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Toxicological studies of ethanol leaf extract of *Cassia fistula* on haematological and biochemical parameters of wistar albino rats

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Abstract
Aim: *Cassia fistula* L. (*Fabaceae*) is an annual herbaceous plant used in folklore medicine for the treatment of a wide range of conditions such as astringent, febrifuge and purgative. But the effects have not been well elucidated. This study was aimed at investigating the effect of *Cassia fistula* on haematological and serum biochemical indices in Wistar rats.
Materials and Methods: Phytochemical constituents from the ethanol extract of *C. fistula* were identified by qualitative techniques and gas chromatography-mass spectroscopy (GC-MS). Twenty male Wistar rats were randomly assigned into four groups of five rats each. Group A, the control, received 0.2ml of corn oil each daily for 7 days; Group B rats were given 100mg/kg b.w. of *Cassia fistula* extract, Group C were given 200mg/kg b.w. of the extract while the rats in Group D were given 300mg/kg b.w ethanol extract of *Cassia fistula* orally for 7 days. Blood samples were collected afterward for determination of haematological parameter while plasma biochemistry was carried on the plasma after 7 days of treatment with the extract. Frequency of micronucleated polychromatic erythrocytes was also determined from blood Giemsa stained smear.
Results: Qualitative analyses revealed the presence of tannins, saponins, alkaloids, anthraquinones, cardenolides, and phenols. In GC-MS analysis, 19 compounds including phytol, oleic, myristic acids etc. were detected from the ethanol leaf extract of *C fistula*.
Oral treatment of *Cassia fistula* was found to be safe up to the 300mg/kg b.w dose because haematological and biochemical parameters in treated rats were comparable to the untreated control. In fact, the extract showed nephroprotective and hepatoprotective activity at 200 mg/kg.
Conclusion: This study showed that ethanol leaf extract of *Cassia fistula* is not toxic upt to 300 mg/kg b.w, instead the extract showed some protective effects on liver and kidney functions at the 200 mg/kg b.w dose. This dosage should therefore be explored further for therapeutic purposes.

Keywords: *Cassia fistula*, haematology, plasma biochemistry, phytochemical screening, toxicity

Introduction
Cassia fistula Linn. (Fabaceae, Caesalpinioideae), commonly known as "Indian Laburnum" has received a considerable attention in the pharmacology of medicinal plants, globally within the last two decades (Abu *et al*., 1999; Akinyemi, 2000; Phongpaichits *et al*., 2004; Danish *et al*., 2011; Bhalerao and Kelkar, 2012, Guruprasad *et al*., 2015) [1-6]. The plant is well distributed in various regions including Asia, South Africa, China, West Indies and Brazil, having been extensively used in Ayurvedic system of medicine for various ailments (Bhalerao and Kelkar, 2012) [5]. The plant is a deciduous tree found in mixed-monsoon forest throughout greater parts of India, ascending to 1300 m in outer Himalaya, where it is widely used in traditional medicine (Gupta, 2010) [7].
Different parts of *C. fistula* have been reported to contain flavonoids, phenolic compounds and proanthocyanidins (Luximon *et al*., 2002) [8]. Beside traditional use, *Cassia fistula* L. has been scientifically reported to possess various pharmacological activities including analgesic (Sheikh *et al*., 2010), anti-inflammatory (Ilavarasan *et al*., 2005) [9], antioxidant (Luximon-Ramma *et al*., 2002; Irshad *et al*., 2012) [8, 10], antidiabetic, hypolipidemic (El-Saadany *et al*., 1991) [11] as well as hepatoprotective activity (Bhakta *et al*., 2001) [12], antibacterial, antifungal (Duraipandivan and Ignacimuthu, 2007; Guruprasad *et al*., 2015) [13, 6] and wound healing properties (Kumar *et al*., 2006) [14], and anticancer activity (Irshad *et al*., 2014) [15].
Given the wide range of actual and suggested use of *C fistula*, there is a need for evaluation of the safety of ethanol leaf extract of *the plant* in order to effectively deploy it for medicinal use without inducing adverse effects. This study was therefore carried out to investigate the effects of different doses of ethanol leaf extract of *Cassia fistula* on haematological and biochemical indices in Wistar rats.

Materials and Methods

Plant Collection, identification and authentication

Fresh leaves of *Cassia fistula* were collected from the Department of Agronomy, University of Ibadan, Ibadan (Nigeria) in the month of May 2014. The plant was identified and authenticated with voucher no: UIH-22396 at the Department of Botany, University of Ibadan, Ibadan, Nigeria.

Preparation of Extract

The freshly collected leaves of *Cassia fistula* plant were air-dried under shade and pulverized.. The dry powder was soaked in hexane for twenty-four hours to de-fat. The ground particles were removed, air-dried and then soaked in 95% ethanol for 72 hours. The soaked particles were removed and the supernatant was filtered to remove other particles. The filtered solution was then passed through rotary evaporator at 40 ^{o}C for drying.

Chemicals

Analytical grade chemicals were purchased from Sigma-Aldrich, Germany; biochemical assay kit obtained from RANDOX Laboratories Ltd., Ardmore, United Kingdom. Freshly prepared stock solution was used for the experiment

Phytochemical screening

The leaf extract of *Cassia fistula* was screened for the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, tannins and phenols using standard methods as described by Harbone, 1984[16] and Evans, 2002[17].

Gas Chromatography Mass Spectrometry (GC-MS) analysis

GC-MS (Aligent Technologies) 5973 Network selective detector with column DB23 model number J & W 1222362 with internal diameter of 60 m × 250 µm × 0.25 µm (250 °C Max) was used for this analysis. The *C. fistula* extract (50 µL) was dispensed into 1mL sample vial and diluted with methanol. The mixture was filtered through a 0.45 µm syringe into GC-MS vials. The injection volume of 1 µL, wash volume of 8 µL injection temperature of 250 °C and flow rate of l mL /min was employed with total flow of 6.4 mL with column flow of 0.57 mL/min. The pressure used was 16.0 kPa. The GC temperature condition was 50 °C for 2 min, increased at °C /min to 100 °C, held for 10 min and increased at 15 °C to 250 °C and held for another 10 min. The starting m/z was 50 and ended with 500. The total runtime was 38 min.

Experimental Animals

Twenty adult male Wistar rats weighing between 160 and 220 g were used for the study. The animals were kept in the experimental animal house, Faculty of Veterinary Medicine, University of Ibadan. The rats were fed with commercial rat feed pellets and potable water was provided *ad libitum* throughout the course of the experiment. They were stabilized and allowed to acclimatize for one week before the commencement of treatment. Standard national and international protocols for the use care of experimental animals were followed throughout the course of the experiment.

Treatment Protocol

The rats were divided into four (A - D) groups of five animals each. The ethanol extract of *Cassia fistula* leaf and corn oil given to experimental animals were administered orally as follows:

Group A: Rats treated with 0.2 ml of corn oil each daily for 7 days.

Group B: Rats treated with plant extract (100 mg/kg) daily for 7 days.

Group C: Rats treated with plant extract (200 mg/kg) daily for 7 days.

Group D: Rats treated with plant extract (300 mg/kg) daily for 7 days.

Sample collection

Blood samples were collected from the retro orbital sinus of the rats into lithium-heparinized tubes 24 hours after the last treatment. Haematological parameters were determined as described by Guyton and Hall (2006) [18]. Packed cell volume (PCV) was determined by microhaematocrit method, haemoglobin concentration (Hb) by cyanmethaemoglobin while red blood cell (RBC) and white blood cell (WBC) were determined using the improved Neubauer haemocytometer. Differential WBCs (lymphocytes, neutrophils, monocytes) were determined in Giemsa stained smear. Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were calculated from the values of haematological parameters as describe by Jain (1985) [19]. Blood samples for biochemical analysis were centrifuged at 3000 rpm for ten minutes to separate the serum. Total protein, albumin, globulin, creatinine, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and blood urea nitrogen were determined by use of automated analysers with the appropriate Randox kit.

Micronucleus assay

The micronucleus test was carried out following Schmid's[20] method. Peripheral blood was obtained by cardiac puncture to prepare a blood film. The slides were fixed with absolute methanol and stained with 3% Giemsa. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) based on the observation of 1,000 PCEs per animal was recorded.

Data analysis

All data are expressed as means and standard deviation while comparison was by one-way ANOVA using the Graphpad® Prism version 5.00 for Windows, Graphpad® Software. Significance was reported at $P < 0.05$.

Results

Table 1 shows the result of the qualitative phytochemical analysis of ethanol leaf extract of *Cassia fistula*. The result revealed the presence of alkaloids, anthraquinones, cardenolides, saponins, tannins, and phenols in the ethanol leaf extract of *C. fistula* whereas flavonoid was absent. The GC-MS analysis of *C. fistula* through the recognition of phytochemicals by the peak areas, molecular formula and molecular formula similarly revealed the presence of 19 compounds (Table 2). Notable among these compounds were 4,5,7,8-Tetra-0-acetyl-2-0-benzyl-3-deoxy-D-manno-2-octulopyranosoate, Phytol, 11-Eicosenoic acid (omega-9 fatty acid), n-Hexadecanoic acid (Palmitic acid), 2,6-Di-O-palmitoyl-L-ascorbic Acid, oleic and Myristic acids and many other long chain fatty acids as well as alpha, beta and gamma

tocopherol (See Table 2 and the chromatogram peaks on Fig 1).

The mean values of haematological parameters are presented in Table 3. There were no significant differences ($P>0.05$) in the mean values of PCV, RBC, HB, basophils, lymphocytes, monocytes and eosinophils across the groups.

The results of the effects of *Cassia fistula* leaves extract on kidney function and lipid profile are presented in Tables 5 and 6. In a manner similar to the observations on the haematological parameters, there were no significant differences ($P>0.05$) in the mean values of creatinine, bicarbonate, potassium ion, urea, Sodium ion and chloride across the groups. However, the lowest Na^+, Cl^-, urea and creatinine were recorded in rats treated with 200 mg/kg b.w. (Group C). Conversely, there were no significant differences ($p<0.05$) in the total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (LDL) across the treated groups when compared with the control.

The results of the micronucleus assay are shown in Fig 2. Although, there increases in the number of micronuclei in rats treated with *C.* fistula extract at 200 and 300mg/kg doses when compared with the untreated control, the increases were not statistically significant, while those rats treated with 100mg/kg dose of the extract had slightly lower micronuclei than the untreated control.

Discussion

Alternative medicine is being widely engaged in the prevention, diagnosis, and treatment of a number of diseases having attracted a sustained increase in public attention over the past two decades due to its accessibility all over the world (Humber, 2002) [21]. Additionally, phytomedicine have shown great promise in the treatment of some intractable infectious diseases (Yesilada, 2005) [22]. Surprisingly however, little emphasis by herbal medicine users is concentrated on possible undesirable side effects associated with the use of plant products, especially the crude extracts. Toxicity and safe dose of any plants and its ingredients are very crucial in the use of health management.

The qualitative phytochemical analysis of ethanol leaf extract of *Cassia fistula* revealed the presence of alkaloids, anthraquinones, cardenolides, saponins, tannins, and phenols whereas flavonoid is absent (Table 1). This shows that the extract may posses some pharmacological activities because of the presence of alkaloids, cardenolides and anthraquinones (Huang *et al*., 2007, Al-Otaibi and Gogary, 2017) [23, 24]. In the GC-MS analysis of *C. fistula, the* 19 compounds that were identified (Table 2) were mainly long chain polyunsaturated fatty acids, ascorbic acid, phytol and tocopherol. The recognition of phytochemicals is determined by the peak area, molecular formula and molecular formula in from of peaks on the chromatogram (Figure 1) Despite the lack of flavonoids in the extract, the presence of ascorbic acid, phytol – a precursor of alpha tocopherol and α, β and γ tocopherol as shown by the GC-MS analysis may confer some antioxidant potentials on the ethanol leaf extracts of *C fistula.* Thus, the extract will be able to help in the mopping up of free radicals and reactive oxygen species during oxidative stress due to the presence of the antioxidants (Traber and Atkinson, 2007) [25]. The plant is also a good food supplement, because of the presence of polyunsaturated and essential long chain fatty acids, especially linolenic and linoleic acid, which were also identified by GC-MS.

Evaluation of the haematological parameters of the rats used for this study showed that there were no significant differences in the mean values of PCV, RBC, HB, basophils, lymphocytes, monocytes and eosinophils across the groups. This suggests that the extract is safe in the rats with no deleterious effect on red blood cells. The safety of the extract at the dosages used in this study is also validated by the results of the micronucleus assay (Fig 2), which showed that the extract did not significantly increase the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in blood smear. The use of micronucleus genotoxicity assay in the evaluation of toxicity by estimating the values of micronucleated polychromatic erythrocytes (MNPCEs) in blood bone marrow smears have been used as a veritable tool in the evaluation of toxicity associated with exogenously introduced toxicant (Matshuyama *et al*., 2018) [26]. This erythrocytes response to toxicity is due to the fact that haematopoietic tissue and processes are very sensitive to toxic compounds and they serve as important indices of the physiological and pathological status in both animals and humans (Adeneye *et al*., 2006) [27].

The mean values of serum biochemistry indices of the wistar rats - globulin, conjugated bilirubin, AST and ALT also showed no significant differences across the groups. However, rats treated with 200 mg/kg b.w. (Group C) showed significant hepatoprotective effect via lowering the serum levels of ALP and GGT. This agrees with previous reports on the hepatoprotective properties of *Cassia fistula* (Bhakta *et al*., 2001; Bhalerao and Kelkar, 2012) [12, 5]. A previous study on the *n-heptane* extract of *C. fistula* leaves also confirmed that *C. fistula* at a dose of 400 mg/kg had significant hepatoprotective activity which was comparable to that of a standard hepatoprotective agent. The liver has been reported as the main target organ of acute toxicity and its exposure to foreign substances results from subsequent absorption of these toxic materials in the small intestine, transportation to the liver where they are metabolized to other compounds, which may or may not be hepatotoxic (Rhiouania *et al*., 2008) [28].

The study also showed that exposure to *C. fistula* up to 200 mg/kg did not affect the lipid profile, however, consumption of the ethanol leaf extract of the plant did increased the values of HDL and LDL significantly. Therefore, *Cassia fistula* leaves extract will not contribute to the establishment of cardiovascular diseases in rats up to the 200 mg/kg dose administered in this study. This is in agreement with the findings of Khatib *et al.,* 2010 [28] that *Cassia fistula* has some cardio-protective activities. However, the highest total cholesterol, triglyceride, high density and low-density lipoprotein were recorded in the rats treated with *Cassia fistula* extract of 300 mg/kg b.w. So, caution must be exercised when the extract is to be used at this dose.'

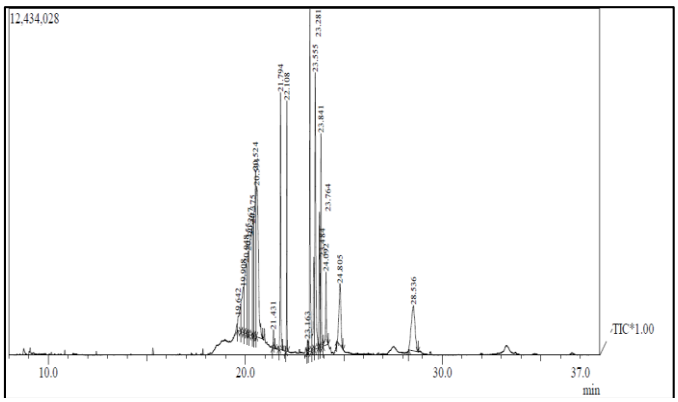

Fig 1: Chromatogram (GC/MS) of ethanol leaf extract of *C. fistula*

Table 1: Qualitative Phytochemical screening of ethanol leaf extract of *C. fistula*

Chemical class of constituents	Qualitative test	Result
Alkaloids	Mayer's reagents	+ve
Anthraquinones	Borntrager's test	+ve
Cardenolides	Killer-killanins	+ve
Flavonoids	Alkaline reagent test	-ve
Saponins	Frothing	+ve
Tannins	Ferric chloride	+ve
Phenols	Sodium hydroxide	+ve

+ = Present; - = Absent.

Table 2: GC-MS analysis of leaf extract of *Cassia fistula*

S/N	RT	MW	Formula	Compound name
1	16.583	496	$C_{23}H_{28}O_{12}$	4,5,7,8-Tetra-0-acetyl-2-0-benzyl-3-deoxy-D-manno-2-octulopyranosoate
2	20.525	296	$C_{20}H_{40}O$	Phytol
3	20.525	310	$C_{20}H_{38}O_2$	11-Eicosenoic acid (omega-9 fatty acid)
4	21.792	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid (Palmitic acid)
5	21.792	652	$C_{38}H_{68}O_8$	2,6-Di-O-palmitoyl-L-ascorbic Acid
6	21.792	270	$C_{17}H_{34}O_2$	Heptadecanoic acid
7	22.033	282	$C_{18}H_{34}O_2$	Ethyl 9-hexadecenoate (Oleic acid)
8	22.108	284	$C_{18}H_{36}O_2$	Hexadecanoic acid, ethyl ester (Ethyl palmitate)
9	23.067	256	$C_{16}H_{32}O_2$	Tetradecanoic acid, ethyl ester (Myristic acid)
10	23.167	292	$C_{19}H_{32}O_2$	9,12,15-Octadecatrienoic acid, methyl ester (Linolenic acid, methyl ester)
11	23.483	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (cis-Linoleic acid)
12	23.483	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Linoleic acid methyl ester)
13	23.483	252	$C_{16}H_{28}O_2$	Oxacycloheptadec-8-en-2-one
14		278	$C_{18}H_{30}O_2$	9,12,15-Octadecatrienoic acid (alpha.-Linolenic acid)
15	24.092	306	$C_{20}H_{34}O_2$	9,12,15-Octadecatrienoic acid, ethyl ester (Meed acid)
16	24.092	312	$C_{20}H_{40}O_2$	Octadecanoic acid, ethyl ester (Stearic acid ethyl ester)
17	24.433	430	$C_{29}H_{50}O_2$	Vitamin E (alpha.-Tocopherol)
18	34.642	416	$C_{28}H_{48}O_2$	Beta.-Tocopherol
19	34.642	416	$C_{28}H_{48}O_2$	Gamma.-Tocopherol

Table 3: Haematological Parameters of Wistar Rats Treated with Ethanol Extract of *Cassia fistula* Leaves.

Parameters	Group A	Group B	Group C	Group D
PCV (%)	47.8±1.92	44.6±2.97	47.8±4.66	44.2±3.77
Hb (g/dl)	15.9±0.60	15.3±0.93	16.2±1.6	14.88±0.88
RBC (x 10^6/µL)	8.38±0.63	8.48±0.79	9.03±1.35	8.14±1.01
MCV (fl)	57.24±4.3	53.06±6.99	53.39±5.14	54.72±5.56
MCH (pg)	19.06±1.17	18.17±1.92	18.07±1.5	18.43±1.59
MCHC (g/dL	33.27±0.7	34.4±2.66	33.89±0.85	33.73±1.13
PLATELETS	707400±121	611400 ±751	578200±767	693400±543
WBC (x 10^3/µL)	13.36±0.22	12.98±0.35	10.03±1.49	10.37±0.14
LYMPH (x 10^3/µL (%)	5.18±0.083 38.8±2.59	4.49±0.12 34.6±4.04	3.65±0.54 36.4±6.66	3.44±0.047 33.2±7.43
NEUTRO (x 10^3/µL) (%)	7.91±0.13 59.2±4.44	8.25±0.22 63.6±3.78	6.44±0.96 64.2±7.56	6.76±0.091 65.2±5.81
MONO (x 10^3/µL) (%)	0.094±0.015 0.7 ± 0.05	0.10±0.03 0.8± 0.03	0.14±0.04 1.4±0.04	0.12±0.002 1.2±0.05
EOSINO (x 10^3/µL) (%)	0.080±0.013 0.6± 0.04	0.10±0.03 0.8±0.01	0.06±0.009 0.6± 0.01	0.02±0.002 0.2± 0.05
BASO (x 10^3/µL) (%)	0.53±0.009 0.004±0.005	0.026±0.007 0.2±0.05	0.004±0.006 0.4±0.0	0.02±0.002 0.2±0.04

PCV = Packed cell volume; Hb= haemoglobin concentration; RBC = red blood cell count; MCV= mean corpuscular volume MCHC = mean corpuscular haemoglobin concentration; WBC = white blood cell count

Table 4: Liver Function Parameters of Wistar rats treated with ethanol extract of *Cassia fistula* leaves.

	TP	ALB	GLB	A/G	TB	CB	AST	ALT	ALP	GGT
Group A	6.6±0.5	3.8±0.2	2.6±0.3	1.4±0.1	0.52±0.19	0.20±0.07	10.6±2.0	8.4± 0.6	36.0±7.2	6.6± 1.1
Group B	6.4±0.3	3.7±0.2	2.8±0.3	1.3±0.2	0.44±0.09	0.18±0.08	10.2±1.4	8.2± 0.3	33.0±8.5	6.0± 1.0
Group C	6.1±0.4	3.6±0.3	2.5±0.3	1.5±0.2	0.34±0.11	0.20±0.12	9.0± 1.6	8.2± 0.9	30.6±3.0*	5.4± 0.8*
Group D	6.7±0.5	3.9±0.2	2.7±0.3	1.4±0.2	0.44±0.11	0.18±0.08	11.8±2.9	8.6±0.6	37.6±7.1	7.4± 0.9

*=Significant value within column. TP=Total Protein; ALB=Albumin; GLB=Globumin; A/G= Albumin: Globulin ratio; TB= Total bilirubin CB=Conjugated bilirubin; AST= Aspartate aminotransferase; ALT=Alanine aminotransferase; ALP=Alkaline phosphatase; GGT=Gamma-glutamyl transpeptidase

Table 5: Kidney Function Parameters of Wistar Rats Treated with Ethanol Extract of *Cassia fistula* Leaves

	Na^+	K^+	Cl^-	$HCO3^-$	UREA	Cr
Group A	136.6±3.51	3.7± 0.34	100±7.91	24.2±2.59	18.8±3.77	0.52±0.15
Group B	136.6±2.41	3.62±0.34	103±5.7	24± 1.41	20.4±3.29	0.54±0.09
Group C	135.6±4.45	3.64±0.42	99± 5.48	24.4±2.07	16.4±4.72	0.44±0.15
Group D	138± 3.08	3.74±0.4	100±7.91	23.8±1.79	20.8±4.97	0.54±0.13

Na^+=Sodium; K^+=Potassium; Cl^-=Chloride; $HCO3^-$=Bicarbonate; Cr=Creatinine

Table 6: Lipid Profile Parameters of Wistar Rats Treated With Ethanol Extract of *Cassia Fistula* Leaves

Parameters	TC	TRG	HDL	LDL
Group A	101.0±8.94	52.2±7.53	30.2±3.19	61.6±5.60
Group B	107.0±13.04	49.0±4.95	34.6±7.27	63.8±10.38
Group C	111.0±14.32	50.6±9.79	32.2±5.63	67.4±12.52
Group D	139.0±25.59*	55.8±7.73*	42.4±9.66*	78.4±6.27*

TC=Total cholesterol; TRG=Triglyceride; HDL=High density Lipoprotein; LDL=Low density lipoprotein

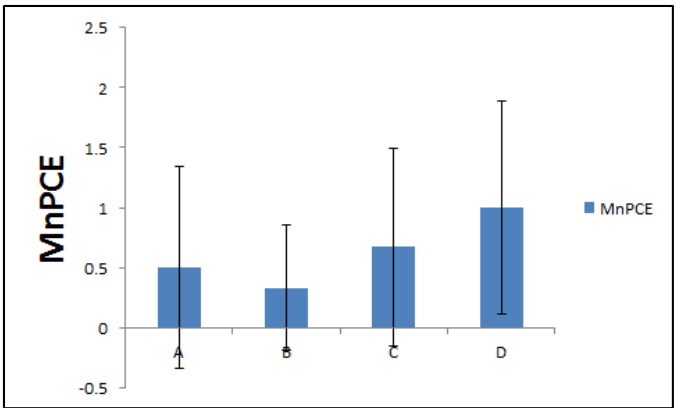

Fig 2: Results of Micronucleus genotoxicity assay in groups of rats treated with 100 (Group B), 200 (Group C) and 300 mg/kg (Group D) ethanol leaf extract of *C. fistula*

Conclusion

This study accentuates the presence of many secondary metabolites in the leaves of *C. fistula* as well as provides an overview of the different classes of molecules that may have pharmacological importance. Furthermore, ethanol extract of *Cassia fistula* up to the dose (200 mg/kg) did not pose any danger to rats, as the extract is both hepato-protective and reno-protective. However, further studies are needed on these phytochemical constituents in order to isolate and elucidate the structures of the compounds with different biological activities.

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