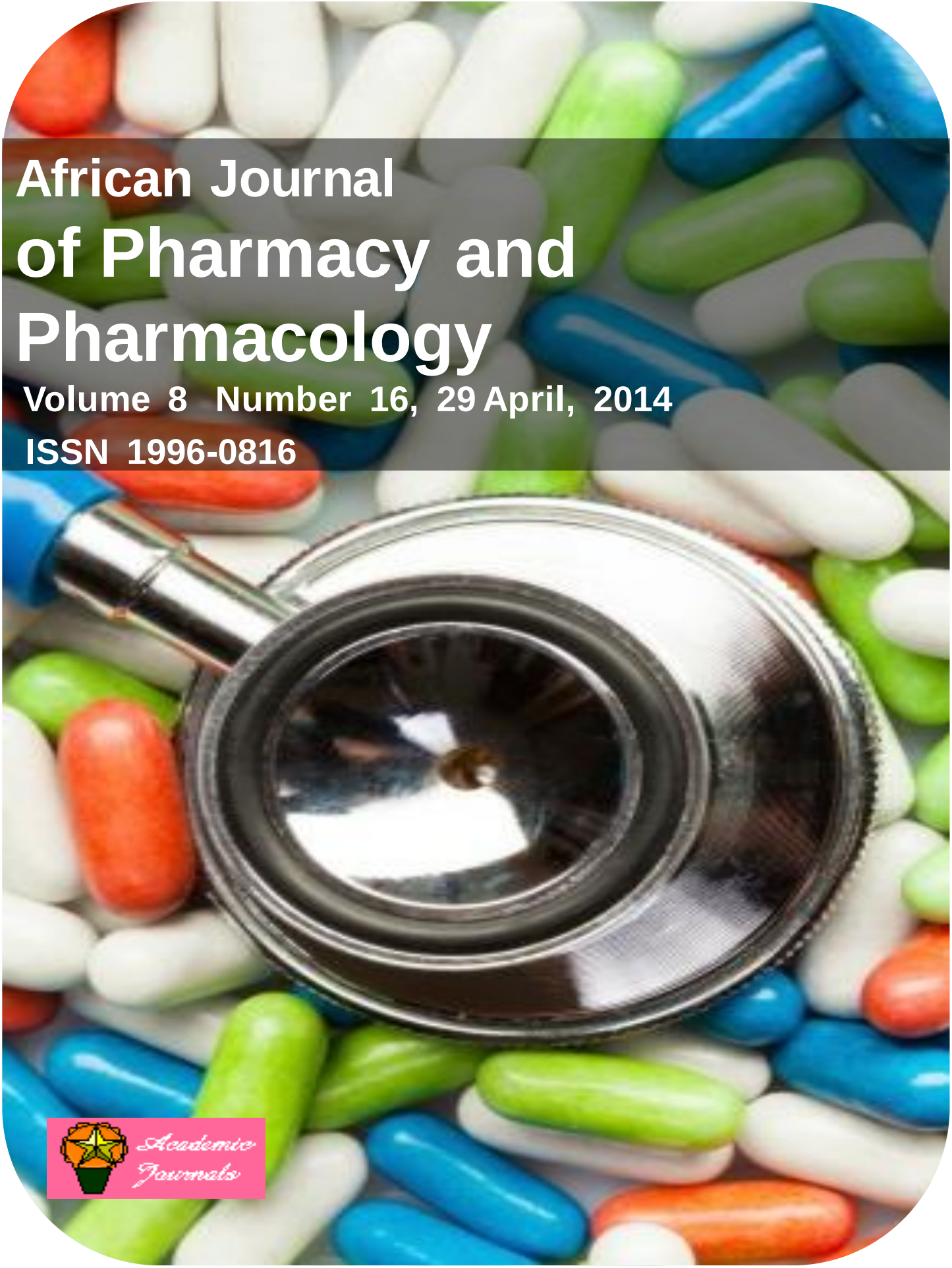

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Full Length Research Paper

Comparative pharmacokinetic study of luteolin after oral administration of Chinese herb compound prescription JiMaiTong in spontaneous hypertensive rats (SHR) and Sprague Dawley (SD) rats

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JiMaiTong (JMT), a Chinese herb compound prescription consisted of *Flos chrysanthemi* Indici, *Spica prunellae* and *Semen cassiae* for anti-hypertension. Luteolin is one of the major bioactivity compositions in *F. chrysanthemi* Indici in JMT. There are some reports about pharmacokinetics of luteolin in extract of *F. chrysanthemi* and husks of peanut in normal rats, but it lacked pharmacokinetic information of luteolin residing in a Chinese herb compound prescription in hypertensive animal models. The present study aimed to develop a high-performance liquid chromatography with photodiode array detection (HPLC-DAD) method for determination of luteolin in rat plasma and for pharmacokinetic study after oral administration of JMT to spontaneous hypertensive rats (SHR) and normal Sprague Dawley (SD) rats. After oral administration of JMT to SHR and SD rats, respectively the content of luteolin in blood samples at different time points were determined by a reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid-liquid phase extraction. This revealed a difference in disposition of luteolin in SHR and SD rats and a relationship between plasma concentration and hypertension of pathological state within the organism. This was the first report on the pharmacokinetic compare of luteolin between normal and hypertension pathological state rats. The hypertension pathological state would effects the disposition of luteolin, and it may provide a meaningful basis for the clinical application of JMT.

Key words: Luteolin, pharmacokinetic, *Flos chrysanthemi* Indici, hypertension, Chinese herb.

INTRODUCTION

JiMaiTong (JMT) is a Chinese herb compound prescription which can effectively depress the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) of spontaneous hypertensive rats (SHR) (Lv et al., 2010), map variability of renal hypertensive rats promote the microcirculation blood flow of RHR by improving nitrogen oxide (NO) content and depress ET-1 and Ang II level in serum (Chen et al., 2012). JMT is composed of *Flos chrysanthemi* Indici, *Spica prunellae and Semen cassia* and contains flavonoids

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compositions such as luteolin, linarin etc. Luteolin (3', 4', 5, 7-tetrahydroxyflavone) is one of the major bioactivity compositions in JMT; it acts as anti-hypertension by relaxing the vascular smooth muscle (Kim et al., 2006; Jiang et al., 2005; Duarte et al., 1993; Ichimura et al., 2006) and blood vessel (Jiang et al., 2005), promotes the opening of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Hou et al., 2008) and protects the cardiac muscle (Lv et al., 2010). Since JMT is a candidate drug for anti-hypertension, it is essential to clarify the pharmacokinetics processes of luteolin *in vivo*.

The reaction of an organism to a similar traditional Chinese medicine would be different under the different physiological or pathological status (Zhao and Zhou, 2008) and the physiological disposition of the compositions in the drug would be changed as the same (Yang et al., 2005; Reng et al., 2006). These would induce the change of the plasma concentration and impact the pharmacological effects of the components. Therefore, a comparative study of pharmacokinetics of compositions in drug in pathological pattern animals and normal animals has more practical significance for determining the efficacy material and guide the clinical application.

There are some reports about pharmacokinetics of luteolin in normal rats after oral administration of *F. chrysanthemi* extract or peanut shells extract and found that luteolin was present mainly as glucuronide conjugates in plasma and bile, and entacapone, a cathechol-O-methyltransrerase (COMT) inhibitor can increase the area under the curve (AUC) of luteolin when co-giving with *F. chrysanthemi* extract (Wan et al. 2008, Chen et al. 2012, Chen et al. 2011). However there was lack of pharmacokinetic information of luteolin in Chinese herb compound in pathological pattern animals. The present study aimed to develop an improved quantitative method to determine luteolin in rat plasma using high-performance liquid chromatography with photodiode array detection (HPLC-DAD) and utilizing it in the pharmacokinetic study of the luteolin in SHR and Sprague Dawley (SD) rats after oral administration of JMT. The pharmacokinetic data could give us more information about the pharmacokinetics of JMT *in vivo* and give some reference for the clinical application.

MATERIALS AND METHODS

Chemicals

The reference standard of luteoln (>99% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Ministry of Health, Beijing, China). JMT was provided by the Institute of Material Medical, Zhejiang Chinese Medical University, China, containing 0.06% (w/w) luteolin determined by HPLC. Methanol was HPLC grade and the water was prepared by Milli-Q system (Millipore, Milford, MA, USA). Others were from standard commercial sources and were of the highest quality available.

Animals

Male SHR rats weighing 280 to 320 g were obtained from Vital River Laboratory Animal Technology Co. Ltd and male SD rats weighing 260 to 280 g were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were according to an approved animal use protocol of Zhejiang Chinese Medical University. They were housed in cages at 25 ± 2°C and exposed to a 12:12 h light-dark cycle with free access to food and water. Animals were fasted but had free access to water for 12 h before experiment and 2 h after drug administration.

Instrumentation

Analysis was performed using high-performance liquid chromatographic system Agilent 1200 series (Agilent Technologies, USA) equipped with an on-line vacuum degasser, a quaternary solvent delivery system, an auto-sampler, a diode array detector (DAD) and a homeothermic column compartment.

Chromatographic condition

The HPLC analysis was performed on an Ultimate XB - C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of 5% glacial acetic acid and methanol (45:55, v/v) at 25 ± 1°C, with a constant rate of 1 ml/min. The injection volume was 20 µl and the wavelength was set at 336 nm for quantitative analysis.

Stocking and working solution

Stock standard solution of luteolin (0.2 mg/ml) was prepared by dissolving 10.02 mg in methanol to 50 ml. A series of working solutions containing luteolin was prepared by subsequent dilution of the stock solution with methanol to 0.1, 1, 2, 5, 20, 100 µg/ml and kept under 4°C.

Sample preparation

After oral administration of JMT, the blood samples were collected from the orbital venous sinus into heparinized tubes according to the specific schedule and then centrifuged at 4,000 g for 10 min at 4°C, the plasmas were collected and treated as the method of Ying et al. (2008), with some modifications. In brief, to detect the total form (free, glucuronidated, sulfated) of luteolin, a 500 µl plasma was hydrolyzed by 0.5 ml hydrochloric acid (10 M) at 80°C for 0.5 h in a 10 ml tube and then 8 ml mixed solution of acetic ether and N-hexane with proportion of 4:1 was added to the mixture. After ultrasonic for 5 min and vortex for 30 s, the tube was centrifuged for 10 min at 4,000 rpm. Then all the supernatant organic phase was carefully transferred to a 1.5 ml tube for eight times and evaporated to dryness with a nitrogen blowing concentrator at room temperature. The residue was reconstituted in 0.2 ml methanol and after centrifuged for 10 min at 12,000 rpm, the concentration of luteolin was determined by the HPLC method described.

Method validation

Specificity

Blank plasma from five rats, blank plasma spiked with luteolin and

sample obtained from rat after oral administration of JMT were processed and assayed as described in 2.6. Interference from endogenous or exogenous materials should not occur at the retention time of luteolin.

Linearity and range

Calibration standard was prepared as described in triplicate and the calibration curve ranging from 0.15 to 15.0 µg/ml was assessed by weighted (1/x) least squares liner regression based on plotting the peak area versus the concentration of the calibration standard. The lowest limit of quantitation (LLOQ) was defined as the lowest concentration that could be accurately and precisely quantitated corresponding to a signal-to-noise ratio of 3.

Precision and accuracy

Standard samples spiked with luteolin at low, medium and high concentrations (5, 20, and 100 µg/ml for all the analytes) were used for accuracy and precision studies (the end concentration was 0.75, 3, 15 ug/ml). Five replicates for each concentration were processed and analyzed as described for accuracy study. The assay recovery and extract recovery were calculated. The intra- and inter-day precisions (relative standard deviations, RSD) were evaluated by analyzing homogeneous samples in five replicates in 1 day. Intra- and inter-day precisions (RSD) were required to be less than15%.

Stability

Stability of luteolin was evaluated by analyzing the standard sample at low, medium and high concentrations. Three replicates were stored at room temperature (about 25°C) for 12 h before sample processing. Sample concentrations were measured at the storage time of 2, 4, 6 and 12 h. The stability was assessed and expressed as remaining (%) of initial determined.

Pharmacokinetics study

3 male SHR and 3 male SD rats were fasted 12 h with free access to water, then a dose of 3.3 g/kg JMT (dissolved in water) was orally administered to SHR and SD rats, respectively. The blood samples were collected from the orbital venous sinus to heparinized tubes at pre-dose and post-dose at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 36 h and then centrifuged at 4,000 g for 10 min at 4°C. The plasma were collected and stored at −80°C until analysis.

Statistical analysis

Pharmacokinetic parameters were calculated with pharmacokinetic software phoenix WinNonlin 6.0. Measurements were expressed as the mean ± standard deviation and *t*-test was used to compare the measures of SHR and SD groups. $P < 0.05$ was considered statistically significant.

RESULTS

Specificity

The specificity of this method to plasma matrix was evaluatedwithplasmafromfiverats.The typical chromatograms of (A) a blank plasma sample, (B) a blank plasma sample spiked with luteolin, (C) a plasma sample from a rat at 15 min after oral administration of 3.3 g/kg JMT are shown in Figure 1. No interferences from endogenous substances in rat plasma were ob-served at the retention times of luteolin (all samples were treated with hydrolysis preparation).

Linearity, range and sensitivity

The peak area of luteolin displayed a good linear relationship over the range of 0.15 to 15 µg/ml. The typical regression equations were as follows: $A = 48.12C + 0.57$. $R^2 = 0.9999$ (A: peak area of luteolin; C: concentration of luteolin in rat plasma). The LLOQ of all analytes was 0.075 µg/ml.

Precision and accuracy

Recovery, precision and accuracy data are presented in Table 1. The extract recoveries for luteolin were 79.4 to 95.8% and the assay recoveries of luteolin were 88.4 to 104.0%. The intra-day precision (RSDs) for luteolin was less than 3.7% and the inter-day precision (RSDs) was less than 6.5%.

Stability

The stability results of luteolin in rat plasma as shown in Table 1 which revealed the luteolin in stocking solution at 25°C for 12 h was stable (the remaining of the initial determined were > 97.0%).

Pharmacokinetic study

The method was successfully applied to analysis of plasma obtained from SHR and SD rats following a single oral dose of 3.3 g/kg JMT. Concentration-time profiles for luteolin of SHR and SD rats after giving JMT is shown in Figure 2. The plasma concentration of luteolin in SHR has double hump and was significantly higher than that of SD rats and the first class elimination rate in SHR was also higher (Table 2).

DISCUSSION

With the characters of moderate and multi-target effects, TCMs are used to treat chronic diseases such as hypertension, either in the form of single or combined herbs. The therapeutic activity of each herb is based on a coplex combination and interaction of its various ingredients, showing an integral effect from those ingredients. The

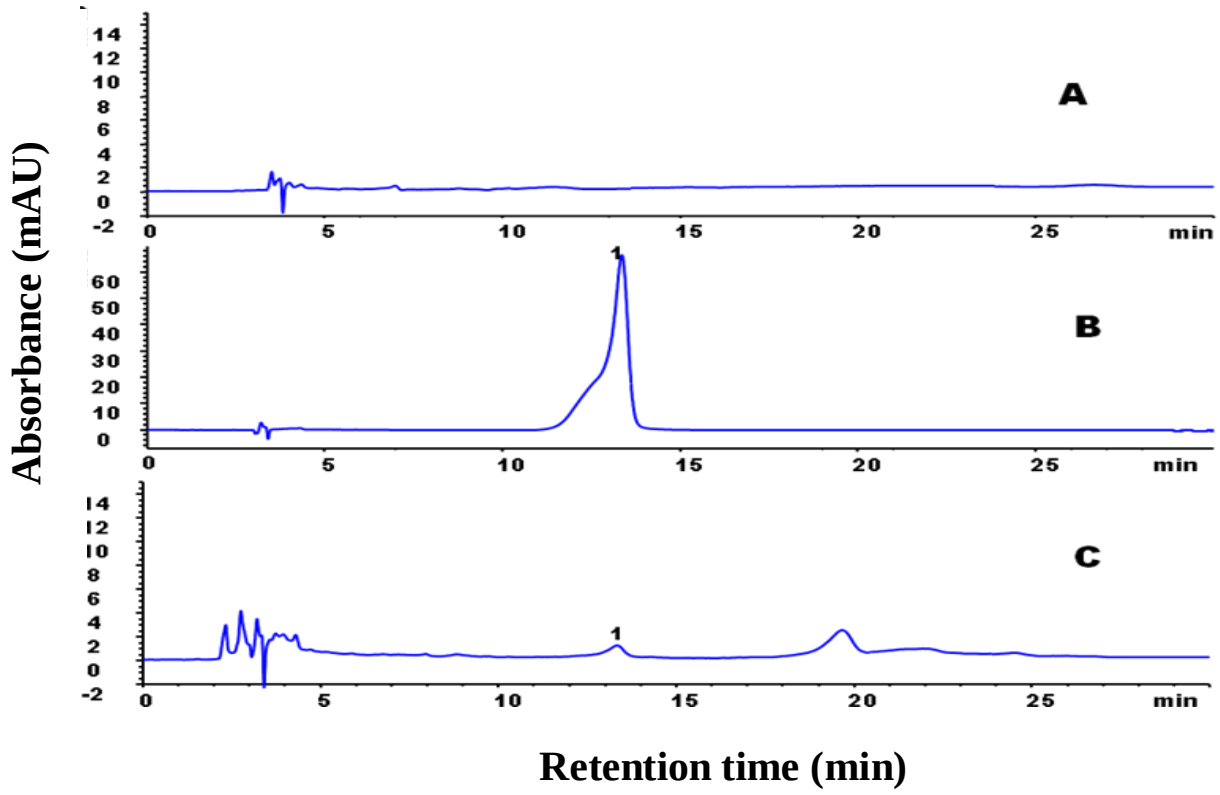

Figure 1. HPLC chromatograms of luteolin in rat plasma .
A: blank plasma; B: luteolin standard (show as 1, the retention time is 13.371min); C: plasma sample 15min after oral administration of 3.3g/kg JMT to SHR. All samples above were treated with hydrolysis.

Table 1. Precision, accuracy and stability of the method (mean ± SD, n = 5).

Spiked conc. (μg/ml)	Intra-day		Inter-day		Assay recovery		Extract recovery		Stability (%)
	Measured conc. (μg/ml)	RSD (%)	Measured conc. (μg/ml)	RSD (%)	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)	
5	4.3±0.11	2.5	4.6±0.24	5.3	88.4±7.92	8.96	79.4±6.99	8.80	99.5±2.16
20	19.4±0.71	3.4	20.4±1.33	6.5	102.6±8.78	8.56	82.8±7.06	8.53	98.1±0.77
100	103.9±3.53	3.7	103.0±6.60	6.4	104.0±4.08	3.93	95.8±3.76	3.92	97.0±3.00

organism is a carrier of medicines to produce a marked effect and an object for drug treatment (Zhang et al., 2008). The pharmacodynamic actions or toxicity of ingredients is closely related with organism state (Ni and Zhang, 2009). Different states of the organism have different response to a medicine and it is important to carry researches on the pharmacokinetic of ingredients in organism with healthy or pathological state (Tian et al., 2012). The luteolin is one of the main active ingredients in the JMT and is important and responsible for the integral effect of JMT on anti-hypertension. The pharmacokinetic study of luteolin in rats with healthy or hypertension state would make for an objective pharmacodynamic evaluation of JMT.

The adopted RP-HPLC method has been develop and successfully applied to the pharmacokinetic analysis of bioactive components from TCM. Thus, with some modifications, the well-developed RP-HPLC coupled with liquid-liquid phase extraction can be considered as a suitable method for this study. However, there are complicated compositions in the Chinese herb compound prescription JMT, so an optimized chromatographic condition was very important in the present study. In this study, an Ultimate XB-C_{18} column (250 mm × 4.6 mm, 5 μm) was applied to separate the luteolin. The mixtures of 5% glacial acetic acid and methanol or 0.5% phosphoric

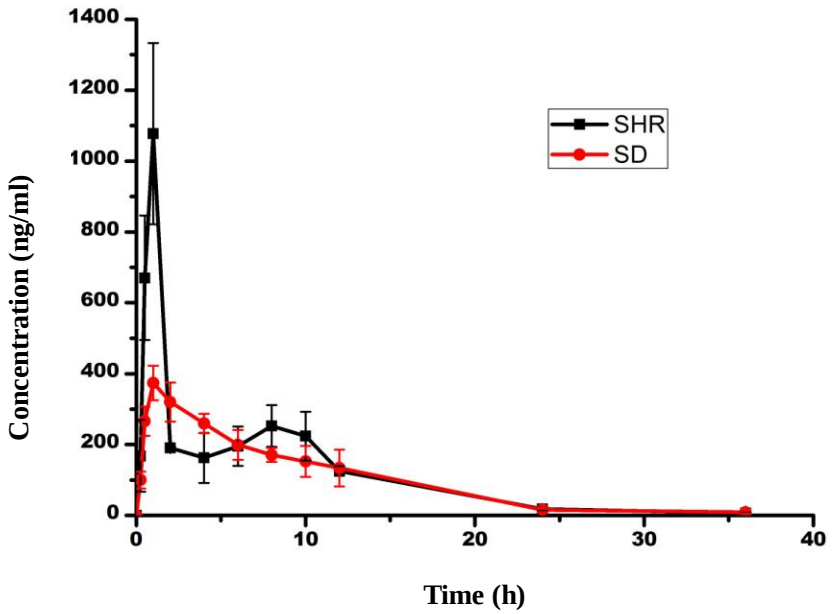

Figure 2. Mean-plasma concentration-time profiles of luteolin after oral administration of 3.3 g/kg JMT to SHR and SD rats. Data were expressed as mean ± SD, n=3.

Table 2. Main pharmacokinetic parameters of luteolin in SHR and SD rats after oral administration of 3.3g/kg JMT (mean ± SD, n = 3).

Parameter	Animals	
	SHR	SD
Lambda_z (1/h)	0.173±0.03*	0.119±0.03
HL_Lambda_z $t_{1/2}$ (hr)	4.08±0.58	6.14±1.59
T_{max} (h)	1.00±0.00	1.33±0.58
C_{max} (µg/ml)	1.08±0.26*	0.39±0.03
AUClast (h µg/ml)	4.19±0.55	3.63±0.67
AUCINF_obs (h µg/ml)	4.23±0.56	3.71±0.65
Vz_F_obs (L/kg)	4693.0±1,181	8254.4±3,595
Cl_F_obs (L/h/kg)	789.0±98	909.7±176
MRTlast (h)	7.38±0.70	8.03±0.51
MRTINF_obs (h)	7.69±0.80	8.93±0.88

*P < 0.05 vs SD rats. Spontaneous hypertensive rats (SHR) and Sprague Dawley (SD) rats.

acid and methanol were compared to get a suitable mobile phase condition and the former was found to make a good separation.

After optimizing the proportion of methanol, a mobile phase of 5% glacial acetic acid and methanol (45:55, v/v) was determined. Luteolin has a free hydroxyl which could be further conjugated by UGTs and existed as a glucuronide form (Shimoi et al., 2001; Liu et al., 1995) and it is difficult to detect free luteolin in plasma; therefore a hydrolysis treatment with hydrochloric acid or α-glucuronidase of sample was very important to assay the methylated metabolites of luteolin *in vivo*. In this study, hydrolysis treatment with hydrochloric acid was selected and after an optimization of extraction conditions with organic solvent for free luteolin, an organic solvent mixture of acetic ether and N-hexane (4:1, v/v) was determined.

In this study, non-compartment model was adopted to perform the evaluation of pharmacokinetics of luteolin in SD rats and SHR after oral administration. An obvious double peak was observed in SHR which indicated that in SHR, enterohepatic circulation may be the way of luteolin's re-absorption (Ying et al., 2008; Chen et al., 2007). As we know, the AUC reflect the relative amount of drug absorbed into the systemic circulation, the large

AUC means the more drug absorption and with the same dosage *in vivo*, the higher blood concentration means the smaller V_d value, which showed the drug is mainly distributed in blood and conversely in tissues. In this study, we found that the C_{max} (1.08 ± 0.26 μg/ml), AUC (4.19 ± 0.55 h μg/ml) and Lambda_z (Ke, 0.173 ± 0.03 1/h) of luteolin in SHR were large than that in SD rats ($P < 0.05$), but T_{max} (1.0 h), V_d (4693.0 ± 1.181 L/kg) and MRTlast (7.38 ± 0.70 h) were smaller. Indicating that in SHR the luteolin after oral administration can be rapidly and effectively absorbed from SHR gastrointestinal tract into the blood and then rapidly eliminated and cleared from the body; while in SD rats, it was absorbed into the blood and widely transported from the blood into tissues and this course prolong the elimination.

Luteolin is a good substrate of catechol-O-methyltransferase (COMT) and *in vivo* it would be methylated to chrysoeriol and diosmetin by COMT (Chen et al., 2011). This metabolic character is contributed to the faster elimination of luteolin in rats (Chen et al., 2012). With the changing of activity of liver metabolic enzyme or the quantity of *β*-glucuronidase, the metabolism of compositions in the animal with pathological status would be changed (Deng et al., 2008; Liu et al., 2012). The pharmacokinetics difference of luteolin in SHR and SD rats observed from this study show that the hypertension pathologic state would affect the metabolism character of luteolin, but the mechanism for this was unclear and needs further investigation.

Taken as a whole, all available pharmacokinetic data indicated that luteolin displayed ideal pharmacokinetic profiles in SHR plasma after oral administration of JMT. Since the JMT is a candidate for the treatment of hypertension, it would be very useful and meaningful to obtain the main active ingredients, such as luteolin, pharmacokinetic information for the better understanding of the pharmacology. To some extent, our pharmacokinetic data presented a substantial evidence for the *in vivo* efficacy of luteolin in the organism with hypertension pathologic state, since a favorable absorption, distribution and elimination of this compound *in vivo* have been demonstrated.

In summary, together with our pharmacological findings, this study could provide some useful clues and guidance, such as dosage regimen and application strategy, for clinical application. The next step will be to clarify this compounds' tissue distribution both in SD rats and SHR and the underlying mechanism of action.

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Short Communication

Effect of *Thespesia populnea* Linn. on dexamethasone induced insulin resistance in mice

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In traditional medicine, plants have been used to treat diabetes mellitus as valuable alternative therapy since synthetic oral hypoglycemic agents are known to exhibit adverse side effects. The present study was planned to investigate the effect of ethanolic extract of bark of *Thespesia populnea* Linn (TP) on plasma glucose, serum triglyceride and body weight in dexamethasone-induced insulin resistance in mice. The animals were divided into diabetic and non-diabetic groups consisting of six animals in each group. TP was administered at doses of 100, 200, and 400 mg/kg per oral (p.o.) in mice which were concomitantly treated with pre-standardized dose of dexamethasone (1 mg/kg intramuscular (i.m.)) for 22 days and effect on plasma glucose, serum triglyceride level and change in body weight were recorded. TP showed significant decrease in plasma glucose ($p<0.01$), serum triglyceride ($p<0.01$) levels and significant increase in body weight ($p<0.01$) as compared to dexamethasone control group. This study revealed that the ethanolic extract of bark of *T. populnea* may prove to be effective in the treatment of insulin resistance owing to its ability to decrease peripheral insulin resistance and can be used in the treatment of type-II diabetes mellitus.

Key words: *Thespesia populnea*, dexamethasone, insulin resistance.

INTRODUCTION

Diabetes mellitus has been a common problem of the world from centuries. It is a disease related to the sweetness, characterized by the presence of excessive sugar in blood and urine due to deficiency in the production of insulin by 'β' cell of pancreas or presence of ineffective insulin. The growth of disease is rapid due to the heredity, endocrine imbalance, dietary imprudence, severe and continued mental stress, and reduction in physical labour and differences in social structure, etc., which is providing a productive atmosphere to diabetes (Gupta et al., 2006).

Insulin resistance has emerged as a far reaching endocrine phenomenon which signifies an impaired biological response and insulin resistance is known to underlie the

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deterioration of glucose homeostasis that lead to typical forms of type-II diabetes mellitus. The development of insulin resistance met initially with compensatory increase in insulin secretion (Bailey, 1999). Worldwide, over 1200 species of plants have been recorded as traditional medicine for diabetes (Bailey, 1999). Some of these plants have been evaluated in laboratories and in a number of cases their efficacy has been confirmed, for instance, *Panax ginseng* (ginseng), *Opuntia cactus* (cactus), *Tecoma stans* (trompeta), *Syzygium cumini* (jambolao) (Boden et al., 2001). Specific chemical constituents of these plants, such as polysaccharides, alkaloids, triterpenoids and xanthones, are believed to be responsible for the hypoglycemic effects and they can be related to actions including increased insulin release and increased glucose metabolism in the body periphery, among others (Wang and Ng, 1999).

The therapeutic potential of *Thespesia populnea* Linn is known from many years, and it has key place in Ayurvedic medicine (Anonymous, 2006). The alcoholic extract of unripe fruits reported for the antibacterial activity (Gaind and Bapna, 1967), anti-hyperglycemic (Satyanarayana et al., 2004), and wound healing potential (Nagappa and Binu, 2001). The bark has been reported for anti-oxidant activity (Ilavarasan et al., 2003), antinociceptive and anti-inflammatory activity (Vasudevan and Parle, 2006), alzheimers disease (Vasudevan et al., 2007), and antidiarrheal activity (Viswanatha et al., 2007). The bark contains thespesone, which is having 2-hydroxy-2, 4, pyranonaphtaquinone as a parent nucleus. Various plants like *Lawsonia alba* having this nucleus are reported for their anti-diabetic potential (Neeli et al., 2007). Hence, considering these correlation and traditional claims related to this plant, the ethanolic extract was evaluated for dexamethasone induced insulin resistance in mice.

EXPERIMENTAL

Plant material and preparation of extract

The bark of *T. populnea* Linn. (Malvaceae) was collected from fields near Dehu Road in Pune, Maharashtra. The specimen was authenticated at Agharkar Research Institute, Pune with voucher specimen No. Auth08-006 and was documented. The bark was dried in shed and then powdered. The ethanol extract was prepared using soxhlet apparatus and concentrated under vacuum. The yield of ethanol extract of whole plant of *T. populnea* (TP) was 13.45% w/w. TP when subjected for phytochemical study showed the presence of flavonoids, beta-sitosterol, terpenoids and tannins (Viswanatha et al., 2007).

Animals

Albino mice weighing 25 to 30 g were used for the study and were kept in animal house at 26 ± 2°C with relative humidity 44 to 56% along with light and dark cycles of 12 h, respectively. Animals were provided with standard diet and water *ad libitum*. The food was withdrawn 18 to 24 h before the start of the experiment.

Design

Acute toxicity study

The acute toxicity study for ethanol extract of *T. populnea* was performed using albino mice. The animals were fasted overnight prior to the experiment and maintained under standard conditions. TP was administrated orally in increasing doses and found safe up to the dose of 2,000 mg/kg (OECD, 2001).

Dexamethasone-induced insulin resistance in mice

All the mice were weighed before treatment, group I (Normal control) received 1% gum acacia (1 ml/kg per oral (p.o.)), and 36 mice were rendered hyperglycemic by daily administration of a prestandardised dose of dexamethasone (1 mg/kg intramuscular (i.m.)) for consecutive 7 days and then divided into six groups of six animals in each. Group II (Dexa-control) continued to receive only dexamethasone and 1% gum acacia (1 ml/kg, p.o.), groups III and IV received ketoconazole (24 mg/kg, p.o.) and pioglitazone (2 mg/kg, p.o.) along with dexamethasone, respectively. Groups V, VI, and VII were treated with dexamethasone along with three different doses of TP 100, 200, and 400 mg/kg, p.o., respectively. Simultaneously, five other groups (Groups VIII, IX, X, XI, and XII), each with six normoglycemic animals, were administered equivalent amount of ketoconazole and pioglitazone and three different doses of TP 100, 200, and 400 mg/kg, p.o., respectively (Gholap and Kar, 2005; Shalam et al., 2006).

Biochemical analysis

On the last day, all the animals were weighed. Blood samples were collected and plasma and serum separated for estimation of glucose and triglyceride, respectively. Biochemical estimation of plasma glucose and serum triglyceride was done by glucose oxidase (GOD)/POD and glycerol-3-phosphate oxidase (GPO)/PAD methods, respectively using standard diagnostic kits (Crest Biosystems, Goa, India).

Statistical analysis

The results were expressed as mean ± standard error of mean (SEM) and statistically analyzed by ANOVA followed by Dunnett test, with level of significance set at $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

Effect on plasma glucose level

In dexamethasone induced insulin resistance, it was found that the Dexa group showed significant increase ($p<0.01$) in plasma glucose level when compared with normal control group. Dexa + Keto and Dexa + Pio groups showed significant decrease ($p<0.01$) in plasma glucose level when compared with Dexa control group. Dexa + TP-400 group showed significant decrease in plasma glucose ($p<0.01$) when compared with Dexa control group. Dexa + TP-200 group showed significant decrease in plasma glucose ($p<0.05$) when compared with Dexa control group. In non-diabetic animals, Keto

Table 1. Effect of *T. populnea* on plasma glucose, serum triglyceride and body weight in dexamethasone induced insulin resistance.

Group	Plasma glucose (mg/dl)	Serum triglyceride (mg/dl)	Body weight change (g)
Normal control	54.68 ± 0.51	83.68 ± 1.17	0.88 ± 0.05
Dexa-control	80.29 ± 0.30##	144.38 ± 2.42##	-2.17 ± 0.13##
Dexa + Keto	65.02 ± 0.65**	92.65 ± 1.96**	0.98 ± 0.05**
Dexa + Pio	55.29 ± 0.94**	85.48 ± 1.91**	0.98 ± 0.07**
Dexa + TP-100	76.98 ± 0.50	139.76 ± 1.16	-1.88 ± 0.11
Dexa + TP-200	76.46 ± 0.21*	138.13 ± 1.22	-1.86 ± 0.08
Dexa + TP-400	71.77 ± 1.31**	121.02 ± 1.92**	0.54 ± 0.10**
Keto	46.73 ± 0 .63##	85.75 ± 1.92	0.99 ± 0.12
Pio	53.51 ± 0.51	81.87 ± 1.83	0.98 ± 0.07
TP-100	56.20 ± 0.68	88.48 ± 1.08	0.96 ± 0.13
TP-200	54.15 ± 0.34	88.76 ± 1.34	1.18 ± 0.13
TP-400	52.35 ± 0.48	89.59 ± 1.04	1.08 ± 0.16

Values are expressed as mean ± SEM (n=6), ANOVA followed by Dunnett test. ##p<0.01 when compared with normal control, *p<0.05, **p<0.01 when compared with Dexa-control. Normal control: Recieved 1% Gum Acacia (1 ml/kg, p.o.); Dexa: Dexamethasone (1 mg/kg, i.m.); Keto: Recieved Ketoconazole (24 mg/kg, p.o); Pio: Recieved Pioglitazone (2 mg/kg, p.o); TP-100, TP-200 and TP-400: Received ethanol *T. populnea* extract 100, 200, and 400 mg/kg, respectively. SEM: Standard error of mean.

group showed significant decrease (p<0.01) in plasma glucose level when compared with normal control group. All the non-diabetic groups treated with TP and Pio did not show any significant decrease in plasma glucose when compared with normal control.

Effect on serum triglycride level

In this study, it was found that Dexa group showed significant increase (p<0.01) in serum triglyceride level when compared with normal control group. Dexa + Keto and Dexa + Pio groups showed significant decrease (p<0.01) in serum triglyceride level when compared with Dexa control group. Dexa + TP-400 group showed significant decrease in serum triglyceride (p<0.01) when compared with Dexa control group. All the non-diabetic groups treated with Pio, Keto and TP did not show any significant decrease in serum triglyceride when compared with normal control.

Effect on body weight

It was found that Dexa group showed significant decrease (p<0.01) in body weight when compared with normal control group. Dexa + Keto and Dexa + Pio groups showed significant increase (p<0.01) in body weight when compared with Dexa control group. Dexa + TP-400 group showed significant increase (p<0.01) in body weight when compared with Dexa control group.

Insulin resistance is defined where a normal or elevated insulin levels produces an attenuated biological response and it refers to acute regulation of carbohydrate metabolism, which leads to impaired insulin sensitivity to insulin mediated glucose disposal (Reaven, 2004). It generally occurs systemically for e.g. in liver or it may occur locally in e.g. adipose tissue or in skeletal muscle (Nandi et al., 2004), and evidence is now accumulating that ectopic lipid accumulation is the central feature of insulin resistance (Phielix and Mensink, 2008). Insulin normally lowers the level of blood glucose through the suppression of hepatic glucose production and stimulation of peripheral glucose uptake, but the dysfunction in any step of this process can result in insulin resistance. In healthy individuals, an increased gluconeogenesis is compensated by a decreased glycogenolysis, due to concomitant hyperinsulinemia, thereby maintaining hepatic glucose output at the same levels, called hepatic auto regulation (Clore et al., 1991). But in type-II diabetes mellitus, a breakdown of hepatic auto regulation is suggested to underlie the increased glucose output (Boden et al., 2001).

The influence of glucocorticoids on insulin sensitivity is the most important in syndromes of cortisol excess (Cushing's syndrome) or deficiency (Addison's disease). In Cushing syndrome, the patient develops glucose intolerance and central obesity, while Addison's disease is associated with increased tissue in insulin sensitivity. There are numerous potential sites of action of glucocorticoids to affect insulin sensitivity. The principal effects of glucocorticoids are to oppose the actions of insulin in the regulation of carbohydrate, lipid and protein metabolism by effects of three main target tissues of liver, skeletal muscle, and fat (Andrews and Walker, 1999). Glucocorticoids increase blood glucose by mobilizing substrates for hepatic gluconeogenesis and stimulate the release of amino acids from skeletal muscle, fatty acids and glycerol from adipose tissue and increase the expression of gluconeogenic enzymes, such as phosphoenolpyruvate

carboxykinase (PEP-CK) (Hanson and Reshef, 1997); hence, enhancing gluconeogenesis in liver. Glucocorticoids stimulate glycogen synthesis by activating glycogen synthase and inactivating glycogen mobilizing enzyme glycogen phosphorylase. Glucocorticoids inhibit peripheral glucose uptake and utilization partly as a result of decreased translocation of glucose transporters (GLUT 4) to cell surface (Dimitriadis et al., 1997). Acute effects of glucocorticoids provide gluconegenic substrates for fat metabolism which result in stimulation of lipolysis. Glucocorticoids increase triglyceride levels causing imbalance in lipid metabolism leading to heperlipidemia with increase in glucose levels leading to hyperglycemia (Shalam et al., 2006). Various pharmacological doses of glucocorticoids induce *ob* gene expression within 24 h which is followed by reduction in body weight, and development of insulin resistance (Shalam et al., 2006).

Intracellular triglycerides and products of fatty acids result in acquired insulin resistance state which results in the lipotoxic effect due to decrease in activity of lipoprotein lipase activity which results in decrease in insulin signalling pathway. There occurs an inhibitory interaction between two major fluids, glucose and free fatty acids which leads to insulin resistance. These interactions constitute a mechanism beyond hormonal regulation for controlling the circulating hormones like insulin, corticosteroid, and adrenalin can modify the control.

In this study, dexamethasone administration resulted in increased blood glucose and triglyceride level and decrease in body weight. TP prevented the rise in blood glucose and triglyceride level and decreases in body weight might be because of significant increase in glucose uptake which might be due to increase in the insulin sensitivity.

In conclusion, it was observed that ethanolic extract of the bark of *T. populnea* may prove to be useful in treatment of conditions like type II diabetes mellitus (NIDDM) probably by overcoming the insulin resistance.

Competing Interests

The authors hereby declare that there are no competing interests.

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Full length research paper

Clinical and microbiological effects of systemic ciprofloxacin and metronidazole in *Aggregatibacter actinomycetemcomitans*-associated periodontitis

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Destructive periodontal disease is a concern because of the potential damage to the dentition and the financial burden of treatment. It is generally agreed that the microorganisms residing in periodontal pockets are responsible for periodontitis. Approximately 500 bacterial taxa inhabit periodontal pockets. Association between *Aggregatibacter actinomycetemcomitans* and destructive and progressive forms of periodontitis has been demonstrated by many authors. The aim of this study was to evaluate the microbiological and clinical effects of systemic metronidazole-ciprofloxacin therapy. In this triple-blind controlled clinical randomized trial, 24 patients with at least 4 sites with ≥4 mm of clinical attachment loss and detection of *A. actinomycetemcomitans* were included. The patients were randomly divided into two groups. The patients in the test group received scaling/root planning (S/RP) and ciprofloxacin plus metronidazole, and the patients in the control group received S/RP and placebo representing the aforementioned antibiotics. Bacteria culturing and recording of clinical Att. loss (CAL), gingival index (GI), plaque index (PI) and bleeding on probing (BOP) were done at the baseline, 10 days, 3 and 6 months after the mechanical therapy. There was no significant difference between test and control groups in *A. actinomycetemcomitans* colony count, CAL, GI, PI and BOP at the baseline. A significant difference was seen in each group between baseline and each of the other research stages for all the recorded parameters. Test group showed a significant difference in *A. actinomycetemcomitans* colony count, BOP and GI on 3rd and 6th months of research as compared to the baseline. No significant difference was seen between two groups in CAL and PI. Application of ciprofloxacin plus metronidazole as an adjunctive to mechanical therapy has significant effects on periodontal clinical parameters and eradication of *A. actinomycetemcomitans* from periodontal tissues.

Key words: Ciprofloxacin, metronidazole, periodontitis, *Aggregatibacter actinomycetemcomitans*.

INTRODUCTION

Destructive periodontal disease is a concern because of the potential damage to the dentition and the financial burden of treatment (Slots, 2002). It is well established that microbiological accumulations on the tooth surface cause inflammatory reactions in the adjacent periodontal tissues (Muller et al., 1998). One of these microorganisms

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is *Aggregatibacter actinomycetemcomitans*, a Gram-negative rod-shaped bacterium (Pavicic et al., 1994). Forms of periodontitis associated with the presence of *A. actinomycetemcomitans* are aggressive periodontitis, sever types of periodontitis and periodontitis as a manifestation of systemic diseases (Marc et al., 2006). Patients suffering from *A. actinomycetemcomitans*-associated periodontitis often respond less favorably to conventional therapy, consisting of mechanical debridement (Pavicic et al., 1994). Renvert et al. (1990) showed that elimination of *A. actinomycetemcomitans* could not predictably be established by repeated mechanical treatment and periodontal surgery in periodontal patients (Renvert et al., 1990). Many other studies showed that *A. actinomycetemcomitans*-associated periodontitis responds poorly to mechanical therapy (Van Winkelhoff and Carolien, 1992). The ability of *A. actinomycetemcomitans* to penetrate epithelial cells and colonize the periodontal tissues around lesion has given support to this pathogen.

Therefore, adjunctive antibiotic has been advised in the treatment of *A. actinomycetemcomitans*-associated periodontitis in an attempt to eradicate the stated microorganism (Karen and Novak, 2006). Thus, the aim of this study was to evaluate the effects of ciprofloxacin-metronidazole therapy as an adjunct to mechanical therapy in treatment of *A. actinomycetemcomitans*-associated periodontitis and eradication of this microorganism.

MATERIALS AND METHODS

Patients

A total number of 24 patients with untreated periodontitis and sub-gingival detection of *A. actinomycetemcomitans* were enrolled into the study. All patients had at least 4 teeth with 4 mm or above of attachment loss. The patients were enrolled if *A. actinomycetemcomitans* culture and bleeding on probing on these four teeth were positive.

Patients with any of the following conditions were excluded from the study: having any systemic diseases, requirement for antibiotic prophylaxis, receiving any periodontal treatment in the previous 3 months, intake of medications which influence the periodontal tissues (such as non-steroid anti-inflammatory drug (NSAIDS), calcium canal blockers, anticonvulsants, cyclosporine A), allergy against ciprofloxacin or metronidazole, pregnancy or lactation or use of antibiotics in the previous month. All patients enrolled into the study signed the informed consent approved by the Ethics Committee of the Dental Faculty, Azad University.

Clinical measurements

Probing depth, attachment loss, plaque index, gingival index and bleeding on probing were assessed in all patients. Sub-gingival samples were also prepared from the sites with 4 mm attachment loss to detect *A. actinomycetemcomitans*.

Probing depth, attachment loss and bleeding on probing were assessed using a William's probe (Hu-friedy, USA). Gingival index assessment was also carried out using the same probe and the criteria demonstrated by Loe and Sillness (1963).

Samples

The subject's tooth has been isolated by sterile cotton roles and sub-gingival sample were obtained with two sterile paper cones (#30) penetrated into the deepest site of the periodontal pocket. After 20 s, the paper cones were transferred to the culturing medium, which will be described later, and have been placed in the center of the plate and sent to the laboratory immediately.

Treatment

All patients that underwent periodontal treatment consisted of sub- and supra-gingival debridement (using conventional periodontal curettes and ultrasonic scaler) and oral hygiene instruction (modified Bass technique). All the treatment steps were carried out by a well-trained dentistry student under the supervision of a periodontist. After this initial therapy, patients were randomly assigned to receive the antibiotic packs labeled either "1" or "2". One of the numbers was antibiotic and the other was placebo. Because this study was triple-blind, neither the researchers nor the patients were aware of the contents of a pack. The regimen was 250 mg metronidazole, three times daily for a week and 500 mg ciprofloxacin, twice a day for 8 days.

All the measurements and samples collecting were repeated 10 days, 3 and 6 months after the mechanical therapy.

Microbiological analysis

In this study, the medium used to culture *A. actinomycetemcomitans* was tryptic soy serum plus bacitracin and vancomycin (TSBV). It consists of trypticase soy agar (40 g/L) and yeast extract (1 g/L) as the base medium. After autoclaving the solution (121°C, 15 pounds for 15 min), horse serum (100cc), bacitracin (75 µg/ml) and vancomycin (2.5 µg/ml) were added to the medium when it reached 45°C temperature. The medium was then dispensed into microbiological plates.

In the laboratory, the paper cones in the center of the plates were rolled to the edges with a sterile microbiological loop to impregnate the microorganisms and then set in an anaerobic jar (with a gaspack). The jar was kept in an incubator for 5 days.

After 5 days of incubation at 37°C, the plates were evaluated for detection of *A. actinomycetemcomitans* colony by a light microscope with 10X lens and star-shaped *A. actinomycetemcomitans* colonies were counted.

RESULTS

All the 24 patients enrolled into the study completed all examinations throughout the 6 months study period. None of the patients reported adverse effects due to taking the antibiotics. The baseline examinations data in test and control patients are shown in Table 1.

As indicated in Table 1, differences between test and control patients in all parameters are inconsiderable. Follow-up examinations took place 10 days, 3 and 6 months after the mechanical treatment.

As shown in Table 2, which is the static outcome of clinical attachment loss data in test and control patients, there was no significant difference between test and control groups at any stage ($P>0.05$). Table 3 shows the *A. actinomycetemcomitans* colony count of patients in each group in all research stages. Significant differences

Table 1. Assessed parameters in patients with *A. actinomycetemcomitans*-associated periodontitis before undergoing metronidazole-ciprofloxacin-therapy.

Parameter	Test	Control	P-values
Mean CC	21.29	21.66	0.956
Mean CAL	4.58	4.58	0.942
Mean PI	96.9	95.1	0.747
BOP +	12 (100%)	12 (100%)	
Mean GI	2	2	

CC: Colony count (colonies of *A. actinomycetemcomitans* seen under microscope), CAL: clinical attachment loss (measured by use of periodontal probe), PI: plaque index (percentage of teeth surfaces with plaque disclose by plaque indicator tablets), BOP: bleeding on probing, GI: gingival index (Loe and Silness, 1963).

Table 2. Mean clinical attachment loss in test and control patients at different stages of the research.

Parameter	Test	Control	P-value
Baseline	4.58	4.58	0.942
10 Days	3.26	3.27	0.944
3 Months	3.27	3.07	0.539
6 Months	3.27	3.08	0.571

Table 3. *A. actinomycetemcomitans* colony count of test and control patients at different stages of research.

Parameter	Group	Min.	Max.	Mean	P-value
Baseline	Test	3.5	54.75	21.23	0.956
	Control	3.75	55.5	21.67	
10 days	Test	0	2.75	0.35	0.086
	Control	0	21.25	4.04	
3 months	Test	0	3.25	0.75	0.022
	Control	1	32.5	9.15	
6 months	Test	0	7.5	1.96	0.008
	Control	1.25	35.25	12.56	

were seen at 3rd and 4th examinations (months 3 and 6) between test and control groups (P<0.05).

Patients bleeding on probing are shown in Table 4. Again, at 3rd and 4th examinations, significant differences were seen between test and control group (P<0.05). Test and control group showed significant differences at 3rd and 4th examinations in gingival index (P<0.05). Table 5 demonstrates gingival indices of test and control patients. In Table 6, plaque indices of patients are shown. No significant difference was seen at any stage of research between test and control group (P>0.05).

DISCUSSION

The aim of the present study was to evaluate the long-term microbiological and clinical effects of mechanical debridement, followed by ciprofloxacin-metronidazole therapy in *A. actinomycetemcomitans*-associated periodontitis. The results show that this combined therapy is effective in suppressing *A. actinomycetemcomitans* below cultivable levels in periodontal pocket, and also in treatment of bleeding on probing and high gingival index. No significant difference was seen in plaque index and attachment loss between test and control groups.

A. actinomycetemcomitans was suppressed below cultivable level in 21 out of 24 patients within the first 10 days of the study, while 9 patients in the control group still had *A. actinomycetemcomitans* in their periodontal pockets. Although, this result did not show significant difference, significant difference was found at 3rd and 6th months of the study.

Similar results have been reported by Muller et al. (1998), Soleymani et al. (2004), Yek et al. (2010), Aimetti et al. (2012), Heller et al., (2011), Soleymani et al. (2004),

Table 4. Number of patients with bleeding on probing in different research stages.

Parameter	Group	No tooth	One tooth	Two teeth	Three teeth	Four teeth	P-value
Baseline	Test	-	-	-	-	12	-
	Control	-	-	-	-	12	
10 Days	Test	12	-	-	-	-	0.319
	Control	9	3	-	-	-	
3 Months	Test	12	-	-	-	-	0.000
	Control	2	5	4	1	-	
6 Months	Test	12	-	-	-	-	0.000
	Control	2	4	4	2	-	

Table 5. Gingival indices of test and control patients in different research stages.

Parameter	Group	Min.	Max.	Mean	P-value
10 Days	Test	0	0.25	0.04	0.101
	Control	0	1.25	0.38	
3 Months	Test	0	0.25	0.06	0.000
	Control	0.25	1.75	1.02	
6 Months	Test	0	0.25	0.10	0.000
	Control	0.5	1.75	1.21	

Table 6. Plaque indices of test and control patients at different research stages.

Parameter	Test	Control	P-value
Baseline	96.92	95	0.747
10 Days	43.58	37.33	0.414
3 Months	69.33	70.17	0.871
6 Months	81.33	81.58	0.964

be considered.

This treatment was also able to decrease bleeding on probing by significant difference between test and control groups in our study. None of the patients in test group had bleeding on probing at any of the research stages.

Lopez et al. (2000), Yek et al. (2010), Aimetti et al. (2012) and Heller et al., (2011) have reported similar results. Adverse result was reported by Bain et al. (1994), since they were unable to show significant difference in bleeding on probing between test and control groups, 24 weeks after the treatment. Different medication seems to be the cause of this controversy, as in Bain's (1994) research, the patients received spiramycin 1.5 million IU bid, and in this study, the patients received ciprofloxacin 500 mg bid plus metronidazole 250 mg tid.

In this study, no significant differences were seen in attachment loss between two groups. Tezel et al. (2005) demonstrated significant differences in attachment loss between test and control groups at the 3rd month of their study. They used different product of ciprofloxacin (Siprosan 500 mg) and in higher dose (tid) and this seems to be the cause of this controversy. Flemmig et al. (1998) also showed significant difference in attachments loss between test and control groups at 12th month of their study. Such controversy may be the result of different attachment loss measurement devices usage. In this study, no significant difference was seen in plaque indices between test and control groups at any stages of the study.

Tinoco et al. (1998) and Bain et al. (1994) were unable to show any significant difference in plaque index between test and control groups, which is similar to the

results of this study. According to the results of this study, we conclude that systemic application of ciprofloxacin plus metronidazole adjacent to mechanical debridement is effective for suppression of A. actinomycetemcomitans and clinical improvement of periodontal tissues.

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ABBREVIATIONS

°C, Degree Celsius; **µg,** microgram; **A.a,** actinobacillus actinomycetemcomitans; **att.,** attachment; **bid,** bis in die (two times a day); **BOP,** bleeding on probing; **CAL,** clinical attachment loss; **cc,** cubic centimeter (milliliter); **GI,** gilgival index; **g,** gram; **IU,** international unit; **mg,** milligram; **ml,** milliliter; **mm,** millimeter; **NSAID,** non-steroid anti-inflammatory drug; **P,** probability value (p-value); **PI,** plaque index; **S/RP,** scaling and root planing; **tid,** ter in die (three times a day); **TSBV,** tryptic soy serum plus bacitracin and vancomycin.

Competing Interests

The authors hereby declare that there were no competing interests.

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