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EVALUATION OF ANTIBACTERIAL AND IMMUNOLOGICAL EFFECTS OF *HIBISCUS CANNABINUS* PLANT EXTRACT IN MICE

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Abstract

Hibiscus Cannabinus is a valuable fibre and medicinal plant native to India and most African countries south of the Sahara. There is paucity of information on the therapeutic usage of this plant in Hausa land. Little is known of its constituents. Key cytokines that bridge the gap between innate and acquired immunity are IL-10 and IL-12. These cytokines are produced in response to microbial infections. In recent years drug resistance to pathogenic bacteria has been commonly reported worldwide. The photo constituents were evaluated using standard phytochemical methods; meanwhile, antimicrobial activities were estimated using the disc diffusion method. Safety and toxicity study were carried out in mice by feeding them methanolic extracts of the plant for 14 days, serum liver enzymes were measured before and alongside liver histology performed at the end. This Research evaluated pro/inflammatory cytokines. Extracts were found safe and non-toxic in mice based on levels of liver enzymes and histology, but showed no antimicrobial activity on the tested organisms. Significant rise was recorded in the

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level of IL-12 in both extracts indicating pro inflammatory potentials. Overall, the methanolic extracts is safe in mice, has phytoactive compounds exhibit no antibacterial activity on test organisms, and possess pro inflammatory potentials.

INTRODUCTION

According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medication for their primary health care needs (Ayoub *et al.*, 2014). It is a normal practice in villages or remote areas to use local herbs or plants to treat and cure various diseases (Ayoub *et al*., 2014). These practices are based on expertise and the use may be as old as mankind (Rojan *et al*., 2006). The field of alternative medicine or folk medicine is under- harnessed because among the estimated 250,000-500,000 plant species, a little fraction or percentage has been duly investigated phytochemicals and submitted for biological or pharmacological investigation and screening (Mahesh *et al.*, 2008).

Historically, natural products and their derivatives have been an invaluable source of therapeutic agents. In vitro and in vivo antimicrobial assays have effectively served as reliable methods to detect several classes of secondary metabolites with potent antimicrobial activity such as penicillin (Ayoub *et al*., 2014).

Hibiscus cannabinus is known as kenaf in English and Rama in Hausa. It is a valuable fibre and medicinal plant native to India and most African countries south of the Sahara (Bukenya *et al.*, 2004 and Wong, 2014). Kenaf grows naturally in grass land and as a weed in fields and waste land (Mukherjee *et al.,* 2010). It is grown from sea level up to an altitude of 2700m but does not do well above 2500. Optimum temperature is from 16°C to 27°C with 500-625m rainfall distributed over a period of 4-5months. It is grown as a fiber crop though grown on a small scale, it is a perennial plant growing to 1.8 (6ft) at fast rate (Bukenya *et al*., 2004).

Stems are erect, slender, and cylindrical. Leaves alternate, they can be simple or pubescent. Flower axillary, solitary and sometimes clustered, near the apex of the plant. (Mukherjee *et al.*, 2010) Once harvested, it can be

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stored or preserved by sprinkling water for 1-2 days to keep fresh, otherwise it can be sun dried. In Nigeria, kenaf leaves are used for soup and local salad. In Uganda, a local delicacy is made from seeds. These are roasted, grinded and pounded and the flower and skin parts are separated in water. The stem is a source of fiber used in the manufacture of twine rope and coarse textiles for sacking and cloth for packaging. Oil extracted from the seed is suitably used as a lubricant and for illumination, for manufacturing of soaps, linoleum and in paints and vanishes (Bukenya *et al*., 2004).

According to previous study, a 100g of kenaf leaves contains 79.0q water 280KJ energy, 5.5g protein 1.2g fat, 12.2g carbohydrate, 2.3g fibre,484mg calcium,18mg potassium, 12.1mg iron and 75mg ascorbic acid. (Bukenya *et al.*, 2004).

The innate immune system is an ancient defense system found in all multicellular organisms. It is comprised of cells and proteins which are able to recognize molecular patterns common to large groups of microorganisms. Another important function of the innate immune system is to activate the more recently developed acquired immune system and to focus its attachment on potentially dangerous antigen (Hessle *et al*., 2000). Two key cytokines that bridge the gap between innate and acquired immunity are interleukin 10(IL-10) and IL-12.Both are produced by monocytes, macrophages, and dendritic cells in response to microbes, but they have largely opposite properties (Hessle *et al*., 2000). Interleukin -12 is a T-cell stimulatory cytokine which activates T cell and NK cells to secrete gamma interferon (IFN-γ) and to lyse target cells. IL-10, in contrast, down regulates T-cell cytotoxicity and IL-12 and IFN-γ production and decreases presentation of antigens for T-cell. Instead,IL-10 stimulates B-cell maturation and antibody production (Hessle *et al*., 2000).

In recent years, a lot of studies are being carried out on various plants and herbs for pharmacological evaluation worldwide, but however, not a lot has been done on toxicology either short term or long term (Idris *et al.,* 2016).

Plant has a nearly unlimited capacity to generate compound that Fascinate research in the quest for new and novel chemotherapeutics. Infectious diseases are the world's leading cause of premature deaths, killing almost

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50,000 people every day (WHO). In Ayurvedic Medicine, the leaves are used in treatment of dysentery and bilious, blood and throat disorders. The powered leaves are applied to guinea worms in Africa (Subi. *Et al*., 2015). In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Ahmad *et al.,* 2001). However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug-resistant bacteria pathogens have further complicated the treatment of infectious disease in immune-compromised, AIDS and cancer patients (Iqbal and Arine, 2001). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants.

There is paucity of information on the therapeutic usage of the plant in Hausa land. The chemical analysis demonstrates its ascorbic acid content, but little is known of other constituents which may have potent antimicrobial and immunological properties (Bukenya *et al*., 2004). Since the seed of the plant has been found to have phytotoxic effect, the leaves which are widely consumed and flower will be assessed for immunological potentials and antimicrobial effect. *Moringa oleifera* is equally widely used in Hausa land as a source of food. However, much attention has been given on its medicinal usage. Such research focus has led to the discovery that it has the potential to boost immunity. It has diverse therapeutic value from management of diabetes mellitus to the treatment of infection (Anwar *et al.,* 2007). Hence the aim of the research is to evaluate Kenaf plants valued as much as *Moringa oleifera* for immunological effect and antimicrobial activities. Selected infectious agents such as *Staphylococcus aureus, Streptococcus pneumonae, Escherecia coli* and *Klebsiella pneumonae* was used to assess the anti-microbial effect of Kenaf, while the level of IL-10 and IL-12 will be used as a marker of immune enhancement.

The aim of the study was to evaluate the antibacterial as well as immunological effect of *Hibiscus cannabinus* in mice. The objectives were to assess the antibacterial activity of *Hibiscus Cannabinus* leaf and flower ethonolic extract on selected bacterial organism, also screen for toxicity

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and safety of the leaf and flower extract in Mice and to evaluate the effects of leaf and flower extract on IL-10 and IL-12

METHOD AND PROCEDURES

The leaf and flower of *Hibiscus cannabinus* were purchased from a garden along Zaria Road, Kumbotso Local Government Area of Kano State. The plant was identified and authenticated at the Plant Biology Department, Faculty of Science, Bayero University Kano, with an Accession number BUKHAN 0251 (Appendix iv)

The leaves and flower were collected, washed and air dried under shade and grinded into powder. Exactly 50g of leave powder was soaked in 500ml of 70% ethanol for 72h and 30g of the flower powder was soaked in 300ml of 70% ethanol for 72h. Each mixture was stirred every 24h using a sterile glass rod. At the end of extraction, each extract was strained, pressed and the combined liquids were clarified by filtration through Whatman's filter paper No.1. as described by (Sukhdev *et al.,* 2008).

Phytochemical screening of the plant extracts was carried out following the standard methods prescribed by Sofowara in (1993) and Trease and Evans (1989) to evaluate the presence of various chemical constituents in the extract.

Media used were Mueller Hinton, Blood and MacConkey agar which were prepared and dispensed according to the manufacturer's instructions. Pure culture isolates of *Staphylococcus aureus, Eschericia .coli, Streptococcus pneumonae* and *Klebsiella pneumonae* were obtained from clinical samples at the Microbiology Laboratory of Aminu Kano Teaching Hospital (AKTH) Kano. Whatman's filter paper was punched into circular disc with the aid of an office punch. The discs (6mm in diameter) were impregnated with the varying concentration of the extracts 1000,500 and 250mg.

Antibacterial activity assay of the various extracts was carried out using Kirby Bauer disc diffusion method. McFarland's (0.5) turbidity standard was used for *Eschericia .coli*, *Klebsiella* and *Staphylococcus* while *Streptococcus* was swabbed directly from a blood agar plate from a 24h old culture. A sterile cotton swab was used to inoculate/swab the surfaces of dried Agar

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plates and the extract impregnated discs were placed. Plates were incubated for 24h at 35°C.

Healthy Albino mice (9) weighing between 17 and 27g were used. The animals were housed individually based on their group of treatment and maintained in a standard laboratory environment at the animal house of Pharmacology Department Bayero University Kano. They were fed with standard rodent feed and water. All mice were maintain at 12-hour light/dark cycle and at room temperature and were randomly selected, tagged and marked for individual identification. Commercially prepared mice feed (grower mash) was used. The composition is given as: Crude protein-15.50%, Crude fiber-7.40%, Calcium-6.59%, Phosphorus-4.85%, Metabolized energy-2500kcal/kg, The factorial design was used and a total of 9 albino mice were grouped into three groups as follows (Anwar MB *et al*., 2016) Group 1: administered leaf extract Group 2: administered flower extract Group 3: control, fed with standard feed All extracts were administered by oral gavage for 14 consecutive days.

According to OECD's (organization of economic cooperation and development) guidelines, dosage should be constituted in appropriate volume usually not exceeding 10mg/kg (1ml/100g) body weight of experimental animals (mice and Rats) for non-aqueous solvent in oral route of administration. However in the case of aqueous solvents, 20mg/kg (2ml/100g) body weight can be considered.

$$\text{Dosage in mg} = \frac{\text{Weight of animal (g)}}{1000\text{g}} \times \text{Dose (mg)}$$

Based on the above formula the mice weighing 26g, 17g, 23g, 22g, 27g and 18g were fed with 0.15ml, 0.09ml, 0.13ml, 0.16ml and 0.10ml of the leaf and flower extract respectively daily for 14 consecutive days while mice weighting 22, 20 and 21gram serve as control. Animals were observed individually at least once during the first 30min after dosing, periodically during the first 24h, and daily thereafter for a total of 14 days for any clinical signs of toxicity or mortality.

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Alkaline phosphates (ALP), aspartate aminotransferese (AST) and Alanine aminotraferase (ALT) were determined by randox (colorimetric) method of Rec (1972). Histological study was carried out at Pathology Laboratory of Aminu Kano teaching Hospital as described by Auwioro (2010). The liver biopsies of the individual mice were fixed with 10% formal saline, dehydrated with ascending grade of alcohol, cleansed with toluene, infiltrated with molten paraffin wax. The microtome sections were stained with haematoxylin and eosin staining technique and examined with leica DM750 microscope and photographed with Leica ICCSOHD camera. Interlukin-10 and Interlukin-12 levels were determined using ELISA method according to manufacturer's instructions before and after administration of extract. The ELISA kit used was Sand which-ELISA method. The microelisa stripplate has been pre-coated with an antibody specific to the test cytokines. Whole blood was collected in EDTA container via Retro-orbital bleeding and was centrifuged at 2,000 rmp for 20 minutes.

All generated data were analyzed using SPSS Software version 20, California, USA was used for statistical analysis. All values were expressed as mean$\pm$SD .The difference between control and treatment groups were tested for significance Using Student t-test. A degree of freedom of 95% p-value of < 0.05 was considered as statistically significant.

RESULTS

Phytochemical screening of the plant crude extract of *hibiscus cannabinus* was carried out to ascertain the presence of bioactive constituents utilizing standards methods. The seven phytoactive compound were saponin, tannin, phenol, alkaloid, flavaliod, cardiac glycoside and terpeniod. Both leaf and flower ethnolic extract exhibited no antimicrobial activity (0mm) at 1000mg, 500mg and 250mg concentration on *staphylococcus aureus, streptococcus pneumonnae, Escherichia coli,* and *klebsiella pnemonnae.* Following the 14days plant crude extract administration to the mice no significant change in weight (gram) was recorded.Of the two cytokines measured (IL-10, IL-12) before and after administration of the plant extract to the mice, only IL-12 show a significant rise. Liver enzyme monitor in the

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course of this study were Alkaline phosphates (ALP), Aspartate aminotransferese (AST) and Alanine aminotraferase (ALT), which were found to be within normal limit before and after extract administration to the mice. Comparing the histology of the liver in leaf and flower extract of *Hibiscus cannabinus* and that of the control group, little/moderate inflammation was seen on the slide carrying the liver of the mice fed with flower extract while mild inflammation and bi-nucleation was seen on that fed with leaf extract. However, unremarkable changes were recorded of that of the control group.

TABLE 1: Phytochemical Screening of the Ethnolic extract of *Hibiscus Cannabinus* Leaf and flower

Phytochemicals	Flower	Leaf	
Saponin	+	+	
Tannin	+	+	
Phenol	+	+	
Alkaloid	+	+	
Flavonoid	+	+	
Cardiac glycoside	+	+	
Terpenoid	+	+	

Key:
+ indicates presence
The antibacterial activities of the ethnolic crude extract of *Hibiscus Cannabinus* leaf and flower (milligram) on test organisms.

TABLE 2: Sensitivity Test Result

Plant Part Unit	*S.aureus*	*E. coli*	*Klebsiella pneumonae*	*Streptococcus Pneumonae*
Leaf				
1000	R	R	R	R
500	R	R	R	R
250	R	R	R	R
Flower				

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1000	R	R	R	R
500	R	R	R	R
250	R	R	R	R
Control				
CIP	S(1.5)	-	S(0.5)	-
AMC	-	S(1.0)	-	-
CH	-	-	-	S(1.0)

KEY:

S-Sensitive (Zone of inhibiion in centimetres)

R-resistant

CONTROL:

C-Ciprofloxacin (5 microgram)

Anc-Ampicillin (10 microgram)

Cpc-chloramphenicol (30 microgram)

TABLE 3 Weigh of mice before and after treatment with leaf and flower extract *Hibiscus Cannbinus*

	Before Treatment	Day 14(after treatment)
Leaf	22.00 ± 4.58	20.33 ±4.93
Flower	22.33 ± 4.51	18.33 ± 2.52
Control	21.00 ± 1.00	20.67 ± 1.53

Result expressed as mean ± SD. P > 0.05 are considered insignificant

There was an insignificant change statistically with respect to IL-10 in group treated with leaf and flower extract while a significant change was recorded in IL-12 level in group treated with leaf and flower as shown in the table below.

Table 4: The level of interleukins recorded before and after administration of crude extract of *Hibiscus Cannabinus* leaf and flower.

Plant part	IL – 10		IL – 12	
	Before	**After**	**Before**	**After**
Leaf	0.54±0.01	0.51±0.01	0.38±0.13	0.40±0.01
Flower	0.56±0.02	0.49±0.01	0.37±0.01	0.40±0.01

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Control	0.55±0.02	0.55±0.02	0.39±0.01	0.39±0.01

Result expressed as mean ± SD. P > 0.05 are considered insignificant

The extract did not induce toxicity activities of liver enzymes Alkaline phosphates (ALP), aspartate aminotransferese (AST) and Alanine aminotraferase (ALT) showed no significant change.

Table 5: Effect of Plant on Liver Enzymes

Plant	AST		ALT		ALP	
part	**Before**	**After**	**Before**	**After**	**Before**	**After**
Leaf	47.33±2.08	56.67±11.59	46.33±2.89	47.33±1.15	44.01±2.65	47.67±8.33
Flower	47.33±2.08	40.67±11.01	46.33±2.89	51.00±4.59	44.00±2.65	49.33±9.29
Control	45.00±0.00	44.67±0.58	46.33±2.89	46.33±2.89	44.00±2.65	44.00±2.65

Result expressed as mean ± SD. P > 0.05 are considered insignificant.

CONCLUSION

The study indicates presence of phytoactive compounds Saponni, Tannin, Phenol, Alkaloid, Falvoniod, Cardiac Glycoside, Terpeniod with the potential of significant increase in IL 12. The role of IL 12 in innate immunity is well established. However, further work is required to establish the therapeutic benefit of the plant in terms of boosting cellular immunity against viral infection via stimulation of IL 12. The liver integrity has not been compromised as demonstrated by normal serum liver enzymes. Determination of serum liver enzymes has been used to assess toxicity of drugs or supplements. Integrity of the liver was not compromised based on histological examination of the liver which mild inflammation.

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