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Full Length Research Paper

Chemotherapeutic efficacy of secnidazole-diminazene aceturate combination therapy in experimental *Trypanosoma brucei brucei* infection in rats

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The chemotherapeutic efficacy of secnidazole-diminazene aceturate (SEC-DA) combination therapy was studied in rats experimentally infected with *Trypanosoma brucei brucei* with a view of eliminating relapse of infection. Twenty rats grouped into 4 groups ($n = 5$) were used for the study as follows: uninfected untreated (A), infected untreated (B), infected and treated with diminazene (DA) (3.5 mg/kg) alone intraperitoneum (IP) once (C), infected and treated with SEC (200 mg/kg, orally) + DA (3.5 mg/kg, IP) (D). Treatment started 8 days post-infection. Parasites disappeared from the blood of the SEC-DA treated rats after 3 days of treatment against 5 days for DA alone. Relapse infection occurred in DA alone, 13 days post-treatment. There was no relapse infection in the SEC-DA-treated rats up to 70 days post-treatment. Hematological profiles of the animals after treatment showed significantly ($p < 0.05$) higher total leucocytes, lymphocytes and neutrophil in the SEC-DA group when compared with DA-treated group. Significant ($p < 0.05$) increase in the hemoglobin concentration and a decrease in the alanine transaminase (ALT) activity were also observed in the SEC-DA group as compared to the DA alone treated group. It is concluded therefore that the SEC-DA combination therapy was more efficacious than DA alone.

Key words: Combination therapy, efficacy, relapse, secnidazole-diminazene aceturate, trypanosomosis.

INTRODUCTION

The use of drugs for control and treatment of African animal trypanosomosis has been in practice for decades, but the rapidity with which trypanosomes develop

resistance to these trypanocides has become a source of worry. Diminazene aceturate at the dose of 3.5 to 7 mg/kg has been recommended for the treatment of

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Trypanosoma brucei brucei infection. But nowadays it seems that this dose (3.5 mg/kg) is only able to get rid of clinical signs but fails to clear the parasites (Desquesnes and Gutiérrez, 2011). Relapse of infection occurred in dogs treated with 7 mg/kg DA (Chukwu et al., 1990). These treatment failures have been attributed to resistance by trypanosomes (Gutiérrez et al., 2013). Thus, due to increasing rates of treatment failure, relapse of previously treated cases where re-exposure has been precluded, drug combination has been suggested as an alternative treatment method to achieve greater efficacy (Onyeyili and Egwu, 1995). It has been suggested that combination of DA with other therapeutically active trypanocides or adjuvants may improve treatment outcome in animals (De dekens et al., 1989). However, effective combination therapies with good clinical application seem elusive. The treatment of dogs infected with *T. b. brucei* and *Trypanosoma congolense* with eflornithine and DA combination was not curative (Onyeyili and Anika, 1990).

Secnidazole (SEC) is effective against anaerobic micro-organisms and protozoa and appears particularly effective in the treatment of amoebiasis, giardiasis, trichomoniasis and bacterial vaginosis (Laurence et al., 2006). Secnidazole is widely distributed in the body and crosses the blood brain barrier in sufficient quantity (Rang et al., 1996). Diminazene aceturate is limited in its distribution in the brain due to its polarity, thus, it does not accumulate in therapeutic concentration (Onyeyili and Anika, 1991). Therefore, sequestered trypanosomes in the brain are not eliminated. This has been shown to be the source of relapse infection in animals (Soeiro et al., 2005). The *in vitro* and *in vivo* antitrypanosomal activities of SEC have been demonstrated, and SEC showed a concentration and dose dependent antitrypanosomal effects *in vitro* and *in vivo* (Eke et al., 2017). It is thought that combination of SEC and DA in the treatment of *T. b. brucei* infection in rats will produce additive synergistic effect, through the combination of their individual trypanocidal effects and pharmacokinetic properties. The aim of this study was therefore to evaluate the therapeutic efficacy of SEC-DA combination in the treatment of experimental *T. b. brucei* infection in rats.

MATERIALS AND METHODS

Trypanosomes

The *T. b. brucei* used in this study was originally isolated from a dog presented at the Veterinary Teaching Hospital University of Nigeria Nsukka. Morphological identification and blood incubation infectivity test were by standard procedure (Rickman and Robson, 1970; Soulsby, 1982). These parasites were maintained in rats from which experimental animals were infected.

Animals

Twenty Sprague Dawley rats obtained from the laboratory

animal facility of the Department of Veterinary Physiology and Pharmacology, University of Nigeria Nsukka were used for the study. They were housed in stainless steel rat cages. They were fed *ad libitum* with pelletized feed (Vital Feeds®). Clean drinking water was also provided *ad libitum*. They were acclimatized for one week before the study. The animal experimental protocol was approved by the Experimental Animal Ethics Committee of the Faculty of Veterinary medicine, University of Nigeria, Nsukka, August 2015 and in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November 24, 1986 (86/609/EEC).

Experimental

The rats were randomly assigned to 4 groups (n = 5). They were treated as follows: Group A: uninfected and untreated, served as the control; Group B: infected untreated; Group C: infected and treated with DA (3.5 mg/kg IP once); Group D: infected and treated with 3.5 mg/kg DA IP once and 200 mg/kg SEC orally.

Secnidazole (SEC) was administered for 6 days. Diminazene aceturate (Lobazene® France) was given immediately after administration of SEC (Secwid® May and Baker Nigeria PLC) on the first day of treatment. The dose of secnidazole used in this study was based on the result of preliminary antitrypanosomal effects of SEC.

Infection of rats

Parasitemia was estimated using the rapid matching technique (Herbert and Lumsden, 1976). All rats were infected intraperitoneally using 1×10^6 *Trypanosoma b. brucei* suspended in 0.2 ml of phosphate buffered saline (PBS). Rats were monitored daily for onset of parasitemia.

Parasitemia was established 5 days post-infection (PI) and treatment starting from day 8 PI, when parasitemia was well established on examination of blood films in all the infected rats. The hematocrit buffy coat technique and stained thin smears were used to confirm total parasite clearance (OIE, 2008).

Parameters for assessing therapeutic efficacy

Parasitemia

This was monitored daily after treatment. Parasitemia level, time of clearance of parasitemia and time of relapse of infection were determined and recorded.

Hematological changes

Samples for hematology were collected into EDTA sample bottles. Samples were collected on days 0, 7, 14 and 21. Full blood count (FBC) was done using the method of Schalm et al. (1975). Packed cell volume (PCV) was done using the microhematocrit method (Coles, 1986). Hemoglobin (Hb) concentration was determined using the cyanomethaemoglobin method (Coles, 1986). Changes in these parameters were measured and recorded.

Alanine transaminase activity

Alanine transaminase (ALT) activity was determined on days 14 and 21 PI using Randox^(R) kits (Randox Laboratories Ltd United Kingdom) according to the manufacturer's guidelines. This is to monitor the effect of treatment on the changes caused by *T. b.*

Table 1. Mean log parasitemia in rats infected with *T. b. brucei* and treated with either SEC-DA combination or DA alone.

	Days post-treatment									
	8	9	10	11	12	13	21	24	35	70
A	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00	0.0±.00	0.0±.00
B	7.9 ± .15 ^b	8.1 ± .09 ^b	8.2±.20 ^b	8.5±.15 ^b	8.6±.12 ^b	8.9±.15 ^b	AD			
C	8.3±.18 ^b	7.9±.20 ^b	4.7±.29 ^c	2.4±.24 ^c	1.1±.20 ^c	0.0±.00 ^a	5.4±.20 ^b			
D	7.9±.20 ^b	6.2±.35 ^c	5.4±.45 ^c	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00 ^a	0.0±.00	0.0±.00	0.0±.00

Different superscripts on the same row vary significantly ($p < 0.05$). AD: all rats in this group died before day 21. Data collection on parasitemia for each group stops as soon as relapse (reappearance of parasites in the blood after initial clearance following treatment) is observed. A: uninfected untreated, B: infected untreated, C: infected and treated with diminazene aceturate (3.5 mg/kg), D: infected and treated with secnidazole (200 mg/kg) + diminazene aceturate (3.5 mg/kg). Day 8 was the first day of treatment.

brucei to the liver.

Statistical analysis

Data collected were presented as mean \pm SEM in tables. Analyses of data were done using one way analysis of variance. Variant means were separated using least significant difference (LSD). Significance was accepted at $p < 0.05$.

RESULTS

Parasitological findings

There was significant ($p < 0.05$) reduction in parasitemia in the two treatment groups following treatment when compared with the infected untreated control. Parasitemia cleared completely 3 days post-treatment (PT) in all the rats treated with SEC-DA combination, while it cleared 5 days PT in rats treated with DA alone. Parasitemia was progressive in the untreated group and all the rats in the group died by day 21 PI. Relapse of infection occurred in group C (DA alone) 13 days PT (day 21 post-infection). There was no relapse of infection in the combination treatment up to the end of the experiment (70 days PT) (Table 1).

Hematological findings

White blood cells

There was significantly ($p < 0.05$) higher level of leukocytes in the uninfected untreated control when compared with the infected rats on day 7 PI. On day 14, PI, significantly ($p < 0.05$) lower level of WBC was observed in the infected untreated control when compared with the groups infected and treated with DA alone, the SEC-DA combination and uninfected untreated. However, by day 21, the WBC count in the group treated with the SEC-DA combination was significantly ($p < 0.05$) higher than that of the group

infected and treated with only DA and the uninfected untreated (Table 2).

Lymphocytes were significantly ($p < 0.05$) higher in the uninfected untreated group on day 7 PI, while significant ($p < 0.05$) increase in the lymphocyte count was recorded in the SEC-DA group on days 14 and 21 when compared with both the uninfected untreated and infected and treated with DA alone. Significantly ($p < 0.05$) higher levels of neutrophil were observed in the uninfected untreated group on days 7 and 14 when compared with the other groups. Nevertheless, on day 21 PI, there was significant ($p < 0.05$) drop in neutrophil in the group infected and treated with only DA (Table 3).

Red blood cell counts, PCV and hemoglobin (Hb) concentration

There was significant ($p < 0.05$) reduction in the RBC number in all infected rats on day 7 PI. Treatment with either SEC-DA combinations or DA alone lead to significant ($p < 0.05$) increase in the number of RBC over the infected untreated group by day 14 PI and by day 21 PI, there was no significant ($p > 0.05$) difference between the uninfected untreated and the infected and treated groups. Significant ($p < 0.05$) reduction in the PCV was observed in all infected animals on day 7 PI when compared with the uninfected untreated control. This however, significantly ($p < 0.05$) increased in all the treated groups by day 14 PI more than the infected untreated. By day 21, there was no significant ($p > 0.05$) variation between the infected and treated groups and the uninfected untreated control. Hemoglobin (Hb) concentrations in all the infected animals were significantly ($p < 0.05$) lower than the uninfected untreated control on day 7 PI. Following treatment, there was significant ($p < 0.05$) increase in all the treated animals more than the infected untreated on day 14. On day 21 PI, the Hb concentration of the SEC-DA group was significantly ($p < 0.05$) higher than both the group treated with only DA and the uninfected untreated group (Table 4).

Table 2. Mean total WBC ($\times 10^3/\mu\text{l}$) count of *T. b. brucei* infected rats treated with SEC-DA combination or DA alone.

	Days post-infection			
	0	7	14	21
A	14.5 \pm 6.6	11 \pm 3.7 ^a	10.9 \pm 18.0 ^a	10.0 \pm 9.9 ^a
B	14.6 \pm 26.2	5.4 \pm 6.5 ^b	7.6 \pm 8.4 ^b	AD
C	14.5 \pm 14.6	5.2 \pm 3.0 ^b	10.6 \pm 12.0 ^a	8.3 \pm 5.2 ^a
D	14.6 \pm 25.3	5.3 \pm 22.4 ^b	11.3 \pm 11.8 ^a	22.1 \pm 16.4 ^b

Different superscripts on the same row vary significantly ($p < 0.05$). AD: all rats in this group died before day 21. A: uninfected untreated, B: infected untreated, C: infected and treated with diminazene aceturate (3.5 mg/kg), D: infected and treated with secnidazole (200 mg/kg) + diminazene aceturate (3.5 mg/kg). Rats were infected on day 0 and treated on day 8.

Table 3. Mean lymphocyte and neutrophil counts of *T. b. brucei* infected rats treated with SEC-DA combination or DA alone.

	Days post- infection			
	0	7	14	21
Lymphocytes ($\times 10^3/\mu\text{l}$)				
A	9.0 \pm 5.7	8.1 \pm 5.0 ^a	8.6 \pm 8.5 ^a	8.6 \pm 85.2 ^a
B	8.7 \pm 20.6	3.7 \pm 4.4 ^b	7.2 \pm 12.9 ^a	AD
C	9.9 \pm 18.4	3.6 \pm 1.2 ^b	8.4 \pm 6.2 ^a	7.9 \pm 15.9 ^a
D	10.0 \pm 6.6	3.1 \pm 14.1 ^b	11.1 \pm 13.5 ^b	19.3 \pm 19.5 ^b
Neutrophil ($\times 10^3/\mu\text{l}$)				
A	3.80 \pm 1.41	1.20 \pm 2.25 ^a	2.50 \pm 2.73 ^a	2.50 \pm 2.75 ^a
B	3.10 \pm 8.13	0.70 \pm 2.38 ^b	0.40 \pm 1.29 ^b	AD
C	2.90 \pm 4.49	0.60 \pm 1.30 ^b	0.40 \pm 0.33 ^b	0.03 \pm 0.28 ^b
D	2.80 \pm 2.12	0.90 \pm 4.56 ^b	0.60 \pm 2.54 ^b	3.60 \pm 10.15 ^a

Different superscripts on the same row vary significantly ($p < 0.05$). AD: all rats in this group died before day 21. Rats were infected on day 0 and treated on day 8. A: uninfected untreated, B: infected untreated, C: infected and treated with diminazene aceturate (3.5 mg/kg), D: infected and treated with secnidazole (200 mg/kg) + diminazene aceturate (3.5 mg/kg).

Serum alanine transaminase activity

The infected untreated rats showed significantly ($p < 0.05$) higher ALT activity on day 14 PI when compared with the uninfected untreated, infected and treated with SEC-DA and DA alone. Nevertheless, the SEC-DA group showed consistently significant ($p < 0.05$) lower levels of ALT activity on both days 14 and 21 PI when compared with the uninfected untreated, infected untreated and the group treated with only DA (Table 5).

DISCUSSION

Combination therapy of SEC-DA was more effective than mono-therapy of DA in rats experimentally infected with *T. b. brucei*. It caused faster clearance of parasitemia than single treatment with DA. The SEC-DA combination therapy also prevented relapse of infection, unlike single treatment with DA. Parasites were absent in the SEC-DA

group after 3 days of treatment, while it cleared 5 days post-treatment (PT) with DA alone. Faster clearance of parasites by SEC-DA combination therapy could be attributed to the additive trypanocidal effects of both SEC and DA. The absence of relapse of infection in the SEC-DA combination could be as a result of the extensive distribution of SEC into organs and tissues where trypanosomes sequester and where DA accumulation is limited (Onyeyili and Anika, 1989; Onyeyili and Anika, 1991). These findings give credence to possible chemotherapeutic synergy between SEC and DA. Trypanosomiasis is characterized by severe reduction of the WBCs and subsequent immunosuppression (Allam et al., 2011). In the present study, there was massive depletion of total leucocyte counts of all infected rats which persisted in the untreated rats. However, after 7 days PT, there was gradual recovery of the leukocytes. The leukocytic response was stronger in the SEC-DA combination therapy. The lymphocytes are the most important cells in the immunological response to

Table 4. Mean RBC, PCV and hemoglobin (Hb) concentration of *T. b. brucei* infected rats treated with SEC-DA combination or DA alone.

	Days post-infection			
	0	7	14	21
RBC (x 10⁶/μl)				
A	8.8 ± 14.6	8.0 ± 61.6 ^a	7.7 ± 35.3 ^a	6.9 ± 483
B	8.8 ± 66.3	7.3 ± 70.1 ^b	4.6 ± 41.3 ^b	AD
C	8.8 ± 28.6	6.7 ± 50.2 ^b	6.7 ± 46.6 ^a	7.0 ± 35.2
D	8.0 ± 26.7	7.3 ± 40.2 ^b	6.9 ± 67.9 ^a	7.3 ± 12.5
PCV (%)				
A	43.2 ± .92	33.8 ± 1.00 ^a	44 ± .84 ^a	38.6 ± 1.30
B	43.4 ± 1.33	25.4 ± 1.17 ^b	31.2 ± 3.02 ^b	AD
C	43.8 ± 1.43	26 ± 1.55 ^b	41.4 ± 0.60 ^b	43.2 ± 1.24
D	44 ± 0.55	25.4 ± 0.93 ^b	42.8 ± 2.08 ^b	39.4 ± 0.93
Hb conc.(g/dl)				
A	28.2 ± 0.80	19.4 ± 0.67 ^a	18.8 ± 0.37 ^a	14 ± 0.71 ^a
B	29.8 ± 0.58	15.2 ± 0.58 ^b	8.75 ± 1.25 ^b	AD
C	28.8 ± 0.73	17 ± 0.68 ^b	17.6 ± 0.4 ^a	15.4 ± 0.51a
D	32.0 ± 3.30	17.2 ± 0.66 ^b	18.75 ± 1.18 ^a	20.2 ± 0.37 ^b

Different superscripts on the same row vary significantly ($p < 0.05$). AD: all rats in this group died before day 21. Rats were infected on day 0 and treatment started on day 8. A: uninfected untreated, B: infected untreated, C: infected and treated with diminazene aceturate (3.5 mg/kg), D: infected and treated with secnidazole (200 mg/kg) + diminazene aceturate (3.5 mg/kg).

Table 5. Serum alanine transaminase activity (ALT) in *T. b. brucei* infected rats treated with SEC-DA combination or DA alone.

ALT (μl)	Days post-infection	
	14	21
A	122.1 ± 7.8 ^a	119.0 ± 12.2 ^a
B	160.9 ± 4.4 ^b	AD
C	105.6 ± 4.1 ^a	118.3 ± 9.9 ^a
D	28.1 ± 2.4 ^c	32.5 ± 9.7 ^b

Different superscripts on the same row vary significantly ($p < 0.05$). AD: all rats in this group died before day 21. Rats were infected on day 0 and treated on day 8. A: uninfected untreated, B: infected untreated, C: infected and treated with diminazene aceturate (3.5 mg/kg), D: infected and treated with secnidazole (200 mg/kg) + diminazene aceturate (3.5 mg/kg).

trypanosomosis in animals (Emeribe and Anosa, 1991). In this study, the SEC-DA combination therapy produced higher lymphocytic response than DA alone. This strong immunological response could be responsible for the earlier clearance of parasitemia and prevention of relapse infection by SEC-DA combination therapy leading to faster recovery of the animals. It could also suggest possible immunostimulatory effect of SEC.

Anemia is a regular and an important feature of

trypanosomosis in animals (Abenga et al., 2016). Trypanosomes cause massive destruction of RBCs and subsequent depletion of the RBC number, severe reduction of the PCV and hemoglobin concentrations. These features were prominent in infected rats in this study. There was recovery of the RBC number, improvement of PCV and increased Hb concentration after treatment with SEC-DA combination and with DA alone. There was no significant difference in the rate of recovery of the hematocrit between the SEC-DA combination therapy and DA alone throughout the period of the study except in the Hb concentration, where there was significant increase in the SEC-DA group over the other groups on day 21 PI. Trypanosomosis caused by *T. b. brucei* is characterized by tissue invasiveness. This adversely affects the serum biochemical parameters (Allam et al., 2011). These changes are associated with some organ damages and monitoring of these changes after treatment could be helpful in prediction of treatment outcome and effectiveness of treatment. Increase in the ALT activity in *T. b. brucei* infection has been reported by various authors (Ezeokonkwo et al., 2012). In this study, expectedly, ALT activity was elevated in infected untreated rats by 14 days post infection. This finding is consistent with the report of Taiwo et al. (2003), who reported elevated ALT in sheep experimentally infected with *T. b. brucei*. However, treatment with DA and SEC-DA combination lowered the ALT activity in infected and

treated rats. Nevertheless, lower ALT activity was recorded in the SEC-DA treated rats compared with DA treated rats. On the other hand, on day 21 PI, elevation of ALT activity was observed in rats treated with DA alone and this coincided with relapse infection in the rats treated with DA alone on the same day. The observed effect of the SEC-DA combination therapy on the serum ALT activity could be due to enhanced parasite clearance from the liver, due to extensive distribution of SEC in the liver thereby preventing further liver damage.

It is therefore concluded that SEC-DA combination therapy in rats was more effective therapy than DA alone in experimental *T. b. brucei* infection. This is because the combination therapy effectively reversed some of the hematological and biochemical changes associated with trypanosomiasis. The combination therapy also caused faster clearance of parasitemia and prevented relapse of infection.

ABBREVIATIONS

SEC, Secnidazole; **DA**, diminazene aceturate; **SEC-DA**, secnidazole-diminazene aceturate; **IP**, intraperitoneum; **PI**, post-infection; **PT**, post-treatment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

In-vivo* evaluation of analgesic, anti-inflammatory and anti-pyretic activity of aqueous methanolic extract of *Jatropha gossypifolia

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Many pathological conditions are associated with pain, fever and inflammation. Synthetic drugs available for the treatment of these ailments accompany many unwanted side effect; most prominent of which is the gastric ulcer. Present study was thus aimed at evaluating the analgesic, anti-pyretic and anti-inflammatory potential of aqueous methanolic extract of leaves of plant *Jatropha gossypifolia*. Anti-inflammatory activity was evaluated using carrageenan induced paw edema. Acetic acid induced writhing test and hot plate method was used to assess the analgesic activity. Anti-pyretic activity was ascertained using brewer's yeast induced pyrexia. The aqueous methanolic extract of the plant *J. gossypifolia* have demonstrated significant analgesic, anti-pyretic and anti-inflammatory activity at 200 mg/kg dose. The data was statistically analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparisons. It has thus concluded that aqueous methanolic extract of leaves of plant *J. gossypifolia* possess analgesic, anti-inflammatory and anti-pyretic properties. These results strongly support the ethno-pharmacological use of this plant as anti-pyretic, analgesic and anti-inflammatory agent.

Key words: *Jatropha gossypifolia*, anti-inflammatory, analgesic, anti-pyretic.

INTRODUCTION

Plant derived chemicals have been used by men for centuries as a source of medicine (Semwal et al., 2010).

A large in number of the population in Pakistan has been using these medicinal plants as their primary source of

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medicine health care system (Shinwari, 2010). The use of such medicinal plants as remedy against various ailments is known by the name of hikmat. Approximately 40,000 of such registered hakims are practicing as health care professional using plant extracts for curing various diseases (Saeed et al., 2011). Practicing as hakim, have no scientific bases therefore needs validation on scientific basis (Khan et al., 2014). *Jatropha gossypifolia* plant belongs to the family "Euphorbiaceae" is locally known as "Lal bherandha". This plant family possesses about 170 different types of species (Kamal et al., 2011). It is extensively found in southern region of Pakistan. The plant has long been used as astringent, anti-cancer, analgesic, anti-inflammatory, diaphoretic and as anti-feedant agent. The leaf bath is used for sores, sprains and rashes. Also the decoction of the leaves is useful for stomach ache, venereal diseases and as a blood purifier (Vickers, 2001). Furthermore this plant was also been used in various skin related ailments and some blood related disorders. The leaf extract has been used as an anticoagulant for biochemical and hematological analysis (Oduola et al., 2004). Whole plant including seeds, flowers, fruit and leaves have medicinal properties. In the present study the aqueous methanolic extract of leaves of the plant *J. gossypifolia* had been used for its potential analgesic, anti-inflammatory and anti-pyretic activity in animal models.

MATERIALS AND METHODS

Chemicals

Indomethacin was purchased from Liometacin Chesi Pharmaceutical, Pakistan. Paracetamol (Provas inj) and Tramadol (Tonoflex inj) were purchased from SAMI Pharmaceuticals Pvt. Ltd. Dimethyl sulfoxide liquid (DMSO), acetic acid, and Brewer's yeast were purchased from Lohari Laboratory Chemicals Pvt. Ltd. Lahore Pakistan; Carrageenan was purchased from Sigma lumba, USA. Sterile water for injection, normal saline (Medisol pharmaceuticals) were obtained from Baber Medicine Company, Kot lakhpatt, Lahore, Pakistan. DMSO was used as control vehicle in all the experiments and all the dilutions of the plant extract were prepared in DMSO.

Animals

The experiments were carried out in albino rats (aging between 40 and 50 days) of species *Rattus norvegicus* of either sex weighing 130 to 150 g. The rats were locally purchased from University of Health Sciences Lahore. The rats were maintained in standard laboratory conditions of 22 to 25°C with alternate light/dark 12/12 h periods. The rats were feed in pellet form and water *ad libitum*.

Plant materials

Leaves of the plant *J. gossypifolia* were collected locally in the month of July to August, 2013. Specimen of the subject plant was identified by Taxonomist in the Department of Botany Govt. College

University Lahore and a specimen was also submitted there in the herbarium with voucher number 2235/Bot. The extract was prepared and standardized as described by Jabeen et al. (2009), briefly; the leaves approximately 700 g were sunshade dried for 14 days and then powdered. The powdered material was then dissolved in 70% methanol with gentle shaking thrice every day for 7 days. The extract was then filtered through filter paper and concentrated using rotary evaporator at low temperature (Jabeen et al., 2009).

Analgesic activity

Acetic acid induced writhing test

Albino rats were divided randomly into five groups comprising of 4 rats each. The rats were deprived of feed 6 h before experiment. Group I received control DMSO liquid 10 ml/kg, group II received indomethacin 10 mg/kg and the III, IV and V groups received *J. gossypifolia* leaves extract 50, 100 and 200 mg/kg body weight through intra-peritoneal route respectively. After 1 h of administration of DMSO, indomethacin and plant extract all the rats were injected with 1% acetic acid solution intra-peritoneal. The number of writhing was counted for 20 min after acetic acid injection as described by (Hajare et al., 2000).

Hot plate test

Experimental rats were acclimatized to laboratory conditions and were randomly divided into five groups consisting to 4 rats in each group. Animals were deprived of feed 1 h prior testing procedure. All the rats were pre-tested for measuring the latency time on hot plate which was maintained at 55±2°C. Animals showing latency time greater than 15 s were rejected from the experiment. Group I received control DMSO liquid 10 ml/kg, group II received tramadol 20 mg/kg and III, IV and V groups received plant extract 50, 100 and 200 mg/kg body weight respectively. Latency time of lifting the paw was then noted by placing each rat on the hot plate maintained at 55±2°C at 0, 20 and 60 minutes after treatment as described by Hajare et al. (2000).

The analgesia percentage was then calculated using the formula:

$$\% \text{ Analgesia} = (\text{Test latency} - \text{Control latency}) / \text{cut off time} - \text{controlled latency} \times 100$$

Anti-inflammatory activity

Carrageenan induced paw edema

The anti-inflammatory activity of aqueous methanolic extract of leaves of the plant *J. gossypifolia* was determined using carrageenan as inflammatory mediator (Amdekar et al., 2012). Albino rats (aging between 40 and 50 days) of species *Rattus norvegicus* of either sex weighing 130 to 150 g were divided randomly into five groups consisting of 4 rats in each group. All rats were deprived of feed 1 h before experimentation. Group I rats were treated with DMSO liquid as negative control, group II rats were treated with standard drug indomethacin 10 mg/kg body weight, group III, IV and V were treated with *J. gossypifolia* extract 50, 100 and 200 mg/kg body weight respectively. After 1 h of intra-peritoneal injection of all the groups, 1% carrageenan solution of approximately 0.5 ml was injected into the left hind paw of each rat. Paw volume of each rat was measured immediately at 0 h and after 3 h of carrageenan immersion injection using liquid immersion method (Fereidoni et al., 2000). The average paw edema in plant

Table 1. Effect of *Jatropha gossypifolia* aqueous methanolic extract 50, 100 and 200 mg/kg in acetic acid induced writhing test.

Animal	Average no. of writhing	Decrease in writhing	Percentage inhibition
Group I negative control (DMSO) 10 ml/kg	25+4.8	0	0
Group II positive control (Indomethacin 10 mg/ kg)	13+1.25*	12	48
Group III JG-Cr treatment group (50 mg/kg)	21+4.5	4	16
Group IV JG-Cr treatment group (100 mg/kg)	19+3.4*	6	24
Group V JG-Cr treatment group (200 mg/kg)	17+1.4*	8	32

The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparisons. The data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control $p < 0.05$.

Table 2. Central analgesic effect of *J. gossypifolia* extract at 50, 100 and 200 mg/kg.

Treatment	Average latency time (s)			
	0 min	20 min	60 min	% Analgesia
DMSO 10 ml/kg	4.85 \pm 0.25	4.6 \pm 0.81	4.6 \pm 0.24	0
Tramadol 20 mg/kg	4.52 \pm 0.43	6.4 \pm 0.26*	8.9 \pm 0.28*	93
JG-Cr 50 mg/kg	4.52 \pm 0.62	4.9 \pm 0.26	5.1 \pm 0.43	11
JG-Cr 100 mg/kg	4.47 \pm 0.40	5.2 \pm 0.37	5.5 \pm 0.33*	29
JG-Cr 200 mg/kg	4.45 \pm 0.53	5.2 \pm 0.25	6.3 \pm 0.4*	58

The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparisons. The data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control * $p < 0.05$.

extract treated groups and group that was treated with the standard was compared with that of negative control group (Gupta et al., 2003).

Inhibition of the inflammation was then measured using following formula:

$$\text{Percentage inhibition} = \frac{(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treated}}}{(V_t - V_0)_{\text{control}}} \times 100$$

Where V_t is paw volume at time t and V_0 is volume at zero time.

Anti-pyretic activity

Brewer's yeast induced pyrexia

Anti-pyretic activity of plant extract was determined using brewer's yeast suspension induced pyrexia in animal models. The normal rectal temperature of each rat was measured with the help of clinical thermometer. All the rats were then injected subcutaneously (at the nape of neck) with 20% brewer's yeast suspension at a dose of 20 ml/kg body weight. After 24 h of injecting yeast suspension rectal temperature of all the rats was again measured using clinical thermometer.

All the rats were randomly placed into five groups consisting of 4 rats each. Group I received negative control DMSO liquid 10 ml/kg, group II received standard drug paracetamol 150 mg/kg and the III, IV and V groups were given plant extract 50, 100 and 200 mg/kg body weight through intra-peritoneal route respectively. Rectal temperature was then measured immediately at 0hr and after 1, 2, 3 and 4 h after drug treatment (Bajpai et al., 2014).

Statistical analysis

The data was statistically analyzed using one way ANOVA followed by post hoc Dunnett's test for multiple comparisons.

Animal experiment ethics

All animal handling procedures received approval from the Animal Management Ordinance of the Pakistan; and all the animal experiment standards approved by the Animal ethics committee of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

RESULTS

Analgesic effect of *J. gossypifolia* extract

The study showed that the plant extracts of *J. gossypifolia* demonstrated significant peripheral as well as central analgesic activity, at a dose level of 200 mg/kg of body weight (Khan, 1992). The analgesic activity of the extract for peripheral analgesic activity was found to be 32% at a dose level of 200 mg/kg as compared to 48% for indomethacin taken as standard treatment (Table 1).

The central analgesic activity was evaluated and the plant extract of *J. gossypifolia* showed dose dependent increase in latency time and the analgesic effect was found to be 58% at 200 mg/kg dose as compared to 93% for reference drug tramadol 20 mg/kg (Table 2). The

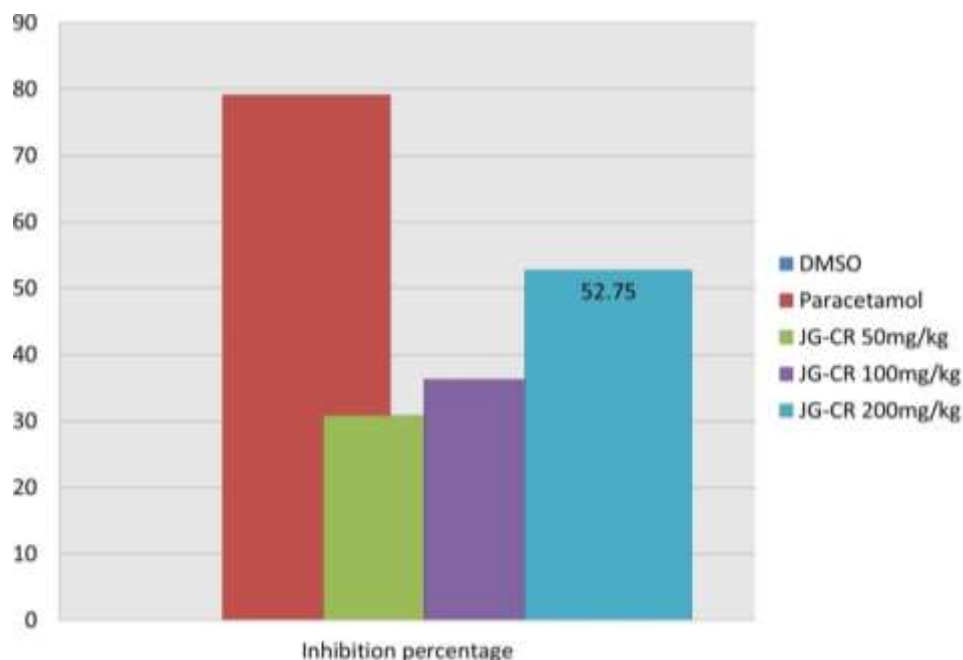


Figure 1. Percentage inhibition of inflammation after three hours of treatment; effect of intraperitoneal administration of *Jatropha gossypifolia* aqueous methanolic extract at 50, 100 and 200 mg/kg doses in carrageenan induced paw edema test. The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparison; the data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control $p < 0.05$.

Table 3. Effect of intraperitoneal administration of *J. gossypifolia* aqueous methanolic extract at 50, 100 and 200 mg/kg doses in carrageenan induced paw edema test.

Animal	Average paw volume cm^3 at 0 h	Average paw volume cm^3 at 3 h	Average increase in paw volume cm^3 after 3 h (vt-vo)	Decrease in oedema as compared to negative CTRL
Group I negative control (DMSO) 10 ml/kg	0.419 \pm 0.04	1.324 \pm 0.04	0.91	0
Group II positive control Indomethacin 10 mg/kg	0.379 \pm 0.045	0.573 \pm 0.05	0.19	0.72
Group III JG-Cr 50 mg/Kg	0.426 \pm 0.03	1.059 \pm 0.03	0.63	0.28
Group IV JG-Cr 100 mg/Kg	0.441 \pm 0.027	1.017 \pm 0.03	0.58	0.33
Group V JG-Cr 200 mg/Kg	0.424 \pm 0.03	0.852 \pm 0.03*	0.43	0.48

The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparison the data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control $p < 0.05$.

percentage analgesia was calculated using the following formula:

$$\text{Percentage Analgesia} = \frac{(\text{test latency} - \text{control latency})}{(\text{cut off time} - \text{control latency})} \times 100$$

Anti-inflammatory effect of plant extract

In carrageenan induced paw edema the plant extract of *J. gossypifolia* showed dose dependent anti-inflammatory

activity. The maximum anti-inflammatory activity of the plant extract *J. gossypifolia* was found at a dose level of 200 mg/kg of about 53% (Figure 1) as compared with the reference drug indomethacin 10 mg/kg where the anti-inflammatory activity was 79% (Table 3).

Anti-pyretic effect of the plant extract

The plant extract at 200 mg/kg showed significant anti-pyretic activity in brewer's yeast induced pyrexia in

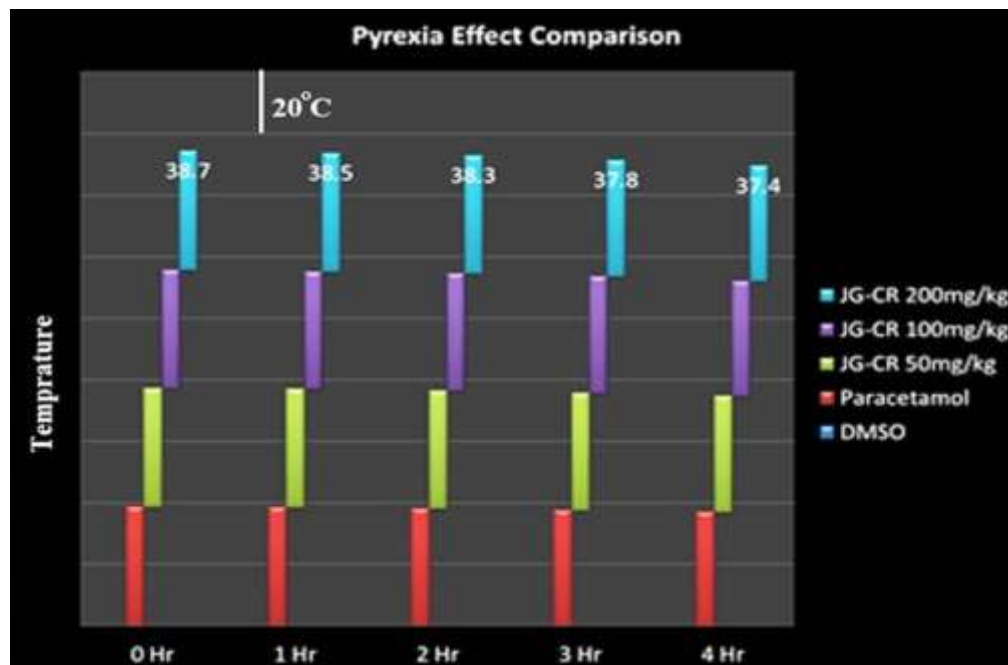


Figure 2. Effect of intraperitoneal administration *J. gassypifolia* aqueous methanolic extract 50, 100 and 200 mg/kg in yeast induced pyrexia in rats. The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparison; the data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control * $p < 0.05$.

Table 4. Effect of intraperitoneal administration *J. gassypifolia* aqueous methanolic extract 50, 100 and 200 mg/kg in yeast induced pyrexia in rats.

Group treatment	Initial rectal temperature (°C)	Rectal temperature after treatment (°C)					% inhibition
		0 hour	1 hour	2 hour	3 hour	4 hour	
DMSO liquid 10 ml/kg	36.7 \pm 0.11	38.6 \pm 0.34	38.5 \pm 0.21	38.4 \pm 0.11	38.4 \pm 0.20	38.2 \pm 0.20	21
Paracetamol 150 mg/kg	36.7 \pm 0.08	38.8 \pm 0.04	38.5 \pm 0.01	38.1 \pm 0.02	37.7 \pm 0.28	37.0 \pm 0.04*	85
JG-Cr 50 mg/kg	36.8 \pm 0.02	38.5 \pm 0.43	38.5 \pm 0.22	38.3 \pm 0.22	38.1 \pm 0.11	37.8 \pm 0.12	41
JG-Cr 100 mg/kg	36.6 \pm 0.14	38.5 \pm 0.11	38.3 \pm 0.02	38.1 \pm 0.25	37.9 \pm 0.11	37.4 \pm 0.41*	58
JG-Cr 200 mg/kg	36.7 \pm 0.12	38.7 \pm 0.20	38.5 \pm 0.31	38.3 \pm 0.21	37.8 \pm 0.10	37.4 \pm 0.23*	65

The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparison The data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control * $p < 0.05$

animal model. The rectal temperature of rats was recorded at 0, 1, 2, 3 and 4 h after the drug treatment (Figure 2). The results thus depicted that the aqueous methanolic extract of *J. gassypifolia* leaves illustrated significant anti-pyretic activity (65%) at dose of 200 mg/kg comparable to the reference drug paracetamol which was 85% (Figure 3) at a dose of 150 mg/kg (Table 4).

DISCUSSION

Results of the present study revealed that leaves of the

plant *J. gassypifolia* possess significant analgesic, anti-pyretic as well as anti-inflammatory activity. Carrageenan induced edema is known to be the acute inflammatory model; sensitive to the cyclooxygenase inhibitors and is used to find the effects of non-steroidal anti-inflammatory agents which inhibit cyclooxygenase involved in prostaglandin synthesis. Although both pathways, that is, cyclooxygenase and lipoxygenase are involved in mediating inflammatory process but cyclooxygenase inhibitors are considered more effective than lipoxygenase inhibitors (Ndebua et al., 2007).

Carrageenan induced paw edema model is well

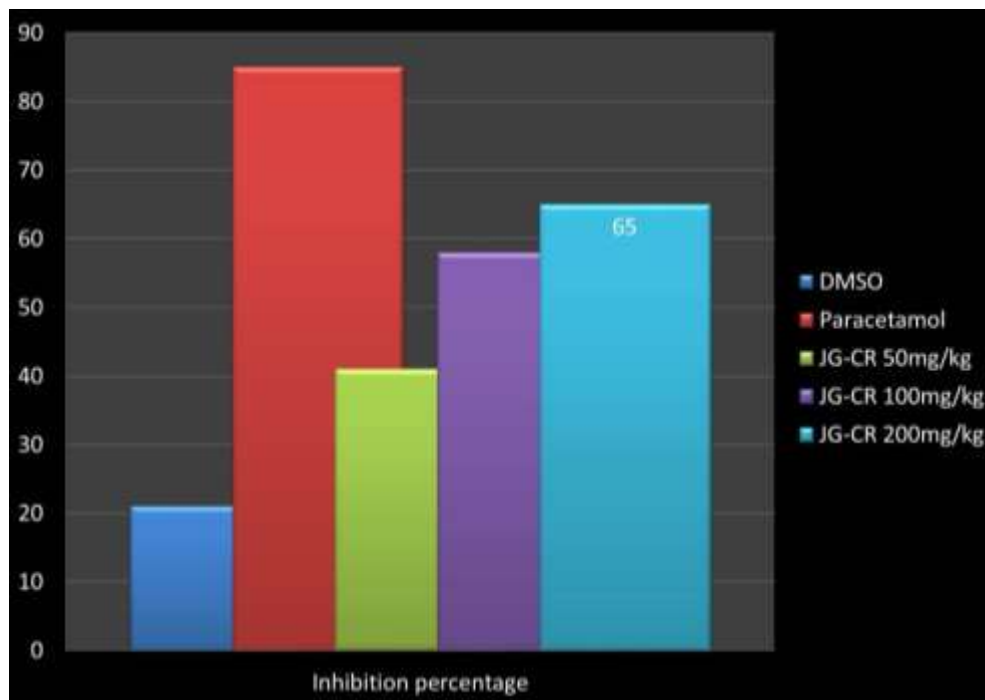


Figure 3. Percentage inhibition of pyrexia after 4 h of the treatment, effect of percentage inhibition of pyrexia with intraperitoneal administration *J. gassypifolia* aqueous methanolic extract 50, 100 and 200 mg/kg in yeast induced pyrexia in rats. The data was analyzed by one way ANOVA followed by post hoc Dunnett's test for multiple comparison; the data are reported as mean \pm S.E.M for group of 4 animals. Asterisks indicated the statistically significant values as compared to control *p <0.05.

established technique for the estimation of anti-inflammatory activity for plant derived natural substances as well as for synthetic drugs. The edema formation is a two phase process; the initial phase of edema involves the action of inflammatory mediators on the vascular permeability, including histamine, serotonin and bradykinin (Toni et al., 2015). The second or the later phase involves the pain mediators like prostaglandins (Chao et al., 2009). Pre-treatment of experimental animals with respective concentration of plant extract reflects that the plant extract was effective in attenuating the early phase of inflammation which involves the release of inflammatory mediators like histamine, serotonin and bradykinin (Muhammad et al., 2012). When the edema was induced by injecting 1% carrageenan solution into the left hind paw of all the experimental rats (Gupta et al., 2003), the aqueous methanolic extract of *J. gassypifolia* showed significant anti-inflammatory activity in both phases of inflammation at a concentration of 200 mg/kg body weight (Figure 1).

The analgesic activities of plant derived natural substances are usually evaluated using acetic acid induced writhing test model (Kumar et al., 2015). The pain induction is caused by the release of certain endogenous substances e.g. bradykinin and also from the production of pain mediators like arachidonic acid via

cyclo-oxygenase pathway and prostaglandin synthesis (Verma et al., 2015). Abdominal writhing usually involves certain pain receptors which are located in the peritoneum (Mbiantcha et al., 2011). When peripheral analgesic activity was determined by acetic acid induced writhing test as described by Hugar et al. (2010), the results demonstrated that the plant extract of *J. gassypifolia* significantly reduces the abdominal writhing in rats at a dose of 200 mg/kg indicating its activity on local peritoneal pain receptors (Table 2).

The hot plate method is a common technique usually used in measuring the central analgesic effects (Singh et al., 2015). The results showed that the plant extract of *J. gassypifolia* at a concentration of 200 mg/kg body weight significantly increased the latency time of lifting the paw which is comparable to tramadol 20 mg/kg used as control. The increase in latency time of lifting the paw as compared with the negative control reflected the decrease in pain threshold which might be due to the inhibition or suppression of thermo receptors in the brain region. The plant extract *J. gassypifolia* at 200 mg/kg dose showed 58% increase in latency time of lifting the paw as compared to the standard drug tramadol which reflected 93% increase in latency time at a dose level of 20 mg/kg (Table 2).

Administration of 20% yeast suspension below the

nape of the neck to the experimental animals induces pyrexia possibly by increasing the synthesis of prostaglandins (Eldahshan and Abdeldaim, 2015). The induction of fever by this method is called pathogenic fever. Induction of pyrexia or fever involves liberation of several mediators and hence antipyretic effect can only be achieved by inhibiting or blocking the release of these mediators. In present study, the plant extract of *J. gassypifolia* has shown its significant anti-pyretic activity (65%) at dose level of 200 mg/kg indicating the presence of chemicals in plant extract that are involved in the inhibition of prostaglandins synthesis confirming the plants potential as antipyretic agent (Figure 3 and Table 4).

In conclusion the extract of the plant *J. gassypifolia* was proved to be a natural and effective remedy for the treatment of pain, inflammation and fever. The study has thus justified the folk use of the *J. gassypifolia* plant extract as a remedy for clinical signs, including pain, inflammation and fever on scientific basis and may require further isolation of active ingredient out of extract for the advancement in pharmaceutical sciences.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A glass dropper with a blue-tinted background. The dropper is centered and has a small amount of liquid inside. The background is a blurred blue light pattern.

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