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Efficacy of methanolic extract of *Terminalia brownii* bark and leaves in treating experimentally infected rabbits

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Abstract

The antimicrobial activity of the methanolic extract of *Terminalia brownii* bark and leaves was tested in rabbits, experimentally infected with *Pasteurella multocida* strain B_2. Two experiments were performed, in one using the bark and in the other using the leaves of the plant. In each experiment 30 rabbits were used and divided into 5 groups 1a, 2a, 3a, 4a, and 5a and 1b, 2b, 3b, 4b and 5b. Each group was injected subcutaneously with 0.2ml of an over night broth culture of *Pasturalla Multocida* strain B_2 ($1x10^{-2}$, $1x10^{-3}$ and $1x10^{-6}$ dilution). Groups 1a and 1b were kept as control. The methanolic extract of *Terminalia brownii* bark and leaves was prepared as a suspension in normal saline at a concentration of 50mg/ml and given orally by stomach tube at a dose rate of 100 mg/kg body weight to each infected rabbit two days before infection and then every day after infection for 12 days. Blood samples were taken before dosing and then every 3 days after doing for counting leuckocytes and finding the percentages of neutrophils and lymphocytes. The plant extracts were found to be effective when the rabbits were infected with low doses of *Pasteurella multocida* strain B_2. In group 5a and 5b only half of the rabbits (50%) died after survival for a number of days, while the other half recovered at the end of therapy. Rabbits of the other infected groups died within 24 hours after infection.

Keywords: *Terminalia brownie*, Methanolic Extract, *Pasteurella multocida*

Introduction

Terminalia brownii (Combertacaea) is known in Sudan as shagarat elsobag. It is widely spread tree in the Sudan. Details of its botanical description were given by Elgazali et al., 1997 and Omer and ElNima, 1999. The maceration of the bark of the plant has been used in traditional medicine for the treatment of cough and bronchitis in west and south east Sudan (Elgazali et al., 1997) and for treatment of diarrhea and gonorrhea (Zakaria et al., 2007).

Chemical analysis of different part of the plant revealed abundance of tannins, flavonoides and saponine (Omer and ElNima, 1999) in which the antimicrobial activity resides. Whereas, *in vitro* studies revealed a high antimicrobial activity residing of the bark and leaves of the plant against pathogens (Omer and ElNima, 1999; Thoria, 2007; Zakaria et al., 2007).

A marked sensitivity to the bark and leaves extract of the plant was exhibited by the clinical bacterial isolates, *Staphlococcus aureus* a coagulanse –positive strain and *Pasteurella multocida* strains B_2 as shown by disc diffusion method (Thoria, 2007). However, the therapeutic value of this plant extracts in treating clinical cases has not been investigated.

In the present study experiments were designed to assess the efficacy of *T. brownii* bark and/or leaves extracts in treating experimental systemic infection in local breed of rabbits.

Material and Methods

Sixty rabbits (30 male and 30 female) of local breed were purchased from Khartoum North vegetable market. Their weight ranged from 1.5-2.00 kg. They were kept in cages within the premises of the Central Veterinary Research Laboratory, Soba, Khartoum. The rabbits were allowed for seven days adaptation period during which clean lucerne was fed *ad-libitum*.

At the end of the adaptation periods, each rabbit was weighed and were divided randomly into 10 groups each of 6 rabbit. Rabbits in group 1a and 1b were kept as uninfected control. Group 2a and 2b rabbits, were injected S/C with 0.2ml of an over night broth culture of *Pasteurella multocida* strain B_2 containing about

$56x10^6$ viable bacterial count per ml. Group 3a and 3b rabbits were injected S/C with 0.2ml of $1x10^{-2}$ diluted broth culture of *Pasturella Multocida* strain B_2 containing about $65x10^4$ viable bacterial count per ml. Rabbits in group 4a and 4b were injected S/C with 0.2ml of the same over night culture of *Pasteurella multocida* B_2 dilution ($1x10^{-3}$) containing about $56x10^{-3}$ viable bacterial count per ml. Group 5a rabbits were injected S/C with 0.2ml of an over night culture of *Pasturella Multocida* B_2 diluted $1x10^{-5}$ containing about $56x10^{-5}$ viable bacterial count per ml.

The fresh bark and leaves of *T. brownii* were collected from Khartoum National Botanical Garden. The plant was identified by the botanists in Medicinal and Aromatic Plant Research Institute, Khartoum, Sudan. 500 gram of the crude ground bark was extracted with methanol using soxhlet extractor. The extract was filtered and the filtrate was evaporated to dry under reduced pressure at 40°C using a rotary evaporator. The methanolic extract was prepared as a suspension in normal saline by dissolving 500 mg of the extract of the bark and/or leaves in 10 ml of sterile normal saline to make a concentration of 50 mg/ml. The bark and leaves methanolic extract was given orally using stomach tube to all rabbits of group 2a, 3a, 4a, 5a and 2b, 3b, 4b and 5b. The extracts were given according to the body weight at a dose rate of 100 mg/kg/body weight to all rabbits of the groups two days before infection. The treatment was continued daily for 12 days after infection for the survived rabbits.

Blood samples from all rabbits were taken from the jugular vein at the end of the adaptation period before infection and before dosing with the plant extract and then every 3 days after infection and treatment with the plant extract for the survived rabbits. Blood samples were examined for the number of leucocytes and finding percentage of neutrophils and lymphocytes, clinical signs and mortality were recorded.

Results

The dosing schedule and time of death of rabbits in different groups are illustrated in table (1a and 1b).

All rabbits in group 2a, 3a and 4a, 2b, 3b and 4b) which received 0.2ml of whole over night culture, $1x10^{-2}$ and $1x10^{-3}$ dilution of the same culture and dosed with the bark or leaves extracts died within 24 hours of the injection and dosing. The survival periods were 10-20 hours, 20-24 hours and 22-24 hours for rabbits in group 2a, 3a and 4a and 2b, 3b and 4b respectively. The prominent clinical signs in those rabbits were weakness, dullness, shallow respiration inappetence and dyspnoea followed by recumbency and death.

Three rabbits died from group 5a and 5b which were injected S/C with 0.2 ml of $1x10^{-5}$ dilution of the broth bacterial culture, showing shallow respiration, dullness, inappetence, conjunctivitis with lacrimation and rhinitis were the conspicuous clinical changes observed in rabbits of this group. The daily oral dosing continued for the survived rabbits in group 5a, 5b for the bark and leaves extracts till the 12^{th} day after infection. Those rabbits gradually returned to normal condition at the end of the treatment course with *T. brownii* bark and leaves extract (Table 1a and 1b).

Examination of blood specimens of those rabbits of groups (5a and 5b) in each batch showed a significant increase in white blood cells two days after infection (Table 2a and 2b). The differential leucocyte counts showed a significant increase in number of neutrophils and drop in the number of lymphocytes starting 2 days after infection, which returned back to normal level at the end of treatment therapy (Table 3a, 3b and 4a, 4b).

Discussion

The susceptibility of *Pasteurlla multocida* serotype B_2 to the methanolic extract of the bark and leaves of *Terminalia brownii* was confirmed *in vitro* tests, by measuring MIC and zones of inhibition of bacterial growth on nutrient agar in the presence of the plant extracts (Thoria, 2007). Yet, those plant extracts failed to combat systemic infection with the same bacterial strain in rabbits. The sensitivity of the organisms to the plant extracts *in vitro* was not parallel by an effective response *in vivo* such discrepancy has been observed in other studies, as quoted by Elbashir (1993) and was experienced clinically in other bacterial infections (Wilkinson, 1976). Rabbits are particularly vulnerable to *Pasteurella multocida*, causing in them fulminating septicaemia (Hagan and Bruner, 1961; Davis et al., 1990). The onset of disease is rapid in rabbits with progressive clinical symptoms that make treatment not always successful (Hagan and Bruner, 1961). This coincides with the results in our experiments in which all rabbits injected with high dose of *P. multocida* serotype B_2 died within 24 hours after infection although oral dosing with the plant extracts had stared 2 days before infections. In large animals, same reports claimed successful treatment of *P. multocida* infection by means of sulphonamide compounds (Carter et al., 1972) and by antibiotic combinations (Karlson and Nystram, 1962). While others stated that treatment was marginally effective after the onset of the disease (de Alwis, 1984). In those studies parenteral routes were used for administering the drugs for treatment while in our experiment only the oral route was used. The latter route may not ensure that high concentration of the crude antibacterial components present in those plant extracts to be rapidly obtained in blood, which is the property of good antibacterial agents (Wilkinson, 1976). The kinetics of absorption, distribution and excretion of the antibacterial ingredients present in

Table 1a: Dosing and time of death of rabbits injected subcutaneously with 0.2ml of an over night broth culture of *Pasteurela multocida* strain (B_2) and $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-5}$ dilutions of the same culture and treated orally with *Terminalia brownii* bark methanolic extract.

Group No.	Animal No.	Age (month)	Weight (kg)	Dose	Fate of goats
Group (1)a	1		1.5	-	Healthy
control	2		1.8	-	Healthy
	3	7-8	1.5	-	Healthy
	4		2	-	Healthy
	5		2	-	Healthy
	6		1.7	-	Healthy
Group (2)a	7		2	0.2ml of an over night nutrient broth culture of *P.*	Died after 10 hours
	8		1.8	*multocida* strain (B_2) injected S/C for each one, and	Died after 18 hours
	9	7-8	2	treated with *Terminalia brownii* bark at dose of 100	Died after 18 hours
	10		1.5	mg/kg body weight.	Died after 18 hours
	11		1.5	The concentration of the extract was 50 mg/ml.	Died after 20 hours
	12		2		Died after 20 hours
Group 3a	13		1.8	Each one was injected subcatcutaneously with 0.2 ml	Died after 20 hours
	14		1.5	of $1x10^{-2}$ diluted over night culture of *P. multocida*	Died after 24 hours
	15	7-8	2	strain (B_2), and treated orally with 100mg/kg body	Died after 24 hours
	16		2	weight with *Terminalia brownii* bark methanolic	Died after 22 hours
	17		1.8	extract concentration of 50 mg/ml.	Died after 24 hours
	18		2		Died after 24 hours
Group 4a	19		2	Each one was injected S/c with 0.2ml of $1x10^{-3}$ diluted	Died after 22 hour
	20		2	over night culture of *P.. multocida* strain (B_2), and	Died after 24 hours
	21	7-8	1.8	treated orally with 100mg/kg body weight with	Died after 24 hours
	22		1.5	*Terminalia brownii* bark methanolic extract at	Died after 22 hour
	23		1.7	concentration of 50 mg/ml.	Died after 24 hours
	24		1.8		Died after 24 hours
Group 5a	25		2	Each one was injected S/C with 0.2ml of $1x10^{-5}$ diluted	Died on day 12
	26		2	broth culture of *P. multocida* strain (B_2) and treated	Treated
	27	7-8	1.7	orally with 100mg/kg body weight with *Terminalia*	Treated
	28		1.8	*brownii* bark methanolic extract at concentration of	Died on day 5
	29		2	50mg/ml.	Treated
	30		2		Died on day 7

these plant extracts are not known, neither their chemotherapeutic index has been calculated, nor the oral daily dose for each infected rabbits was blindly estimated. However, it appears from the results that the extract of this plant is effective when the rabbits are infected with low doses of *Pasteurella multocida* serotype B_2. In group 5a and 5b rabbits which were infected with few organisms, only half of the animal died after survived for a number of days, while the other half recovered completely from infection at the end of therapy with the plant extract, which continued for 12 days after infections. In rabbits of the other groups, which were injected with large number of organisms most of the toxic symptoms observed seemed to be due to the effect of free bacterial endotoxin (Rimler and Brogden, 1986). In such circumstances, the endotoxin will be liberated in large amounts by the disintegrated bacterial cells in the animal's body (Davis et al., 1990) and usually causes severe fatal shock syndrome (Sleigh and Timbury, 1998). It is known that bacterial endotoxins, which are liberated only from gram-negative bacterial cells, can not be neutralized by antibiotics (Davis et al., 1990) or even by immune serum (Kheng & Phay, 1963). This may explain failure of these plant extracts to combat disseminating bacteraemia caused by gram-negative *Pasturella Multocida*, even though extract contain active antibacterial agents.

In conclusion more *in vivo* studies are needed to elucidate the antibacterial efficacy of the methanolic extracts of the bark and leaves of *Terminalia brownii*. These studies should include experimental infections with gram-positive bacteria, which normally do not possess an endotoxin within their cell walls (Davis et al., 1990). The nature of these antibacterial components present in the plant extracts should be verified clearly, particularly their absorption and obtainable blood levels after oral dosing, their ability to bind to proteins and their metabolism in the animal body (Wilkinson, 1976) before being exploited as a therapy for treatment of systemic bacterial infection.

Table (1b): Dosing and time of death of rabbits injected subcutaneously with 0.2ml of an over night broth culture of *Pasteurela multocida* strain (B_2) and $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-5}$ dilutions of the same culture, and treated orally with *Terminalia brownii* leaves methanolic extract.

Group No.	Animal No.	Age (month)	Weight (kg)	Dose	Fate of goats
Group (1)b	1		1.8	-	Healthy
control	2		2	-	Healthy
	3	7-8	2	-	Healthy
	4		1.5	-	Healthy
	5		1.8	-	Healthy
	6		2	-	Healthy
Group (2)b	7		2	Each one was injected S/c with 0.2ml of an over night	Died after 10 hours
	8		2	broth culture of *Past. multocida* strain (B) and treated	Died after 20 hours
	9	7-8	1.7	orally with 100mg/kg body weight of *Terminalia*	Died after 20 hours
	10		1.8	*brownii* leaves methanolic extract at concentration of	Died after 20 hours
	11		1.8	50mg/ml.	Died after 15 hours
	12		2		Died after 20 hours
Group 3b	13		2	Each one was injected s/c with 0.2ml of diluted culture	Died after 24 hours
	14		2	$1x10^{-2}$ of *Past. multocida* strain (B), and treated orally	Died after 20 hours
	15	7-8	1.7	with 100mg/kg body weight with *Terminalia brownii*	Died after 24 hours
	16		1.8	leaves methanolic extract concentration of 50mg/ml.	Died after 24 hours
	17		2		Died after 24 hours
	18		1.8		Died after 24 hours
Group 4b	19		2	Each one was injected S/c with 0.2ml of diluted culture	Died after 22 hour
	20		1.7	$1x10^{-3}$ diluted over night broth culture of *Past.*	Died after 24 hours
	21	7-8	2	*multocida* strain (B), and treated orally with *Terminalia*	Died after 24 hours
	22		1.5	*brownii* leaves extract 100mg/kg body weight, at	Died after 24 hour
	23		2	concentration of 50mg/ml.	Died after 24 hours
	24		2		Died after 24 hours
Group 5b	25		2	Each one was injected S/c with 0.2ml of $1x10^{-5}$ diluted	Treated
	26		2	over night culture of *Past. multocida* strain (B) and	Died on day 2
	27	7-8	1.7	treated orally with 100mg/kg body weight with	Treated
	28		1.8	*Terminalia brownii* leaves methanolic extract at	Treated
	29		2	concentration of 50mg/ml.	Died on day 3
	30		2		Died on day 6

Table 2a: Changes in white blood cell counts in blood of rabbits infected subcutaneously with diluted broth culture $1x10^{-5}$ of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* bark methanolic extract for 15 days

Days	Groups	
	G1	G5a
0	4.283 ± 0.062^{d}	3.983 ± 0.62^{e}
3	4.560 ± 0.82^{d}	9.780 ± 1.19^{a}
6	4.659 ± 0.85^{d}	9.260 ± 1.18^{a}
9	4.300 ± 0.91^{d}	7.000 ± 0.77^{b}
12	4.380 ± 0.67^{d}	6.040 ± 1.06^{c}
15	4.295 ± 0.65^{d}	4.240 ± 0.60^{d}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different ($P\leq0.05$); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* bark methanolic extract.

Table 2b: Changes in white blood cell counts in blood of rabbits infected subcutaneously with 0.2ml diluted broth culture $1x10^{-5}$ of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* leaves methanolic extract for 15 days

Days	Groups	
	G1	G5b
0	4.593 ± 0.38^{d}	4.492 ± 0.47^{d}
3	4.593 ± 0.39^{d}	11.358 ± 2.18^{a}
6	4.579 ± 0.32^{d}	9.683 ± 1.31^{b}
9	4.650 ± 0.51^{d}	7.467 ± 1.18^{c}
12	4.640 ± 0.55^{d}	5.433 ± 0.84^{d}
15	4.596 ± 0.41^{d}	4.475 ± 0.46^{d}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different ($P\leq0.05$); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* leaves methanolic extract.

Table 3a: Changes in percentage of neutrophils in blood of rabbits infected subcutaneously with diluted broth culture 1x10^{-5} of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* bark methanolic extract for 15 days.

Days	Groups	
	G1	G5a
0	39.667±3.39^{f}	40.167±2.52^{e}
3	40.220±2.13^{e}	64.600±6.99^{a}
6	39.825±3.38^{f}	60.400±6.01^{b}
9	39.792±3.37^{f}	56.200±3.87^{c}
12	39.785±3.15^{d}	48.000±2.53^{d}
15	39.667±3.39^{f}	40.800±1.95^{e}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different (P≤0.05); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* bark methanolic extract.

Table 3b: Changes in percentage of lymphocytes in blood of rabbits infected subcutaneously with diluted broth culture 1x10^{-5} of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* bark methanolic extract for 15 days.

Days of treatment	Groups	
	G1	G5a
0	55.333±4.08^{a}	54.833±3.31^{a}
3	55.350±4.08^{a}	29.000±6.75^{a}
6	55.410±4.08^{a}	35.600±4.32^{c}
9	55.352±4.07^{a}	38.000±2.61^{c}
12	55.347±4.08^{a}	48.200±2.48^{b}
15	55.365±4.08^{a}	54.200±2.60^{a}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different (P≤0.05); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* bark methanolic extract.

Table 4a: Changes in percentage of neutrophils in blood of rabbits infected subcutaneously with 2.0 ml of diluted broth culture 1x10^{-5} of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* leaves methanolic extract for 15 days

Days	Groups	
	G1	G5b
0	40.667±1.97^{e}	40.167±1.72^{e}
3	40.750±1.98^{e}	71.000±2.53^{a}
6	40.770±1.97^{e}	61.333±2.50^{b}
9	40.700±1.99^{e}	52.000±2.53^{c}
12	40.677±1.97^{e}	47.167±2.56^{d}
15	40.732±1.98^{e}	40.000±0.63^{e}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different (P≤0.05); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* leaves methanolic extract.

Table 4b: Changes in percentage of lymphocytes in blood of rabbits infected subcutaneously with 2.0 ml of diluted broth culture 1x10^{-5} of *Pasteurella multocida* (strain B_2) and treated with *Terminalia brownii* leaves methanolic extract for 15 days.

Days	Groups	
	G1	G5b
0	54.333±3.27^{a}	54.333±1.63^{a}
3	54.348±3.25^{a}	25.500±2.17^{e}
6	54.350±3.29^{a}	34.167±2.14^{d}
9	54.430±3.21^{a}	42.833±2.14^{c}
12	54.338±3.24^{a}	48.667±2.50^{b}
15	54.432±3.80^{a}	55.500±0.55^{a}

Mean values (±SD) having different superscript letters in columns and rows (between groups and days) are significantly different (P≤0.05); G1: Control; G2: Infected with *Pasteurella multocida* and treated with *Terminalia brownii* leaves methanolic extract.

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