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Phytochemical, antibacterial and toxicity studies of the aqueous extract of *Euclayptus camaldulensis* Dehnh

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ABSTRACT

The aqueous extract of the bark of E. camaldulensis was investigated for the presence of bioactive substances, antibacterial activity against Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa and Salmonella Typhi, and the acute toxicity in mammals. Investigation reveals the presence of saponins and tannins which contribute to its medicinal value. The demonstration of antimicrobial activity against both gram-negative and gram-positive bacteria is an indication that the plant is a potential source for production of drugs with a broad spectrum of activity. The extract was bactericidal against S. aureus (50mg/ml), P. aeroginosa (50mg/ml), E. coli (100mg/ml) and B. subtilis (25mg/ml). It was however bacteriostatic against S. typhi and K. pneumoniae at 100mg/ml but had MIC of 12.5mg/ml and 25mg/ml respectively. Investigation also reveals that the plant is pro-inflammatory. The LD_{50} of the extract is ≥5000mg/kg b. wt. the heamatological and blood chemistry test shows that the PCV and marker enzymes (SGPT, SGOT and ALP) levels were all within the reference range. These results support the traditional application of the plant and suggests that the plant extracts possess compounds that are not acutely toxic with antibacterial properties that can be used as antibacterial agents in novel drugs for the treatment of gastroenteritis, supperative infections, urinary tract infections, typhoid fever and wound infections.

Keywords: *E. camaldulensis, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella typhi*.

INTRODUCTION

Plant is man's friend in survival, giving him food and fuel and medicine from the days beyond dawn of civilization [1]. Plants continue to be a major source of medicine, as they have been throughout human history [2]. Some medicinal plants such as opium poppy have been long recognized and widely used, while others, such as Pacific yew, the original source of the cancer drug, taxol, are relatively new arrivals to the mainstream medicine. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value.

In all countries of the world there exists traditional knowledge related to the health of humans and animals [3]. Herbal remedies are widely available for the treatment and prevention of various diseases and often contain highly active pharmacological compounds [4]. Many plants synthesize substances that are useful for the maintenance of health in humans and other animals. These substances, most of which are phenols or their oxygen substituted derivatives such as tannins, secondary metabolites of plants which have been isolated are at least 1200 [5]. In many cases, these substances (particularly alkaloids) serve as plant defence mechanism against predation by microorganism, insects and herbivores [6, 7].

Presently in developing countries, synthetic drug are not only expensive and inadequate for the treatment of disease but are often with adulterations and side effects [8]. The search for newer sources of antibiotics is a global challenge preoccupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs [9]. In Africa, traditional healers and remedies made from plants play an important role in the health of millions of people. Africa has a long and impressive list of medicinal plants [10]. Many African plants are used in traditional medicine as antimicrobial agents but only a few are documented [11, 12, 13]. However 80% of the world's population use plant as their primary source of medication [14, 15] in view of the fact that antibiotics are sometimes associated with adverse side effects to the host including hypersensitivity, immunosuppressive and allergic reactions, it is of interest to develop alternative antimicrobial drugs such as medicinal plants for treatment of infectious diseases [16].

The fact that herbal medicines are natural does not mean that they do not contain some toxic substances which could be dangerous when consumed by humans. Herbal medicine can also cause undesired effects. Therefore, the common belief that anything "natural is safe" is not correct [17]. Many herbal additives have some toxic side effects, and may be fatal in some doses. Toxicity related to herbal medicine is becoming more widely recognized as these remedies become popular in most parts of the world. Toxic effect of herbal medicine ranges from allergic reactions to cardiovascular, hepatic, renal, neurological and dermatological toxic effect [18].

The plant, *Eucalyptus camaldulensis*, commonly known as the "River red gum", belongs to the family of myrtaceae. It is a large evergreen plant, 20 - 40m high, with a stout trunk, widely spreading, irregular smooth bark shedding at intervals throughout the year to show white, yellow and grey, becoming rough at the base. It is regarded as one of the most planted Eucalypts, plantations occur in Argentina, Arizona, California, Egypt, Kenya, Morocco, Nigeria, Pakistan, Senegal, Sierra Leone etc [19]. In folk medicine, *E. camaldulensis* is used as anaesthetic, astringent and antiseptic. It is also used as remedy for cough, diarrhea, sore throat, cold, dysentery, hemorrhage and wound [20]. The Igala people call it "Ogwu iba" meaning fever remedy, and it is used for the treatment of a wide range of ailments.

In many of the countries of the developed world, infectious diseases – of childhood in particular – have been generally conquered. This, however, is far from being the case in the developing countries, where ther are responsible for 45% of all deaths. The leading killers are acute respiratory diseases, HIV/AIDS, diarrhoeal diseases, tuberculosis, malaria and measles [21]. Infectious diseases are characterized by the existence of a living infectious agent, which is transmissible. Some infectious agents have been selected for study for a possible development of a new antibiotic against them.

Pseudomonas aeruginosa is a gram-negative bacterium with unipolar motility [22]. *P. aeruginosa* is an opportunistic pathogen of human and plants. *Salmonella* Typhi is a gram negative bacteria and it is the cause of the disease typhoid fever [23, 24]. *Escherichia coli* is a

Gram – negative bacterium that is commonly found in the lower intestine of warm-blooded animals. Virulent strains of *E. coli* can cause gastro enteritis [25]. Urinary tact inflections and neonatal meningitis. In rare cases virulent strains are also responsible for haemolytic uremic syndrome (HUS) peritenitis septicemia and gram-negative pneumonia. *Klebsiella pneumoniae* is a gram negative bacterium found in the normal flora of the mouth, skin and intestines [26]. It can cause bacterial pneumonia, typically due to aspiration by alcoholics though it's more commonly implicated in hospital acquired urinary tract and wound infection particularly in immunocompromised individuals.

Staphylococcus aureus is a gram positive spherical bacterium that colonizes mainly the nasal passages. *S. aureus* causes a variety of supperative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furuculosis, more serious infections such as pneumonia, mastitis, phlebitis, meningitis and urinary tract infections and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical deviices. *S.aureus* causes food poisoning by releasing enterotoxins into the food and toxic shock syndrome by release of super antigens into the blood stream. *Bacillus subtilis* is a gram-positive non-pathognenic bacteria. They can contaminate food and could sometime result to food poisoning.

MATERIALS AND METHODS

The plant was collected from the Professorial Quarters, Kogi State University, Anyigba. They were identified by Prof. COC Agwu of the Biological sciences Department, Kogi State University, Anyigba, Nigeria. The test organism: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* (ATCC 10145), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi* were obtained from the Microbiology Department of Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. Reagents and media were obtained from reputable names like BDH Poole, England and Lab M ltd Lancashire, England.

Phytochemical Screening

Qualitative phytochemical analysis of the plant was determined as follows:
Determination of alkaloids: 0.5g of the sample was accurately weighed and defatted with 5% ethyl ether for saponins and cardenolides for 15mins. The defatted sample was extracted for 20mins with 5.0ml of aqueous HCl on a steam bath. The resulting mixture was centrifuged for 10mins at 3000rpm to remove filtrate (Supernatant). 1.0ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1.0ml portion was treated similarly with Dragendorff's reagent. Turbidity or precipitation with either of these reagents was taken as evidence for the presence of alkaloids [27, 28].

Test for saponins: The ability of saponins to produce frothing in aqueous solution was used as screening test for the sample. 0.5g of dried extract was shaken with water in a test tube, frothing which persist on warming was taken as evidence for the presence of saponins.

Test for tannins: 5.0g of dried extract was stirred with 10.0ml of distilled water. This was filtered and ferric chloride reagent was added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins [28].

Test for anthraquinones: Borntrager's test was used for the detection of anthraquinones. 5.0g of dried extract was shaken with 10.0ml of benzene. This was filtered and 5.0ml of 10% ammonia

solution was added to the filtrate. The mixture was shaken and the presence of violet colour in the ammoniacal (lower) phase indicated the presence of free hydroxy anthraquinones [28].

Test for cardiac glycosides: 0.5g of dried extract was dissolved in 2.0ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under laid with 1.0ml of concentrated H_2SO_4. A brown ring obtained at the interface indicated the presence of a cardenolides.

Aqueous Extraction

The plant materials were washed and air dried at room temperature for some days. They were then pulverized using high speed Creston grinder.

For the aqueous extract, 100g of leaf sample were macerated in 750 ml aliquots of distilled water in conical flasks. The mixture was rocked gently to form a homogeneous suspension and allowed to stand for 24 hours. This was then filtered through Whatman No 1 filter paper using a Speedvac vacuum pump, Buchner funnel and Buchner flask. The filtrate was then evaporated to dryness in a water bath to obtain the aqueous crude extract (yield = % starting material).

Antibacterial Screening

The method of [28] was used for the standardization of inoculum. The antimicrobial activity of the aqueous extract of *Eucalyptus camaldulensis* was determined by using a modification of the agar well diffusion technique described by [29] and [30]. 0.2ml of the standardized microbial suspension of the test organisms was seeded into 600ml of molten Mueller-Hinton agar at a temperature of 40^{o}C. The seeded agar was poured aseptically into sterile Petri dishes allowed to set at room temperature. The solidified agar was bored with a sterile 8mm cork borer to create 5 wells on the agar plate about 10mm deep. The wells were filled with 0.1ml of 12.5mg/ml, 25mg/ml, 50mg/ml, and 100mg/ml of the reconstituted crude extract and the fifth well was filled with distilled water to serve as solvent control. Similarly, 3 plates were prepared for the standard antibiotics, gentamycin, amoxicillin and chloramphenicol used as control. The plates were incubated for 24 hours at 37^{o}C. The resulting zones of inhibitions in mm were then measured using a transparent ruler. The experiment was carried out in triplicates and results recorded as mean values.

The MIC was determined using the serial dilution method. Four test tubes were prepared for each of the extracts. The test tubes contained 3ml of 12.5mg/ml, 25mg/ml, 50mg/ml and 100mg/ml of reconstituted crude extract in nutrient broth agar. 1 drop (0.025ml) of the standardized bacteria was then suspended in each of the test tubes. Similarly, four test tubes prepared for each of the standard antibiotics and inoculated with the standardized bacteria. They were then incubated at 37^{0}C for about 15 to 24 hours. The experiment was carried out in triplicates and results recorded as mean values. The MIC was determined as the lowest concentration of crude extracts that inhibited visible growth (turbidity) of the test organisms after 15hours.

One milliliter of sample from the test tubes used in MIC which did not show any visible growth was streaked out on Nutrient agar plates to determine the minimum concentration required to kill organisms. These concentrations were indicated by the failure of the test organism transferred to the Nutrient agar plates to grow. The concentration of the extract indicating a bactericidal effect after 24 hours of incubation at 37^{0}C was regarded as the Minimum bactericidal Concentration (MBC).

Toxicological Screening

The LD_{50} test was carried out using the fixed dose procedure (FDP). A single dose of 3000mg/kg b. wt. was administered to four healthy albino rats. If the mortality is more than two, the dose is reduced, but if there is no mortality observed, the dose is increased to 5000mg/kg b. wt. but thereafter there would be no need to increase the dose.

Acute Toxicity Test: A modification of [31] was used. 28 healthy albino rats where randomized into seven groups of 4 animals each. Group 1 was administered daily with 800mg/kg b. wt. of the crude extracts for 14 days. Similarly, Groups 2, 3, 4, 5, and 6 were administered with 1200mg/kg, 1600mg/kg, 2000mg/kg, 3000mg/kg and 500mg/kg b. wt. of the crude extracts respectively. Group 7 which served as the control was administered with normal saline, the control vehicle, for the same period of time. The test animals were observed for lethargy, restlessness, ulceration, weight loss, appetite and deaths. After 14 days, the packed cell volume of the surviving animals was carried out by the Heamatocrit capillary method, and the animals were then necropsized. Organs like the heart, liver, kidney and lung were observed for physical signs of abrasions, and their weight were also taken. Marker enzymes like SGOT/AST, SGPT/ALT and alkaline phosphatase were investigated using Dialab ready to use kits and the total protein was determined using the Randox protein kit.

RESULTS

Phytochemistry: The phytochemical test shows the presence of certain phytocomponents as presented in Table 1

Antibacterial Activity: The antimicrobial activities of the aqueous extract of *E. camaldulensis* with reference to some standard antibiotics is as presented in Tables 2 – 7

Toxicological Studies: LD_{50} of *E. camaldulensis* is >5000mg/kg b. wt. using albino rats. There were no apparent pharmacotoxic signs observed in animals that received p.o. doses of *E. camaldulensis* and animals that received 5 ml of normal saline in the duration of the test. There were also no changes in the skin colour, fur and eyes in the test period. However, there was loss of appetite observed in animals that were treated p.o. with doses of extracts of *E. camaldulensis*, from 12 hours post administration to about 24 hours. This was not observed in the animals that received only normal saline.

Mortality check revealed that within the test period, one animal died each from the group that received normal saline and the group of male treated p.o.with 5000 mg/kg b. wt. of extract of *E. camaldulensis*. All the animals gained weight over the test period. The weight changes were random. Animals that received normal saline had a higher percentage weight gain than those treated p.o. with extract of *E. camaldulensis* (Table 8).

The surviving test animals were sacrificed on the 15th day with chloroform anaesthesia. Organs like liver, heart, kidney and lungs were removed, the weight taken and physically inspected for signs of lesion. The organ weight with respect to the body weight of the animals before dosing was not significantly different from those of control. Physical signs of abrasion were seen in some organs of the test group animals but not the control animals. The PCV value of the test animals ranged from 46-57% while that of the control was 58%.

The blood chemistry parameters such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total protein treated animals were similar to those of the control animals and were all within the reference range (Table 9).

DISCUSSION

The plant studied was found to contain saponin and tannin, alkaloids, anthraquinone and cardiac glycosides were however absent. This agrees with the findings of [13], [32] and [8] who have reported the presence of these components and others in the members of Myrtaceae family. Saponins are glycosides of steroids i.e. steroid alkaloids; they are generally regarded as anti-nutrients but are also believed to be useful in human diet for controlling cholesterols. Its presence in this plant therefore could suggests that the plant is of medicinal value, There is evidence of the presence of saponins in traditional medicine preparations [33, 34], where oral administrations might be expected to lead to hydrolysis of glycoside from terpenoid (and obviation of any toxicity associated with the intact molecule). Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins, tannins are distributed all over the plant kingdom. Tannins have traditionally been considered antinutritional but it may be employed medicinally in antidiarrheal, hemostatic, and antihemorrhoidal compounds. Its presence in the plant suggest it to be of medicinal value because tannins have shown potential antiviral [35], antibacterial and antiparasitic effects [36].

The aqueous extract of *E. camaldulensis* was active against *S. aureus* (gram positive bacteria) even from a low concentration (12.mg/ml). The zone of inhibition increased in a dose dependent manner and it was comparable with those of standard antibiotics used. It had an MIC of ≤12.5mg/ml and bactericidal with an MBC of 50mg/ml. This suggests that the *E. camaldulensis* extract contains components which may be effective in treating a variety of supperative (pus-forming) infections, superficial skin lesions such as boils, and more serious infections such as pneumonia, mastitis, phlebitis, meningitis and urinary tract infections and deep-seated infections, such as osteomyelitis and endocarditis caused by *S. aureus* .

Although *B. subtilis* (gram positive bacteria) is non-pathognenic, they can contaminate food causing spoilage. Aqueous extract of *E. camaldulensis* was found to be bactericidal against *B. subtilis* (MIC = 25mg/ml, MBC = 25mg/ml). This suggests the importance of aqueous extract of *E. camaldulensis* in nutrition too for the preservation of food from bacterial spoilage.

The aqueous extract of *E. camaldulensis* was very effective against S. *typhi* (gram negative bacteria). Result reveals that it was more effective than amoxicillin and chloramphenicol and with comparable activity with gentamycin. This suggests that the plant could be a source of novel anti-typhoid drug. However, even though it had a low MIC of ≤12.5mg/ml, it was bacteriostatic at 100mg/ml. this may mean that the plant is either bacteristatic or that the MBC is >100mg/ml.

At low concentration, the crude extract of *E. camaldulensis* was not active against *P. aeroginosa* (gram negative bacteria), but at high concentration, it began to show some activity, and this was much lower than those of the standard antibiotics used. This suggests that aqueous extracts of *E. camaldulensis* may not be effective in treating diseases caused by *P. aeroginosa* at low concentrations (<50mg/ml).

Even though the crude extract of *E. camaldulensis* did not exhibit as much activity against *E. coli* as it did against *S. aureus* and *S. typhi*, it showed activity that even surpassed those of two standard antibiotics used, this suggests that at concentrations ≥50mg/ml, the aqueous extract of *E. camaldulensis* may be effective in the treatment of diseases caused by virulent strains of *E. coli*.

K. pneumoniae (gram negative bacteria), can cause bacterial pneumonia, is more commonly implicated in hospital acquired urinary tract and wound infection particularly in immunocompromised individuals *Klebsiella* ranks second to *E.coli* for urinary tract infections in older persons. *K. pneumonia* is also an opportunistic pathogen for patients with chronic pulmonary disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoseleroma. This makes the result obtained which shows that aqueous extract of *E. camaldulensis* have comparable activity with some standard antibiotics against *K. pneumoniae* interesting. It is bacteristatic at 25mg/ml. This suggests that it could serve as a source for novel drug against the ailments caused by *K. pneumoniae.*

Table 1: Phytochemical component of *Eucalyptus camaldulensis*

alkaloid	-
saponins	+
tannins	+
anthraquinones	-
cardiac glycosides	-

+: present; -: absent

Table 2: Zone of inhibition of the aqueous extract of *E. camaldulensis* on at 12.5mg/ml

	Zone of Inhibition (mm)			
Test Organism	*E. camaldulensis*	Amoxicillin	Chloramphenicol	Gentamycin
S. aureus	3	1	5	2
S. typhi	4	4	0	10
P. aeroginosa	0	5	1	1
E. coli	0	0	0	0
K. pnemuoniae	0	1	2	1
B. subtilis	1	1	1	0

Table 3: Zone of inhibition of the aqueous extract of *E. camaldulensis* on at 25mg/ml

	Zone of Inhibition (mm)			
Test Organism	*E. camaldulensis*	Amoxicillin	Chloramphenicol	Gentamycin
S. aureus	4	2	10	6
S. typhi	5	4	0	10
P. aeroginosa	1	8	1	5
E. coli	1	1	4	5
K. pnemuoniae	4	3	5	3
B. subtilis	2	5	3	0

Table 4: Zone of inhibition of the aqueous extract of *E. camaldulensis* on at 50mg/ml

	Zone of Inhibition (mm)			
Test Organism	*E. camaldulensis*	Amoxicillin	Chloramphenicol	Gentamycin
S. aureus	6	3	11	9
S. typhi	7	7	1	12
P. aeroginosa	4	10	6	8
E. coli	4	2	7	7
K. pnemuoniae	6	6	7	5
B. subtilis	3	7	5	2

The LD_{50} of the plant extract (>5000mg/ml) suggests that the plant may be considered safe for consumption [37]. The deaths that occurred in the animals were in a dose dependent manner, but we can not conclude based on that that the death was due to the administration of the extract. The inflammation observed at the site of administration also shows that the extract is pro-inflammatory. Changes in physical behaviour, body weight and internal organ weight are critical

for objective evaluation of effect of compound on test animals, since such changes are often the first sign of toxicity [38]. The treated rats lost weight, whereas the control animals gained weight, this may beh attributed to the loss of appetite observed in the treated animals.

Table 5: Zone of inhibition of the aqueous extract of *E. camaldulensis* on at 100mg/ml

	Zone of Inhibition (mm)			
Test Organism	*E. camaldulensis*	Amoxicillin	Chloramphenicol	Gentamycin
S. aureus	10	8	17	11
S. typhi	10	9	7	18
P. aeroginosa	7	14	11	10
E. coli	8	4	9	10
K. pnemuoniae	8	10	10	8
B. subtilis	6	11	7	3

Table 6: MIC of the aqueous extract of *E. camaldulensis* on some selected bacteria

	MIC (mg/ml)			
Test Organism	*E. camaldulensis*	Amoxicillin	Chloramphenicol	Gentamycin
S. aureus	12.5	25	12.5	12.5
S. typhi	12.5	12.5	100	12.5
P. aeroginosa	50	12.5	50	25
E. coli	50	50	25	25
K. pnemuoniae	25	12.5	12.5	12.5
B. subtilis	25	25	25	50

Table 7: MBC of aqueous extracts of *E. camaldulensis* on some selected bacteria

Bacteria	**MBC (mg/ml)**
S. aureus	50
S. typhi	-
P. aeroginosa	50
E. coli	100
K. pnemuoniae	-
B. subtilis	25

Table 8: Percentage weight change in test animals after administration of aqueous extracts of *E. camaldulensis* for 14 days

Groups	Dose (mg/kg b. wt.)	Mean weight[a] (kg)	Mean weight[b] (kg)	weight change
Group 1	800	210.27	214.54	2.03%
Group 2	1200	192.45	196.11	1.90%
Group 3	1600	186.90	191.20	2.30%
Group 4	2000	181.64	183.87	1.23%
Group 5	3000	180.48	183.21	1.51%
Group 6	5000	181.54	184.79	1.79%
Control	normal saline	186.48	190.98	3.07%

[a]: before administration; [b]: after administration

Bone marrow is one of the target sites for the adverse effects of the tests substances. Blood cells are mainly produced in the bone marrow; any test substance that affects the bone marrow could inhibit the certain enzyme activities involved in the production of heamoglobin in red blood cells and reduce the ability of the blood to distribute oxygen. The result of the PCV shows that there was gradual progress of anaemia from group 1 to group 4 in a dose dependent manner (56 to 46). However, groups 5 and 6 had an increase in the PCV level that is comparable with that of the control (58%). These may mean that all these are due to variation in the body weight and organ size of the rats.

Blood chemistry examinations are carried to ascertain damage to liver, kidney, heart and other internal organs. An elevated level of SGOT is a biochemical marker for myocardial infarction and acute liver damage. Result obtained shows that the treated animals and control had SGOT within the reference range. Result obtained from another indicator of the liver health, SGPT, shows that the treated animals had levels within the reference range. Elevated level of ALP may be due to liver damage and bone diseases. Result shows that the treated animals and control had ALP within the reference range. These suggest that the extract does not have acute deleterious effects on the heart and liver. However, the total protein levels were found to be decreased in a dose dependent manner in the treated animals. Total proteins are tissue building complex which have been stored in the liver on its way to the cell. Elevated levels is an indication of liver damage, sepsis, rheumatic fever etc, but decreased level is an indication of chronic alcoholism, starvation, heart failure, prolonged immobilization, malabsorption or malnutrition and pregnancy. The lower levels observed may be due to the loss in appetite of the animals.

The presence of secondary plant products in the bark of *E. camaldulensis* that are biologically important e.g saponins and tannins contribute to its medicinal value. The demonstration of antimicrobial activity against both gram-negative and gram-positive bacteria is an indication that the plant is a potential source for production of drugs with a broad spectrum of activity. Investigation reveals that the plant is not acutely toxic at ≥5000mg/kg b. wt. The results of the study also supports the traditional application of the plant and suggests that the plant extracts possess compounds with antibacterial properties that can be used as antibacterial agents in novel drugs for the treatment of gastroenteritis, supperative infections , urinary tract infections, typhoid fever and wound infections.

Table 9: Blood chemistry results

Groups	AST (µKatal/L)	ALT (µKatal/L)	ALP (µKatal/L)	Total Protein (g/L)
Group 1	21.69	24.73	73.82	65.60
Group 2	27.15	34.59	89.33	64.50
Group 3	26.11	31.83	186.28	67.86
Group 4	26.06	28.58	112.51	64.06
Group 5	23.49	24.43	85.61	64.54
Group 6	20.36	23.02	97.23	69.12
Control	22.79	30.62	72.45	66.50
Standard value	<37	<41	<448	64 - 83

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