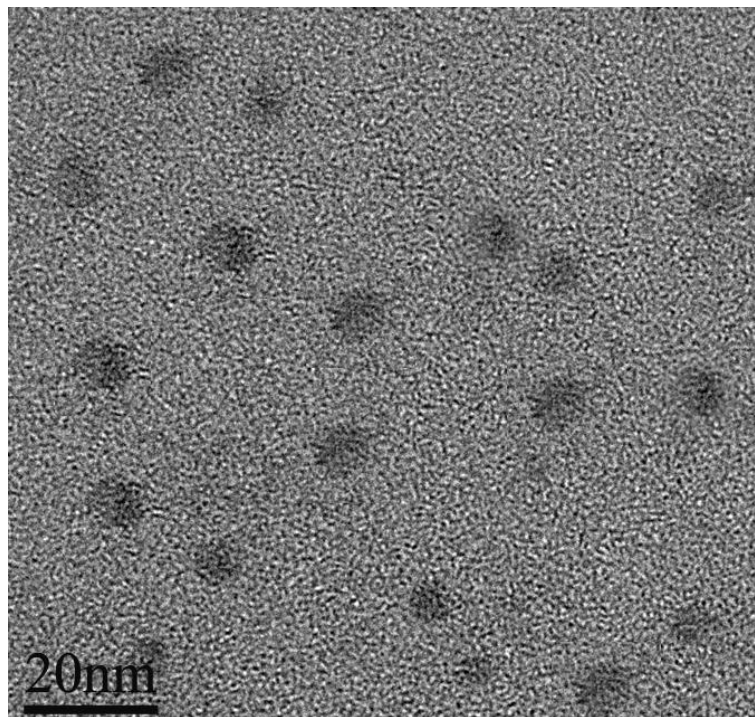
Measurement data are expressed as mean  $\pm$  standard deviation and were analyzed using statistical package for social sciences (SPSS 13.0 software). Differences among two groups were compared using analysis of random sample t test

## **RESULTS AND DISCUSSION**

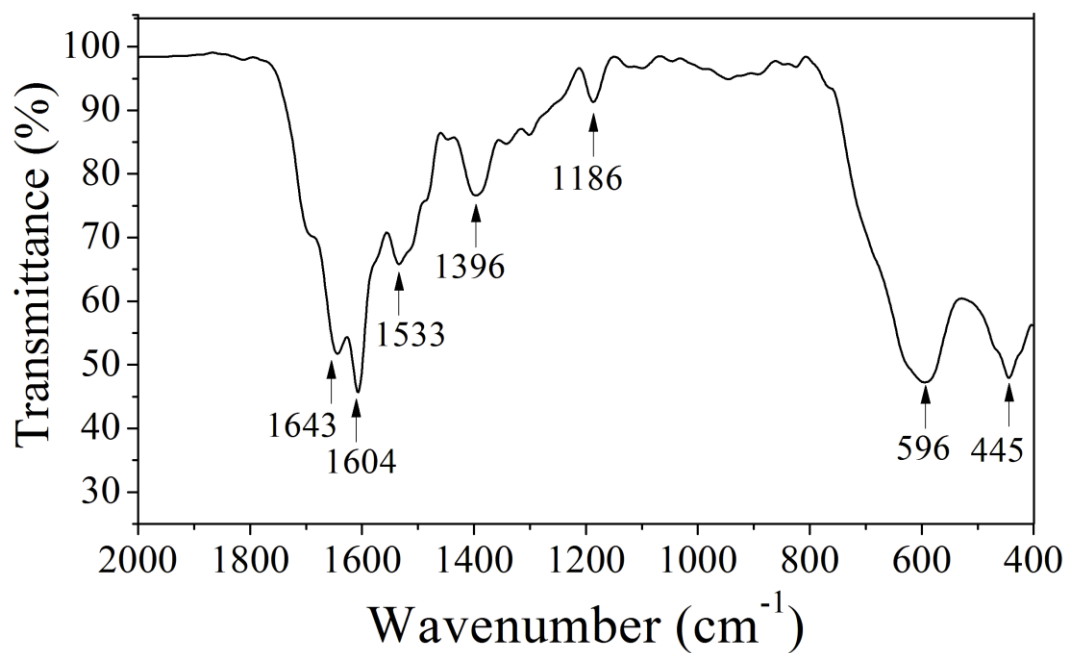
### **Folic acid-modified superparamagnetic $\text{Fe}_3\text{O}_4$ nanoparticles characterization**

The folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles were characterized with X-ray diffraction analysis as shown in Figure 2. There are seven diffraction peaks at  $2\theta$  value of 30.1, 35.4, 43.1, 53.2, 57.0, 62.7 and 74.0°, corresponding to the cubic phase of  $\text{Fe}_3\text{O}_4$  (220), (311), (400), (422), (511), (440) and (533) planes. The diffraction peak position and relative intensity are well matched with the standard card (JCPD No. 82-1533). There are no other impurity peaks observed, indicating that folic acid-modified  $\text{Fe}_3\text{O}_4$  nanoparticles have a cubic structure. The particle size is about 8 nm, estimated by the Scherrer formula.

Transmission electron microscopy image of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles is shown in Figure 3. Most of the nanoparticles are spherical and the average size of particles is initially estimated at 8 nm, which is consistent with the X-ray diffraction analysis. The surface groups of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles are characterized by FTIR (Figure 4). The absorption peak at 596 and 445  $\text{cm}^{-1}$  are attributed to Fe-O bond absorption.



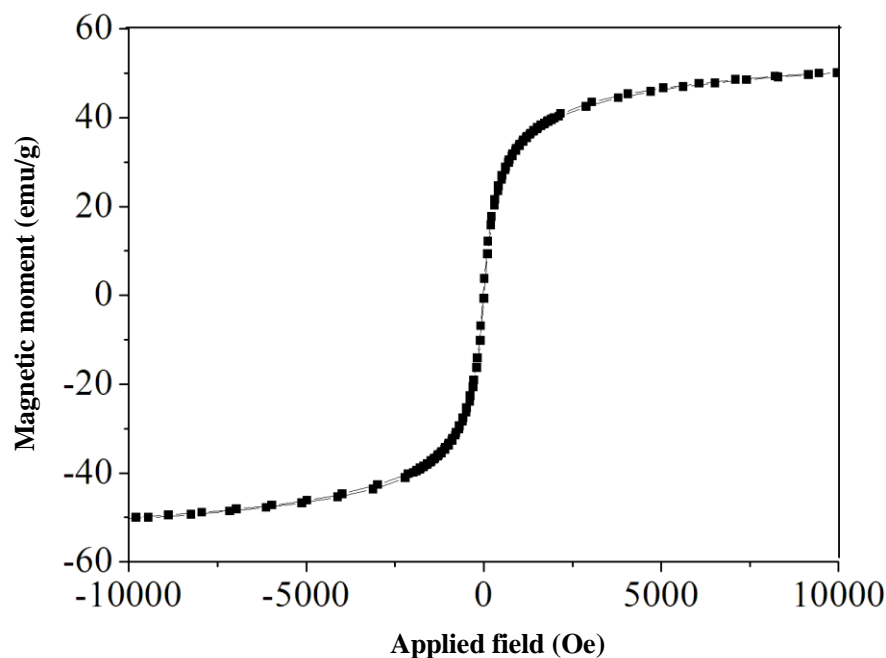
**Figure 3.** TEM image of folic acid- modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles.



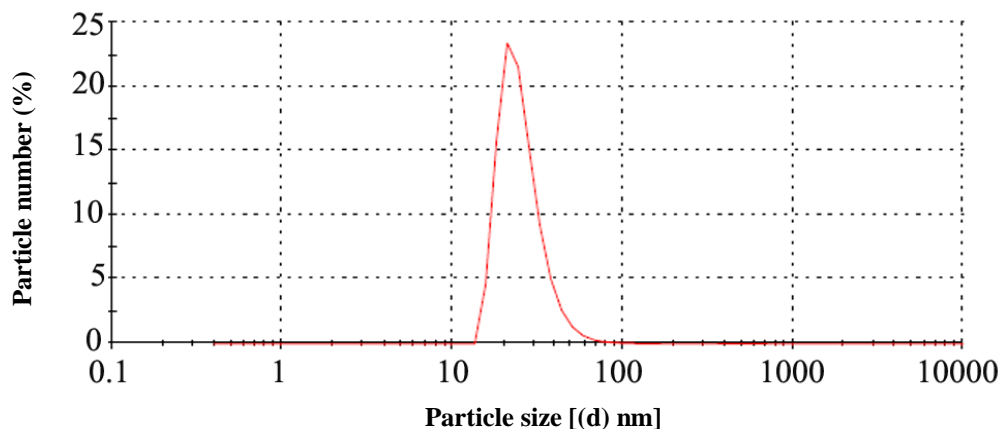
**Figure 4.** FTIR image of folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles.

Absorption at 1643 cm<sup>-1</sup> can be attributed to N-H bending vibration, which is also favorable evidence for the existence of primary amine in molecules. Absorption at 1604 cm<sup>-1</sup> corresponded to the aromatic ring stretching

vibration in the folic acid molecule, absorption at 1396 cm<sup>-1</sup> corresponded to the benzoic vibrations in folic acid molecule (Sun et al., 2006), and absorption at 1533 cm<sup>-1</sup> can be attributed to the characteristic absorption of



**Figure 5.** Magnetic hysteresis loop of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles.



**Figure 6.** The particle size distribution of superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles.

amide. Infrared data proved that folic acid molecules have successfully modified the surface of  $\text{Fe}_3\text{O}_4$  nanoparticles. Magnetic hysteresis loop of folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nanoparticles is shown in Figure 5. The hysteresis loop passes through the original point, and the coercivity and remanence are close to zero, indicating that folic acid-modified  $\text{Fe}_3\text{O}_4$  nanoparticles were superparamagnetic. Saturation magnetization is 51 emu/g. The particle size distribution of folic acid-modified  $\text{Fe}_3\text{O}_4$  nanoparticles is shown in Figure 6. Results show that the particle size distribution is

relatively narrow, with an average of 25.7 nm.

#### **Preliminary safety assessment of folic acid-modified superparamagnetic $\text{Fe}_3\text{O}_4$ nano-magnetic fluid**

The results of *in vivo* acute toxicity test of Wistar rats show that 3 days after folic acid-modified superparamagnetic  $\text{Fe}_3\text{O}_4$  nano-magnetic fluid was injected into tail vein of Wistar rats, there was no abnormal activities, no rat death, or no marked changes of major organs after

**Table 1.** Effect of folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid on the growth of human skin fibroblasts by SRB assay.

Group	Absorbance			
	Fe <sub>3</sub> O <sub>4</sub> group	1.64±0.12	1.35±0.25	1.47±0.27
Control group	1.57±0.23	1.51±0.14	1.51±0.27	1.68±0.24

Random sample *t*-test, *P* < 0.01, indicating no significant difference between Fe<sub>3</sub>O<sub>4</sub> group and control group.

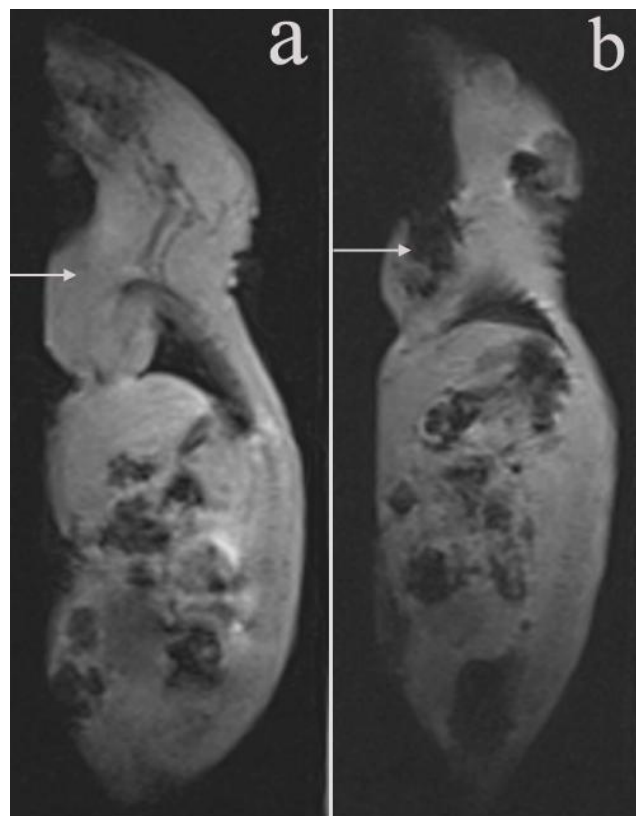
death. There was no significant difference between two groups.

#### Cell safety test of folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid by SRB assay

Cell safety test of folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid by SRB assay (Table 1) show that the folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid and superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluids have the similar influence on the growth on human skin fibroblasts as normal saline, they cannot modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluids significantly inhibit cell growth compared to normal saline. Statistical analysis showed no significant difference between the two groups. This is evidence that folic acid-modified superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluids have no significant toxicity on human skin fibroblasts.

#### Tumor cell labeling results

Figure 7 shows the unenhanced T2WI image of the leg of tumor-bearing rats (Figure 7a) and the enhanced T2WI image of the leg of tumor-bearing rats 40 min after injection of folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid (Figure 7b, arrows refer to the position of the tumor). In MRI unenhanced T2WI imaging of the leg of tumor-bearing rats, the MRI signals showed no significant differences between the tumor and surrounding normal tissue, and the tumors were hard to detect. However, the enhanced T2WI imaging displayed significantly more MRI signal contrast in the tumor and surrounding normal tissue, and the MRI scan could detect the presence of the tumor. This enhanced contrast occurred because Fe<sub>3</sub>O<sub>4</sub> nanoparticles modified by folic acid could bind to folate receptors that are highly expressed in tumor cells. The folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nanoparticles were then transported into the tumor cells and labeled as the tumor cells, thus the MRI T2 signals of the labeled tumor were significantly negatively enhanced. Accordingly, the imaging contrast between normal tissue and tumor tissue became apparent, and the labeled tumor was clearly visible. This is evidence that folic acid-modified Fe<sub>3</sub>O<sub>4</sub>



**Figure 7.** The unenhanced T2WI image of the leg of tumor-bearing rats (a) and the enhanced T2WI image of the leg of tumor-bearing rats at 40 min after injection of folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid (b). Arrows refer to the position of the tumor.

nanoparticles can be used for targeted labeling of human liver tumors in tumor bearing rats.

#### Conclusion

In summary, the folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nanoparticles are easy to prepare and effectively transduce into tumor cells and label tumor cells *in vivo*, which is conducive for tracing tumor cells. Folic acid-modified Fe<sub>3</sub>O<sub>4</sub> nano-magnetic fluid is a very promising substance for targeting tumor cells.

## ACKNOELEDGEMENTS

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Full Length Research Paper

## Hepatoprotective and antioxidant effect of corosolic acid on carbon tetrachloride induced hepatotoxicity

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The present study was designed to investigate the hepatoprotective and antioxidant properties of corosolic acid (CRA) on carbon tetrachloride (CCl<sub>4</sub>)-induced liver damage in rats. Liver damage was induced by giving a single oral dose of CCl<sub>4</sub> (1:1 in liquid paraffin) at 1.25 ml/kg body weight (BW). Rats were pretreated with CRA dose of 10, 20 and 40 mg/kg BW (once daily for 7 days before CCl<sub>4</sub> intoxication). Pretreatment with CRA showed significant hepatoprotection by reducing the aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) enzymatic activities which had been raised by CCl<sub>4</sub> administration. The levels of lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) were significantly increased by CCl<sub>4</sub> administration and pretreatment with CRA; the levels of lipid peroxidative markers were reduced. The activities of enzymic antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) and the levels of non enzymic antioxidants (Vitamins C, Vitamins E and reduced glutathione (GSH)) were decreased by CCl<sub>4</sub> administration and those pretreated with CRA above enzymic and non enzymic antioxidants were increased. The present study concluded that CRA possesses hepatoprotective and antioxidant properties against CCl<sub>4</sub>-induced hepatotoxicity in rats.

**Key words:** Hepatotoxicity, carbon tetrachloride (CCl<sub>4</sub>), corosolic acid, lipids peroxidations, antioxidant.

### INTRODUCTION

The liver is a vital organ present in vertebrates and some other animals. It has a wide range of functions such as drug metabolism, amino acid metabolism, lipid metabolism and glycolysis. Hepatotoxic chemicals cause the liver damages which are induced by lipid peroxidation and other oxidative damages (Muhtaseb et al., 2008; Appiah et al., 2009). Carbon tetrachloride (CCl<sub>4</sub>), a well-known model compound for producing chemical hepatic injury and it is biotransformed by hepatic microsomal cytochrome P450 (CYP) 2E1 to trichloromethyl-free radicals (CCl<sub>3</sub>• and/or CCl<sub>3</sub>OO•) (Brattin et al., 1985; Rechnagel and Glende, 1973; Rikans et al., 1994). Generally, these metabolites react with antioxidant enzymes such as glutathione (GSH) and catalase and superoxide dismutase (SOD) (Rikans et al., 1994). However, overproduction of trichloromethyl-free radicals is consi-

dered the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis (Basu, 2003; Brautbar and Williams, 2002; Weber et al., 2003; Williams and Burk, 1990).

Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for the treatment of liver disorders (Somasundaram et al., 2010). There is a great demand for the development of an efficient hepatoprotective drug from the natural resource (Tandon et al., 2008). Corosolic acid (CRA), a triterpenoids, which is isolated from *Actinidia valvata* Dunn and also has been discovered in many Chinese medicinal herbs, such as the *Lagerstroemia speciosa* L (Fukushima et al., 2006) and banana leaves (Yamaguchi et al., 2006). It has been

reported that CRA produces an excellent anti-diabetic activity in some animal experiments and clinical trials, including improvement of glucose metabolism by reducing insulin resistance in a mice model and lowering effect on post-challenge plasma glucose levels in human (Fukushima et al., 2006; Miura et al., 2006). It was also reported that CRA displayed some cytotoxic activities against several human cancer cell lines (Ahn et al., 1998; Yoshida et al., 2005). In the present study, we investigated the hepatoprotective and antioxidant properties of CRA used against CCl<sub>4</sub>-induced liver damage in rats.

## MATERIALS AND METHODS

### Chemicals

CCl<sub>4</sub> was purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. CRA was purchased from Mansite Pharmaceutical Co., Ltd (Chendu, China). All other chemicals used were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

### Experimental animals

Male albino Wistar rats (180 to 200 g) were housed in clean cages at a 20 to 24°C temperature, 12-h light/12-h dark cycle and 52% relative humidity in the animal house at the College of Medicine, King Saud University. Ethics approval was obtained from the ethics committee of the College of Medicine Research Center at King Saud University, Riyadh, Saudi Arabia (11/3215/IRB). The animals were given free access to water and received a standard pellet diet. Animals care was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH, 1985).

### CCl<sub>4</sub>-induced hepatotoxicity

Hepatotoxicity was induced a single oral dose of CCl<sub>4</sub> (1:1 in liquid paraffin) at 1.25 ml/kg BW at an interval of 6 h after the administration of last dose of CRA on the 7th day.

### Experimental design

#### Dose determination study

The animals were divided into six groups of six animals in each group. CRA was suspended in 0.1% dimethylsulfoxide (DMSO), and fed to rats via an oral route at 10, 20 and 40 mg/kg body weight (BW) for 7 days. Takagi et al. (2010) reported that CRA treated mice at 10 mg/kg BW inhibit hypercholesterolemia and hepatic steatosis caused by dietary cholesterol in KK-Ay mice. Hence these three different doses were fixed based on previous report. Then a single oral dose of CCl<sub>4</sub> (1:1 in liquid paraffin) at 1.25 ml/kg BW was given at an interval of 6 h after the administration of last dose of CRA. Group I served as control rats that received 0.1% DMSO only, Group II served as control rats treated with 40 mg/kg BW CRA. Group III was administered CCl<sub>4</sub> (negative control). Groups IV, V and VI were administered CRA at 10, 20 and 40 mg/kg BW and also administered CCl<sub>4</sub> at an interval of 6 h after the administration of last dose of CRA on the 7th day. Animals were sacrificed after 24 h of CCl<sub>4</sub> administration. Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min and used for estimated hepatic marker enzymes. Among the three different

doses 20 mg/kg BW showed the maximum activity as compared to other two doses. So, the 20 mg/kg BW was fixed as an optimum dose and used for further study.

### Experimental protocol for further study

The animals were divided into six groups of six animals in each group. CRA was suspended in 0.1% DMSO, and fed to rats via an oral route at 20 mg/kg BW for 7 days. Then a single oral dose of CCl<sub>4</sub> (1:1 in liquid paraffin) at 1.25 ml/kg BW (Saba et al., 2010) was given at an interval of 6 h after the administration of last dose of CRA. Group I served as control rats received 0.1% DMSO only, Group II served as control rats treated with 20 mg/kg BW CRA. Group III was administered CCl<sub>4</sub> (negative control). Groups IV was administered CRA at 20 mg/kg BW and also administered CCl<sub>4</sub> at an interval of 6 h after the administration of last dose of CRA on the 7th day. Animals were sacrificed after 24 h of CCl<sub>4</sub> administration. Blood sample was collected in tubes containing a mixture of ethylene diamine tetra acetic acid (EDTA) for the estimation of plasma lipid peroxidation and antioxidants. Tissue was sliced into pieces and homogenized in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate. The homogenate were centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

### Biochemical assays

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined in accordance with the method provided by Reitman and Frankel (1957) and King (1965a). The estimation of plasma and tissue thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) were done by the methods of Niehaus and Samuelson (1968) and Jiang et al. (1992), respectively. The activities of SOD, CAT and GPx in erythrocyte and tissue were measured by the methods of Kakkar et al. (1978), Sinha (1972) and Rotruck et al. (1973), respectively. The levels of vitamins C and E and GSH in plasma and tissue were estimated by the methods of Roe and Kuether (1943), Baker et al. (1980) and Ellman (1959), respectively.

### Statistical analysis

Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Statistical Package of Social Science (SPSS Inc., Chicago, IL, USA) 10.0 for Windows. Significance level was set at  $P < 0.05$ .

## RESULTS AND DISCUSSION

The present study demonstrates the hepatoprotective, curative and antioxidant effects of CRA against CCl<sub>4</sub>-induced liver injury in rats. Liver injury induced by CCl<sub>4</sub> is a common model for screening the hepatoprotective activity of drugs, because this chemical is a potent hepatotoxin and a single exposure can rapidly lead to severe hepatic necrosis and steatosis (Brautbar and Williams, 2002; Brent and Rumack, 1993; Manibusan et al., 2007). Table 1 shows the activities of hepatic function marker enzymes AST, ALT and ALP in the serum of control and CCl<sub>4</sub>-induced hepatotoxicity in rats. Pretreat-



**Table 1.** Effect of CRA on the activities of hepatic marker enzymes in the serum of CCl<sub>4</sub>-hepatotoxic rats.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	69.21 ± 4.11 <sup>a</sup>	36.13 ± 3.11 <sup>a</sup>	85.37 ± 6.69 <sup>a</sup>
Control + CRA (40 mg/kg BW)	70.36 ± 4.14 <sup>a</sup>	38.84 ± 2.71 <sup>a</sup>	86.39 ± 6.61 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	136.24 ± 10.51 <sup>b</sup>	108.16 ± 5.23 <sup>b</sup>	180.09 ± 12.18 <sup>b</sup>
CCl <sub>4</sub> + CRA (10 mg/kg BW)	106.68 ± 8.19 <sup>c</sup>	78.30 ± 5.39 <sup>c</sup>	136.23 ± 10.57 <sup>c</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	75.54 ± 5.51 <sup>a,d</sup>	42.16 ± 3.10 <sup>a,d</sup>	90.15 ± 6.20 <sup>a,d</sup>
CCl <sub>4</sub> + CRA (40 mg/kg BW)	92.39 ± 7.99 <sup>e</sup>	63.26 ± 4.66 <sup>e</sup>	120.24 ± 10.77 <sup>e</sup>

Values are expressed as means ± standard deviation (SD) for six rats in each group. Values not sharing a common superscript differ significantly at  $P < 0.05$  (DMRT).

**Table 2.** Effect of CRA on levels of TBARS and LOOH in plasma and liver of CCl<sub>4</sub>-hepatotoxic rats.

Group	TBARS		LOOH	
	Plasma (mmol/dl)	Liver (mmol/100 g wet tissue)	Plasma (mmol/dl)	Liver (mmol/100 g wet tissue)
Control	0.20 ± 0.02 <sup>a</sup>	0.86 ± 0.05 <sup>a</sup>	6.60 ± 0.58 <sup>a</sup>	80.66 ± 5.15 <sup>a</sup>
Control + CRA (20 mg/kg BW)	0.22 ± 0.02 <sup>a</sup>	0.83 ± 0.05 <sup>a</sup>	7.10 ± 0.66 <sup>a</sup>	81.15 ± 3.18 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	0.56 ± 0.05 <sup>b</sup>	2.35 ± 0.15 <sup>b</sup>	28.47 ± 2.11 <sup>b</sup>	187.21 ± 10.20 <sup>b</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	0.31 ± 0.03 <sup>c</sup>	0.94 ± 0.08 <sup>c</sup>	10.19 ± 1.08 <sup>c</sup>	94.52 ± 6.88 <sup>c</sup>

Values are expressed as means ± standard deviation (SD) for six rats in each group. Values not sharing a common superscript differ significantly at  $P < 0.05$  (DMRT).

ment with CRA showed significant hepatoprotection by reducing the AST, ALT and ALP enzymatic activities which had been raised by CCl<sub>4</sub> administration. Increases in serum AST, ALT and ALP levels by CCl<sub>4</sub> have been attributed to hepatic structural damage, because these enzymes are normally localized to the cytoplasm and are released into the circulation after cellular damage has occurred (Recknagel et al., 1989). The present study showed that pretreatment with CRA dramatically suppressed CCl<sub>4</sub>-induced hepatic injury. We used at three different doses such as 10, 20 and 40 mg/kg BW of CRA and estimated hepatic marker enzymes. Among the three different doses, 20 mg/kg BW showed maximum activity and plays an important role in protecting against CCl<sub>4</sub>-induced acute liver injury in rats. The lower dose of CRA (10 mg/kg BW) was not effective, because its concentration might not have been enough to counteract the CCl<sub>4</sub>-induced toxicity. The higher concentration of CRA (40 mg/kg BW) might have resulted in the production of by-products that are interfering with the hepatoprotective activity, and consequently, decreasing its effect. Hence, 20 mg/kg BW of CRA is optimum for hepatoprotective activity. Hence, further study used optimum dose of 20 mg/kg BW only. Restoration of increased hepatic serum enzyme levels to normal levels reflects protection against the hepatic damage caused by hepatotoxins (Vogel, 2002).

The levels of TBARS and LOOH, respectively in the plasma and liver of control and CCl<sub>4</sub>-induced hep-

totoxicity in rats are shown in Table 2. The levels of lipid peroxidation markers such as TBARS and LOOH were significantly increased by CCl<sub>4</sub> administration and pretreatment with CRA, reduced the levels of lipid peroxidative markers. Lipid peroxidations as well as altered levels of some endogenous scavengers are taken as indirect *in vivo* reliable indices for oxidative stress (Comporti, 1987). The levels of lipid peroxidation in the CCl<sub>4</sub> treated rats were assessed by measuring the TBARS and LOOH in the liver tissue (Ganie et al., 2011). The increased TBARS and LOOH levels in the liver of CCl<sub>4</sub> treated animals indicate enhanced lipid peroxidation leading to tissue injury. CRA significantly lowered the levels of TBARS and LOOH could be related to its antioxidant capacity to scavenge reactive oxygen species (ROS). This result demonstrates the antiperoxidative and antioxidant effects of CRA. Drugs with antioxidant properties may supply endogenous defense systems and reduce both initiation and propagation of ROS (Bergendi et al., 1865).

We further studied the *in vivo* antioxidant activity of CRA by estimation of erythrocytes and liver. The activities of enzymic antioxidants (SOD, CAT and GPx) and non enzymic antioxidants (Vitamins C, Vitamins E and GSH) in the erythrocyte, plasma and liver of control and CCl<sub>4</sub>-induced hepatotoxicity in rats are described in Table 3, 4, 5 and 6. The activities of enzymic antioxidants (SOD, CAT and GPx) and the levels of non enzymic antioxidants (Vitamins C, Vitamins E and GSH) were

**Table 3.** Effect of CRA on the activities of SOD, CAT and GPx in erythrocytes of CCl<sub>4</sub>-hepatotoxic rats.

Group	Erythrocyte (U/mg Hemoglobin)		
	SOD	CAT	GPx
Control	6.82 ± 0.48 <sup>a</sup>	180.11 ± 13.15 <sup>a</sup>	14.58 ± 1.22 <sup>a</sup>
Control + CRA (20 mg/kg BW)	7.08 ± 0.50 <sup>a</sup>	179.23 ± 13.12 <sup>a</sup>	14.97 ± 1.32 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	3.32 ± 0.24 <sup>b</sup>	105.65 ± 8.45 <sup>b</sup>	6.29 ± 0.48 <sup>b</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	5.92 ± 0.34 <sup>a,c</sup>	176.63 ± 10.31 <sup>a,c</sup>	13.21 ± 1.24 <sup>a,c</sup>

U- Enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition. U-μmole of H<sub>2</sub>O<sub>2</sub> consumed/min. U-μg of GSH utilized/min. Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

**Table 4.** Effect of CRA on the activities of SOD, CAT and GPx in liver of CCl<sub>4</sub>-hepatotoxic rats.

Group	Liver (U/mg protein)		
	SOD	CAT	GPx
Control	9.16 ± 0.72 <sup>a</sup>	85.32 ± 5.10 <sup>a</sup>	8.55 ± 0.56 <sup>a</sup>
Control + CRA (20 mg/kg BW)	8.96 ± 0.65 <sup>a</sup>	84.75 ± 4.86 <sup>a</sup>	8.23 ± 0.60 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	4.30 ± 0.35 <sup>b</sup>	39.09 ± 3.21 <sup>b</sup>	3.87 ± 0.34 <sup>b</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	8.31 ± 0.62 <sup>a</sup>	79.21 ± 4.99 <sup>a,c</sup>	7.22 ± 0.51 <sup>a,c</sup>

U- Enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition. U-μmole of H<sub>2</sub>O<sub>2</sub> consumed/min. U-μg of GSH utilized/min. Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

**Table 5.** Effect of CRA on the levels of GSH, vitamin C and vitamin E in plasma of CCl<sub>4</sub>-hepatotoxic rats.

Group	Plasma (mg/dl)		
	GSH	vitamin C	vitamin E
Control	32.96 ± 1.88 <sup>a</sup>	3.12 ± 0.24 <sup>a</sup>	1.82 ± 0.10 <sup>a</sup>
Control + CRA (20 mg/kg BW)	32.04 ± 2.30 <sup>a</sup>	2.98 ± 0.26 <sup>a</sup>	1.79 ± .15 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	16.24 ± 1.42 <sup>b</sup>	0.88 ± 0.05 <sup>b</sup>	0.48 ± 0.04 <sup>b</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	27.30 ± 2.45 <sup>a,c</sup>	2.48 ± 0.21 <sup>c</sup>	1.35 ± 0.3 <sup>c</sup>

Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

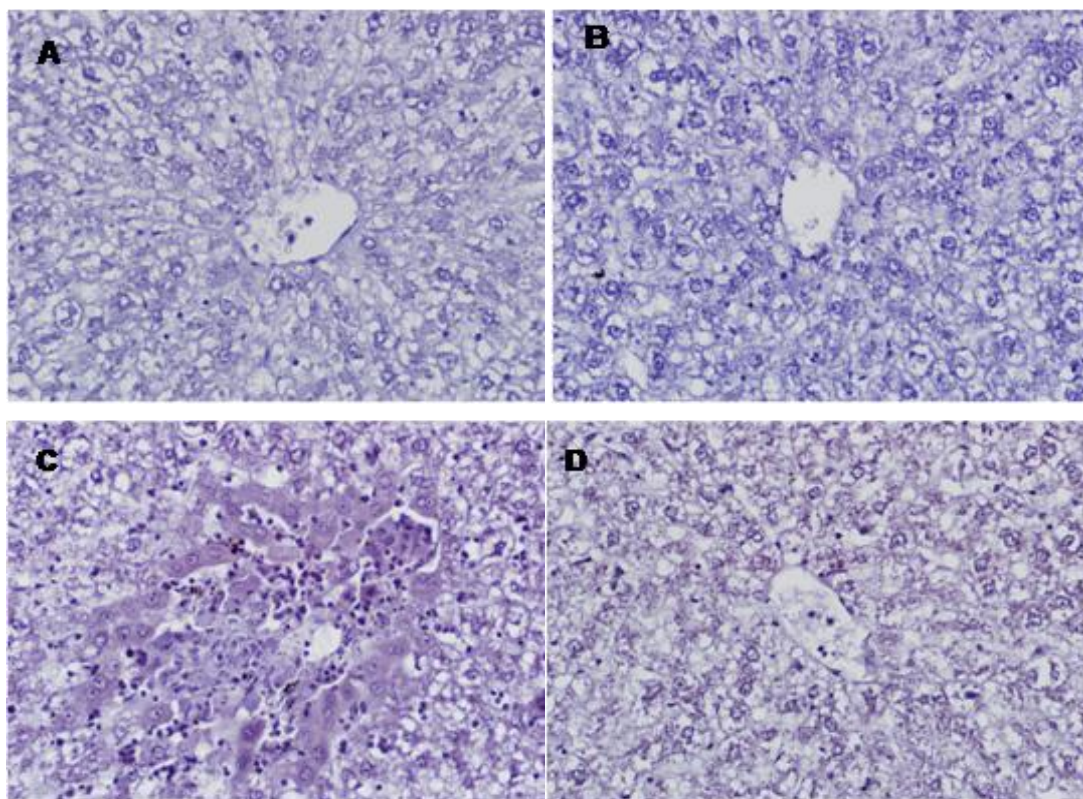
**Table 6.** Effect of CRA on the activities of GSH, vitamin C and vitamin E in liver of CCl<sub>4</sub>-hepatotoxic rats.

Group	Liver (mg/100 g wet tissue)		
	GSH	Vitamin C	vitamin E
Control	112.21 ± 10.45 <sup>a</sup>	0.86 ± 0.05 <sup>a</sup>	6.45 ± 0.51 <sup>a</sup>
Control + CRA (20 mg/kg BW)	111.19 ± 10.06 <sup>a</sup>	0.84 ± 0.05 <sup>a</sup>	6.32 ± 0.56 <sup>a</sup>
CCl <sub>4</sub> -hepatotoxicity (1.25 ml/kg BW)	61.56 ± 5.15 <sup>b</sup>	0.36 ± 0.03 <sup>b</sup>	2.62 ± 0.18 <sup>b</sup>
CCl <sub>4</sub> + CRA (20 mg/kg BW)	107.67 ± 8.11 <sup>a,c</sup>	0.72 ± 0.05 <sup>c</sup>	5.48 ± 0.42 <sup>a,c</sup>

Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

decreased by CCl<sub>4</sub> administration and those pretreated with CRA above enzymic and non enzymic antioxidants were increased. CCl<sub>4</sub> not only initiates lipid peroxidation,

but also reduces tissue SOD, CAT and GPx, activities, and this depletion may result from oxidative modification of these proteins (Augustyniak and Wazkilwicz, 2005).



**Figure 1.** Histopathological changes of liver (H&E, 40 $\times$ ). (A) Control rats showing normal hepatocytes; (B) Control rats treated with CRA showing normal hepatocytes and central vein; (C) CCl<sub>4</sub>-induced hepatotoxic rats showing degeneration of hepatocytes with nuclei pyknosis, increased vacuolation of cytoplasm and mononuclear cellular infiltration; (D) CCl<sub>4</sub>-induced hepatotoxic rats treated with CRA showing normal hepatocytes and central vein.

Cells have a number of mechanisms to defend themselves from the toxic effect of ROS including free radical scavengers and chain reaction terminators such as SOD, CAT, and GPx systems. SOD removes superoxide radicals by converting them into H<sub>2</sub>O<sub>2</sub> which can be rapidly converted into water by CAT and GPx. Cellular injury occurs when ROS generation exceeds the cellular capacity of removal (Wu et al., 2009). CRA administration effectively protected against the loss of these antioxidant activities after CCl<sub>4</sub> administration. Excessive liver damage and oxidative stress caused by CCl<sub>4</sub> depleted the levels of GSH, vitamin C and vitamin E in our study. Oxidative stress induced by CCl<sub>4</sub> results in the increased utilization of GSH and subsequently the levels of GSH is decreased in plasma and tissues. Utilization of vitamin E is increased when oxidative stress is induced by CCl<sub>4</sub> and this shows the protective role of vitamin E in mitigating the elevated oxidative stress. Vitamin C scavenges and destroys free radicals in combination with vitamin E and glutathione (George, 2003). It also functions cooperatively with vitamin E by regenerating tocopherol from the tocopheroxyl radical (Kaneto et al., 1999). A decrease in the levels of vitamin C may indicate increased oxidative stress and free radical formation in CCl<sub>4</sub>-induced liver

injury. CRA treatment effectively restored the depleted levels of these non enzymic antioxidants. In the present study, the elevation of GSH levels in plasma and tissues was observed in the CRA treated rats. This indicates that the CRA can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH or have both effects. Increase in GSH levels could also contribute to the recycling of other antioxidants such as vitamin E and vitamin C (Exner et al., 2000). The histological changes in the liver of control and CCl<sub>4</sub>-induced hepatotoxic rats are as shown in Figure 1. CCl<sub>4</sub>-induced hepatotoxic rats showed degeneration of hepatocytes with pyknosis of their nuclei, increased vacuolation of their cytoplasm and some mononuclear cellular infiltration was also seen in and around the damaged areas. Treatment with corosolic reduced these changes to near normalcy.

## Conclusions

The results of this study demonstrate that CRA has a potent hepatoprotective action upon CCl<sub>4</sub>-induced hepatic damage in rats. Our results show that the hepatoprotective

tive and antioxidant effects of CRA may be due to its antioxidant and free radical scavenging properties. The biochemical findings were supported by histopathological study.

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Full Length Research Paper

## Study on utilization and efficacy of commonly used anthelmintics against gastrointestinal nematodes in naturally infected sheep in North Gondar, North-Western Ethiopia

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Anthelmintic utilization and efficacy of commonly used anthelmintics against gastrointestinal nematodes in naturally infected sheep were assessed in North Gondar, North-Western Ethiopia. Anthelmintic utilization data were collected with a semi-structured questionnaire. An efficacy trial was conducted on 28 naturally infected sheep. The animals were randomly allocated into seven groups (four in each). Groups were treated with albendazole, ivermectin, tetramisole, levamisole, albendazole, ivermectin plus albendazole, albendazole plus levamisole, and a no-treatment control group. The faecal egg count reduction test (FECRT) was used to evaluate the efficacy of anthelmintics and identification of parasites was done by the faecal culture examination method. Data from the survey showed that different anthelmintics were used and some improper utilization was also recorded. Livestock owners had a tendency to deworm their animals throughout the year, but most commonly at the beginning of the rainy season. The highest FECRT (100%) was observed in animals receiving combined therapy, followed by albendazole (99.08%), ivermectin (96.69%), levamisole (90.06%) and the lowest reduction percentage was observed in the tetramisole group (89.51%). Parasite species surviving treatment were: albendazole, *Trichuris*; ivermectin, *Trichuris* and *Haemonchus*; levamisole and tetramisole, *Trichuris*, *Haemonchus* and *Oesophagostomum*. In summary, the different anthelmintics used in the study area did not have equal efficacy. Therefore, proper utilization and selection of anthelmintics are necessary for effective control of these parasites in Ethiopia.

**Key words:** Anthelmintics, efficacy, gastrointestinal nematodes, sheep, Ethiopia.

### INTRODUCTION

In Ethiopia, small ruminants are important sources of income for rural communities whose livelihood is largely based on livestock production (Abebe and Esayas, 2001; Biffa et al., 2006). However, sheep production in the country is hindered by many factors including animal health constraints, inadequate nutrition and poor

husbandry systems (Sissay et al., 2006). Studies in different parts of the country have shown that gastrointestinal nematodes are major problems in sheep production, causing mortality and production losses (Tembely et al., 1997; Abebe and Esayas, 2001; Biffa et al., 2006). Livestock owners mainly rely on the utilization

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of anthelmintics to control these parasites. Several anthelmintic preparations are imported and distributed in the country via legal or illegal agents without proper efficacy testing, control or registration. Furthermore, majority of anthelmintic preparations used today have been utilized for two or more decades. These conditions favour the development of resistance by the parasites (George et al., 2011). Thus, there is concern among professionals and livestock owners about the efficacy of anthelmintic products available in this region.

Anthelmintic resistance in sheep is a global problem (Kaplan and Vidyashankar, 2012; Chandrawathani et al., 1999; Sangster, 1999; Papadopoulos, 2008; George et al., 2011). In Ethiopia, it has been reported by Sissay et al. (2006) in Eastern, and Eguale et al. (2009) and Kumsa and Abebe (2009) in Southern part of the country. There is no published report on the utilization and efficacy of commonly used anthelmintics to control gastrointestinal nematodes of sheep in North Gondar. This study was designed to assess the pattern of anthelmintic utilization and evaluate the efficacy of commonly used anthelmintics against gastrointestinal nematodes of naturally infected sheep in North Gondar.

## MATERIALS AND METHODS

### Study area

This study was conducted from January to September, 2011 in North Gondar, Amhara regional state, North-Western Ethiopia. The area includes highland, midland and lowland agro-ecologies. These diverse agro-ecological zones are attributable to the high range of altitudinal differences, which varies from 4620 m at the Simen mountains in the North to 550 m in the West. The mean minimum and maximum temperatures vary with altitude. In the highlands, temperature varies from 11 to 32°C, whereas in the lowlands, temperature may reach 44.5°C especially in the month of April or May. Rainfall varies from 880 to 1772 mm. The area has two main seasons: the wet season from June to September when the area receives its major rainfall and the dry season from October to May with sparse and erratic rainfall. The humidity also varies with altitude. Many of the dwellers, both rural and urban, are involved in animal production and the area has an estimated sheep population of 524,087 (CSA, 2009).

### Questionnaire survey

A questionnaire-based survey was conducted in three districts which represent highland, midland and lowland areas of the zone. From these districts, data were collected from 15 randomly selected governmental veterinary clinics and 12 private veterinary pharmacies. Ninety livestock owners (farmers) having 5 to 25 animals randomly selected and included in the study. Veterinary professionals in the selected districts and owners or workers of private veterinary pharmacies were informed about the purpose of the study and data collection was started after getting their permission and co-operation. Farmers and veterinary professionals were asked to fill the questionnaire about the pattern of anthelmintic use including details of the estimated number of doses used per year, the estimated cost of anthelmintics used annually and the names of products that they usually used.

### Study animals and experimental design

Twenty-eight sheep of the same sex (male), breed (local) and age (about 6 months) were purchased from the market at Gondar town. The sheep were brought to the Faculty of Veterinary Medicine, University of Gondar. The animals were then acclimatized for one month and allowed to graze in parasite-contaminated pasture. Each animal was identified by ear tag. The animals were randomly allocated into seven groups (four in each). The first group was treated with albendazole, the second with ivermectin, the third with tetramisole, the fourth with levamisole, the fifth with albendazole plus ivermectin, the sixth with albendazole plus levamisole and the last group was left untreated (control). All anthelmintics were purchased from private veterinary pharmacies in and around Gondar town. Each animal was weighed by automatic balance for dose calculation. Dose rate and route of administration were based on manufactures' recommendation and anthelmintics were chosen based on the frequency of utilization in the study area (Table 1).

### Faecal sample collection and examination

Faecal samples were collected from each experimental sheep for coproscopic examination by using pre-labelled universal bottles on day 0 (before treatment) and again on day 10 post-treatment (Coles et al., 1992). Samples were examined for parasite eggs using saturated salt solution as a flotation fluid in the Veterinary Parasitology Laboratory. Faecal eggs per gram (EPG) of strongyle-type nematodes were determined for each sample using the modified McMaster technique according to Coles et al. (1992). The efficacy of the drugs was assessed by the percentage reduction of mean egg excretion on the 10th day of post-treatment, following the methods described by Kochapakdee et al. (1995) and Arece et al. (2004).

### Larval identification

About 10 g faecal samples from each sheep were collected before and after treatment (11 days). Samples were finely disrupted using a mortar and pestle, a small amount of water was added to moisten, and the samples left for 14 days at room temperature in a Petri dish (Waghorn et al., 2006), adding small amounts of water as necessary. Third stage, larvae (L<sub>3</sub>) were collected using the Baerman technique as described by Urquhart et al. (2003). L<sub>3</sub> were identified based on the morphological keys given by MAFF (1977) and van Wyk et al. (2004).

### Data analysis

Data were recorded in Excel spreadsheet and descriptive statistics (means, standard error and percentages) were calculated. Post-treatment faecal egg counts (FEG) were transformed to the natural logarithm (count plus one) and means were compared among groups through analysis of variance (ANOVA) and difference between treatments was compared using least square method of multiple comparisons. All data were analyzed using Statistical Package for Social Sciences (SPSS) version 17 statistical software. Probability (P) value less than 0.05 was used to determine the level of significance.

## RESULTS

### Questionnaire survey

The survey indicated that the most commonly utilized anthelmintics were albendazole, followed by ivermectin,

**Table 1.** Detail information about anthelmintics used in the trial.

Generic name	Trade name	Manufacturer	Formulation, route of administration	Recommended dose and route	Batch /Lot number	Manufactured date	Expired date
Albendazole	Albenda-QK 300 mg	Chengdu Quiankun Veterinary Pharamaceutical Co. Ltd. China	Bolus, oral	7.5 mg/kg oral	B11905	09/12/08	09/11/11
Ivermectin	Ivermectin 1% solution	Shandong Jinyang Biological Pharmaceutical Co. Ltd. China	Injectable solution, subcutaneous	0.5 ml/25 kg subcutaneous	09YW0706	12/07/09	11/07/12
Levamisole	Terazole Y	Ashish life science Pvt. Ltd, India	Bolus, oral	7.5 mg/kg oral	ALT/1288	06/2008	05/2012
Tetramisole	Doxanish sheep	Ashish life science Pvt. Ltd, India	Bolus, oral	15 mg/kg	ALT/2218	02/2010	01/2014

**Table 2.** The most commonly utilized anthelmintics to deworm sheep in North Gondar.

Generic name	Trade name	Manufacturer	Composition/Formulation
Albendazole	Albenda-QK	Chengdu Qiankun Veterinary Pharmaceuticals Co., Ltd., China	Albendazole 300 mg bolus
	Ashialben 600	Ashish Life Science Pvt. Ltd, India	Albendazole 600 mg bolus
	Alzole®	East Africa Pharmaceuticals, Ethiopia	Albendazole 300 mg bolus
	Albendazole	Star laboratories, Pakistan	Albendazole 300 mg bolus
Tetramisole	Ashitetra	Ashish Life Science Pvt Ltd, India	Tetramisole HCl bolus
	Doxanish	Ashish Life Science Pvt Ltd, India	150 mg Tetramisole HCl bolus
	Tetsole®	East Africa Pharmaceuticals, Ethiopia	Tetramisole HCl bolus
Levamisole	Terazole R	Ashish Life Science Pvt Ltd, India	Levamisole HCl 300 mg bolus
	Terazole Y	Ashish Life Science Pvt Ltd, India	Levamisole HCl 200 mg
Ivermectin	Ivermic	Laboratorios Microsules Uruguay S.A	Ivermectin 1% injectable solution
	Ivermectin	Hebei Yuanzheng Pharmaceuticals, China	Ivermectin 1% injectable solution

tetramisole, and levamisole, respectively. Several brands were utilized, but Chinese and Indian brands predominated (Table 2). These anthelmintics were sold by private veterinary pharmacies, governmental veterinary clinics and paravets. Respondents said that drugs were also sold in supermarkets and ordinary shops (34,29.06%), in human pharmacies (12,10.26%), with agricultural pesticides (11,9.40%) and open markets (7,5.98%). Livestock owners went to these places and bought the type and amount of drug(s) they wanted. Of the 90 interviewed farmers, 57 (63.33%) had anthelmintics in their possession during the time of interview. Most farmers (44.44%) dewormed their animals twice a year (Table 3) and spent about 51.67 Ethiopian birr (equivalent to 3 US dollars) on average per year to purchase anthelmintics. They have a tendency to

**Table 3.** Response of livestock owners on their deworming practices (frequency per year, number and percent of respondents).

Frequency per year	No. of respondents	Percentage
None	3	3.33
One	11	12.22
Two	40	44.44
Three	12	13.33
Four	24	26.67

deworm throughout the year but most commonly at the beginning of rainy season, followed by end of rainy season and during rainy season. Fewer anthelmintics

**Table 4.** Mean faecal egg count and percent reduction after treatment of sheep using different anthelmintics or their combinations.

Anthelmintic	Mean FEC $\pm$ SEM		Reduction (%)			95% confidence interval	
	On the day of treatment	After 10 days post treatment	Mean $\pm$ SEM	Minimum	Maximum	Lower	Upper
Albendazole	1362.50 $\pm$ 338.04	12.50 $\pm$ 12.50	99.08 $\pm$ 0.69	97.22	100.00	97.95	100.00
Ivermectin	1887.50 $\pm$ 280.09	62.50 $\pm$ 31.46	96.69 $\pm$ 1.54	92.68	100.00	93.42	99.46
Tetramisole	2025.00 $\pm$ 612.88	212.50 $\pm$ 85.09	89.51 $\pm$ 6.14	74.29	95.45	73.95	98.01
Levamisole	2012.50 $\pm$ 333.15	200.00 $\pm$ 54.01	90.06 $\pm$ 2.17	85.71	97.14	86.29	94.77
Albendazole and ivermectin	1487.50 $\pm$ 383.72	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00	100.00	100.00	100.00
Albendazole and levamisole	1962.50 $\pm$ 134.44	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00	100.00	100.00	100.00
Untreated control	1687.50 $\pm$ 177.92	1950.00 $\pm$ 235.41	NA	NA	NA	NA	NA

FEC: Faecal egg count; SEM: standard error of the mean; NA: not applicable.

**Table 5.** Survivor parasites after treatment with different anthelmintics.

Group	Anthelmintics	Survived parasite
1	Albendazole	<i>Trichuris</i>
2	Ivermectin	<i>Trichuris, Haemonchus</i>
3	Tetramisole	<i>Trichuris, Haemonchus, Oesophagostomun</i>
4	Levamisole	<i>Trichuris, Haemonchus, Oesophagostomun</i>
5	Albendazole and Ivermectin	none
6	Albendazole and Levamisole	none
7	Untreated control	<i>Haemonchus, Cooperia, Chabertia, Trichuris, Oesophagostomun</i>

were utilized during the dry season (Figure 1). Livestock owners also responded that deworming was needed if the animal showed decreased appetite (66.67%), loss of body condition and emaciation (56.67%), diarrhoea (20.00%), coughing (23.33%) and finally if there was sneezing and/or nasal discharge (13.33%). None of the farmers interviewed weighed their animals to calculate the dose. Simple guessing and information obtained from the professional were the basis for determining the dose.

#### Pre-treatment faecal examinations and larval identification

Faecal examination for the presence of parasite eggs in the pre-treatment faecal samples revealed that all sheep (100%) were positive for strongyle eggs. Other gastrointestinal parasites, including *Trichuris* species (28.57%) and *Nematodirus* species (17.85%), were also observed. *Haemonchus* was the dominant parasite identified after larval culture, occurring in all experimental sheep.

#### Faecal egg count reduction test (FECRT)

The results of the FECRT for each anthelmintic are shown in Table 4. No parasite egg was recorded on the 10th day of post-treatment in groups receiving anthelmintic combinations. Considerable numbers of eggs were

found in groups treated with levamisole and tetramisole.

There was a significant difference ( $P < 0.05$ ) in mean FEC among the groups of sheep treated with different anthelmintics. Pair wise comparisons revealed that there were no significant ( $P > 0.05$ ) differences among the albendazole, ivermectin and anthelmintic combination groups, whereas post treatment FEC were significantly ( $P < 0.05$ ) lower in the albendazole, ivermectin and anthelmintic combination groups than in the tetramisole and levamisole groups.

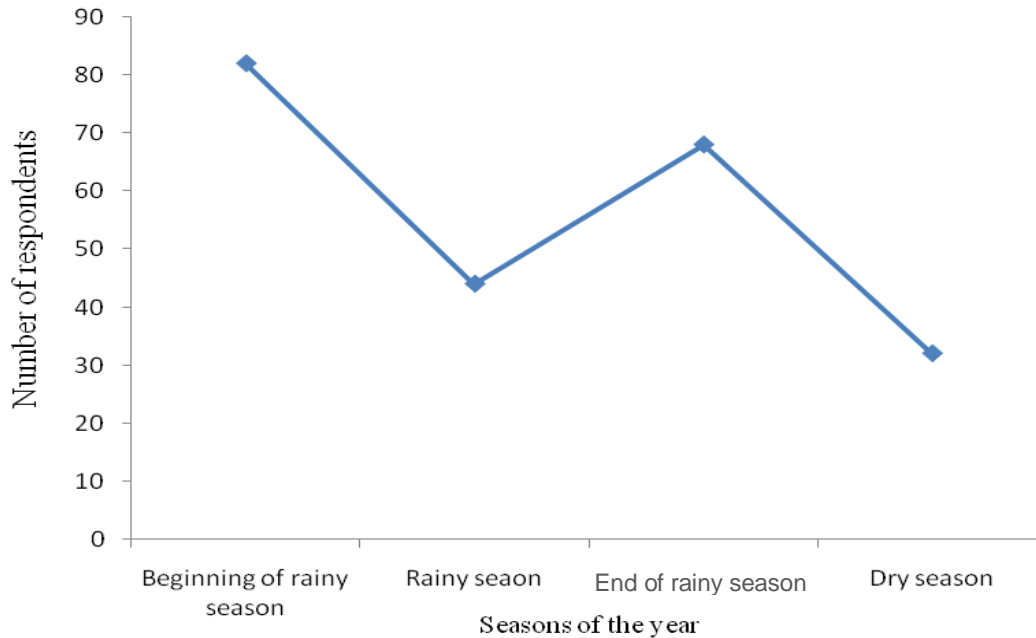
#### Post treatment parasite identification

The types of gastrointestinal nematode parasites that survived in each group after treatment are listed in Table 5. No parasite was recovered in groups receiving combined anthelmintics. Only *Trichuris* was found in faecal samples of animals treated with albendazole. The most pathogenic gastrointestinal nematode (*Haemonchus*) was observed in animals that were treated with ivermectin, levamisole and tetramisole.

#### DISCUSSION

The study revealed that anthelmintic drugs are quite commonly but improperly utilized in the area. Three group of anthelmintics namely benzimidazoles





**Figure 1.** Pattern of deworming by livestock owners in relation to season.

(Albendazole), imidazothiazole (Tetramisole and Levamisole) and macrocyclic lactone (Ivermectin) were used. Other alternative anthelmintic were not available in the market. Utilization of limited group of drugs for a long period may favour the development of resistance (Papadopoulos, 2008). Benzimidazoles group of anthelmintics especially albendazole was the most commonly used by livestock owners to deworm their sheep. Similar finding was also reported by Kumsa et al. (2010a, b) in the Southern part of the Ethiopia and Arece et al. (2004) in Cuba.

Availability of anthelmintics in the hand of livestock owners and selling together with other commodities in supermarkets and ordinary shops may also promote resistance as under dosing is eminent. Most farmers deworm their animals twice a year and spent about 3.00 US dollars for anthelmintic purchase. This is a rough estimate as most of them did not keep proper records. Anyway, deworming practice should not be routine task. It has to be conducted strategically based on the epidemiology of the parasites and after confirming the presence of the parasite in a given animal. Syndromes mentioned by livestock owners for deworming may not be induced by gastrointestinal parasites alone in which use of anthelmintic may be useless.

The anthelmintic trial study revealed that the anthelmintic drugs used in the area does not have equal efficacy on reduction of helminth infection the sheep. Albendazole and ivermectin formulations were found to be more efficacious for gastrointestinal nematodes in sheep at a recommended dose than others. Similar finding was also reported by Arece et al. (2004).

The observed predominance of *Haemonchus* in the

pre-treatment coprocultures was also reported by Chandrawathani et al. (1999), Sissay et al. (2006), Kumsa and Wossene (2009) and Kumsa et al. (2010a). *Haemonchus* was also identified from the coproculture samples after treatment with ivermectin, tetramisole and levamisole. This finding was supported by many previous studies that reported the association between *Haemonchus* species and reduced efficacy of anthelmintics (Chandrawathani et al., 1999; Arece et al., 2004).

Albendazole has a very good efficacy in reducing gastrointestinal nematodes. Only one parasite (*Trichuris*) was found in one of the treated sheep. Other most pathogenic nematodes like *Haemonchus* were effectively treated. This finding is in agreement with previous research outputs like Chaka et al. (2009) as they confirmed that most anthelmintics sold on Ethiopian markets were effective to treat *Haemonchus* in experimentally infected sheep. Good state of efficacy of albendazole was also reported by Sheferaw and Asha (2010) in the Wolaita (Southern Ethiopia). However, resistance of *Haemonchus* against albendazole were reported by Kumsa and Abebe (2009) in goat in Hawassa (Southern Ethiopia) and a high level of anthelmintic resistance to albendazole in goat was also reported by Sissay et al. (2006) in Eastern Ethiopia. These may be related with the difference in anthelmintic utilization in different parts of the country.

Ivermectin was also effective in reducing FEC but in lesser extent than albendazole (99.08 versus 96.69%, respectively). Especially the recovery of *Haemonchus* in ivermectin treated group needs a special attention. Similarly, Sissay et al. (2006) reported ivermectin resis-

tance in *Haemonchus contortus* and *Trichostrongylus* species dominated infection in goats. In contrast to this finding Arece et al. (2004) reported a 100% efficacy of different formulations of ivermectins in Cuba. This may be related to the difference in the frequency and ways of utilization of the drug among localities.

Tetramisole and levamisole were less efficacious than others. The lower efficacy may also be related to the fact that the resistance can be developed against one anthelmintic in a group which may also occur in the other members of the group (Sangster, 1999) since these two anthelmintics are related drugs (Imidazothiazoles). Lowest efficacy of imidazothiazoles was also reported by Arece et al. (2004) and Sissay et al. (2006). The quality of the preparation of these anthelmintics may also be the contribution factor for their lowest efficacy which necessitates further investigation and comparison with standard products.

The 100% efficacy result obtained by the use of two anthelmintics together is promising. It can be recommended to be used by farmers despite the cost and safety. Reducing resistance by the administration of combined anthelmintics was also mentioned by Papadopoulos (2008). However, the benefit and cost has to be thoroughly investigated.

In conclusion, the results of this study showed the high efficacy of albendazole and ivermectins; however, the low efficiency of levamisole and tetramisole. For a more sustainable control of helminth parasites, the strategy has to be directed to the integration of different parasite control methods. To maintain the efficacy of anthelmintics, proper utilization should be practiced.

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