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Removal of Phorbol Ester from Jatropha Curcas Seed Cake Reduces Toxicity in Rats Blood Plasma Studied through Various Parameters

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ABSTRACT

Jatropha curcas in spite of having many medicinal effects is not used usually as medicine because of the presence of toxic components like phorbol ester. In this study, we have compared many chemical and biochemical parameters between plant extract (MEMJC) Methanolic extract of mechanical cake of *Jatropha curcas* seeds and without the presence of phorbol ester (MEHJCAT**).** Alkali treated mechanical cake of *Jatropha curcas* seeds .The phorbol ester was removed by alkali treatment. Phytochemical analyses were done to determine composition. The survival study was evaluated using Kaplan Meier Chart. SOD activity was assayed by the method of McCord and Fridovich and Catalase activity was assayed by Aebi's method. Urea, Creatinine, triglyceride level in plasma was measured using their respective kits. The removal of phorbol ester through alkali treatment makes the MEHJCAT less toxic or less vulnerable to generate free radical in rats. Through oral fat tolerance test, we have found that MAHJCAT reduces the absorption of triglyceride in blood. The results of renal function test also favored MEMJC as more toxic then MEHJCAT. Also from kaplen meier survival curve, it has been found that both drugs MEMJC and MEHJCAT have no mortality significance proved by the measurement of $LD_{50.}$ So, we have concluded that MEHJCAT as better medicinal plant product through the study of all biochemical parameters.

Keywords: *Jatropha curcas* seed cake, Oral fat tolerance test, Hyperglycemic, Phytochemicals.

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INTRODUCTION

Jatropha curcas is a multipurpose plant adapted to barren or affected areas and can be exist in poor stony soils, belongs to the family of Euphorbiaceae having a lot of economic and medicinal aspects. It contains high amount of oil and upon trans esterification this oil can be converted into high quality biodiesel. On the other hand, this seed cake or kernel meal leftover has also been utilized in feed formulation[1]. The kernel meal is also rich in protein[2]. The various parts of this plant like leaves, fruit, seed, stem bark, branches, twigs, latex and root were used for the medicinal purposes [2]. It has been found that the main cause of *Jatropha curcas* toxicity is the presence of phorbol ester which prevent its utilizations as feed ingredients and as medicine[3, 1]. Phorbol esters have tigliane skeletal structure called diterpenes. There are mainly six phorbol esters that has been found.[4] .Phorbol esters as amphiphilic molecules has tendency to bind phospholipids membrane receptors. During normal signal transduction process, PKC (protein kinase C) has activated by DAG (diacyl glycerol), which is involved in various other signal transduction pathways. The phorbol ester has found to be act as analogue of DAG and is strong PKC activators. Phorbol esters do not directly induce tumors but promote tumor growth following exposure to sub carcinogenic dose of carcinogen through hyper activation of PKC and trigger cell proliferation. So through detoxification by removal of phorbol ester we can prepare more beneficial plant products which would be more beneficial to individual's health.

MATERIALS AND METHOD

Plant Material

Jatropha Curcas seeds cake *(*identification of the plant was done by Prof. K.N. Diwedi Reference number "DG/KND/11-12/603" was given to plant sample). was obtained from, Surya Pharmaceutical Company D-17 Industrial areas Ramnagar Chandauli.

Ethical Considerations

The studies were conducted under the rules and regulation of Institute Ethical Committee, IMS, BHU. (Ethical committee letter no-Dean /12-13/CAEC/15)

Preparation of seed cake extract

The cake was dried in oven at 50^0C. Cake was subjected to methanolic extraction by reflux method. Its powdered form had been prepared in iron vessel. For extract preparation, weight the amount of sample was refluxed in round bottom flask by two hour on water bath. The solvent was filtered out and the process was repeated two times. The solvent from all the steps were collected

and distilled in vacuum distillation plant. The solvent free extract was prepared by drying the solvent on water bath and by desiccation in the vacuum desiccator till constant of the weight.

Phytochemical analysis of two cakes of *Jatropha curcas seeds*

Different cakes of *Jatropha curcas*-were dissolved in suitable solvents and analyzed for the presence of different phytochemical such as phenols, tannins, terpenoid, flavonoids and saponins 5. Different cakes of *Jatropha curcas seeds* showed presence of various Phytochemical as given below in table 1.

Quantification of phorbol ester was done by HPLC analysis

Phorbol ester concentration in both extract was determined by HPLC-UV method. Both extracts aliquot was loaded separately on an HPLC-UV reverse phase C18 LiChrophere 100, 5 µm (250x4 mm ID. from Merck, Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (35 °C) and the flow rate was 1 ml/min using isocratic elution of 1:1 (v/v) deionized water mixed with acrylonitrile as mobile phase[6].

Phytochemical analysis for secondary metabolites

The preliminary Phytochemical screening of secondary metabolites was done by Harborne method (1973).

Animal Design

All rats were kept in 6 hrs fasting condition. The rats were divided in four groups (n=6). Having 6 rats in each.

Group 1 Rats consume only water (Ethical committee letter no-Dean /12-13/CAEC/15)

Group 3 for each conc. fed only *drug (*MEMJC and MEHJCAT*)* at the concentrations of 6.25 mg/100 g bw and 3.125 mg /100 g bw ,

Group 2 Consume only HFD (high fat diet) Plus Drug Vector orally,

Group 4 for each conc. fed HFD plus *drug (*MEMJC and MEHJCAT*)* at the concentrations of 6.25 mg/100 g BW and 3.125 mg /100 g BW.

Dose Preparation

After alkali treatment, known concentrations in the ratio of 1:16 w/v was boiled till the volume of require is ¼ then it was filtered and then it was concentrated to the dryness on the water bath. Its fixed amount was dissolved in water to make stock solution to get further dilution Preparation of various concentrations of *Jatropha curcas* press mechanical cake and alkali treated cake was made by water decoction method.6 (6.25 mg/100 g BW) and (3.125 mg /100 g BW to get a appropriate working solution .So that volume of oral administration showed be within 1 ml for all tested doses.

Antioxidant Enzymatic activity

SOD activity was assayed by the method of McCord and Fridovich and Catalase activity was assayed by Aebi's method.

Measurement of Urea, Creatinine in plasma

Urea and Creatinine level in plasma was measured using an kits (Accurex).

High fat diet preparation

High fat diet contains 40% Lard, 40% Glucose, 18 % Soybean, 1% Vitamin B and 1% Methionine.

Oral Fat Tolerance Test

On the day preceding the experiment, the rats were appropriately grouped and placed in the experiment room for acclimatization. The rats were fasted for 6 hrs. The doses for the study were fixed at 6.25 mg/kg and 3.125 mg/kg BW. The extracts were dissolved in a vehicle containing 2% Tween-20**.** Normal rats weighing 150 ± 5 g were used in this study. At 0 and 20 days after treatment the OFTT were done. Just before experiment nearly 0.5 ml blood was collected from the occular vein of each rat (for measuring basal value of Triglyceride) in ependorf tube .Within a minute, they were given their drugs and after half an hour they were given high fat diet. The 0.5 ml bloods were taken again at 2hrs, 4hrs and 6 hrs after HFD. The amount of HFD given to each rats was 12.5 ml/ kg body weight. The plasma was separated at 3000 rpm for 10 min. and stored at -20^{o} C. for the determination of OFTT (oral fat tolerance test). The triglyceride concentration was measured by their own Kits (Accurex).

RESULTS AND DISCUSSION

Quantitative Estimations

In both MEMJC and MEHJCAT, the test for the presence of alkaloid, saponins, tannin, terpenoid, phenols, flavonoids, glycosides was found to be positive. The steroids were absent (Table 1).

Table 1: Qualitative analysis of two cakes of *Jatropha curcas seeds*

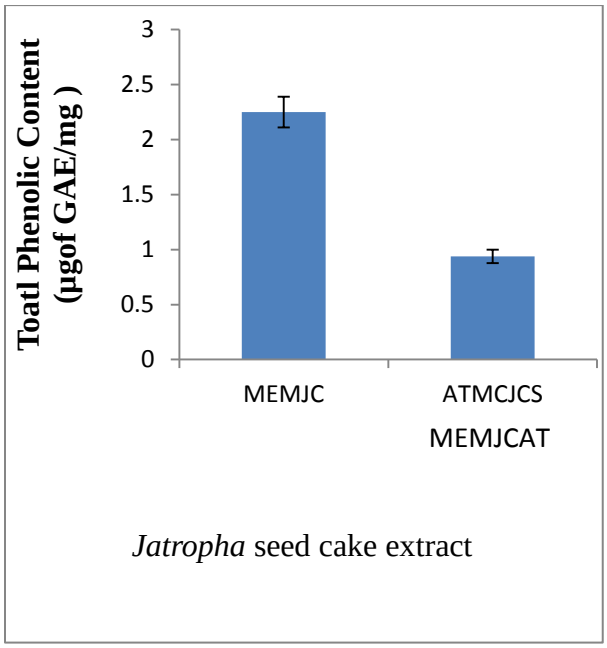
Class of Phytochemical	MEMJC	MEHJCAT
Alkaloid	+	+
Saponin	+	+
Tannin	+	+
Terpenoids	+	+
Steroid	–	–
Phenol	+	+
Flavonoids	+	+
Glycosides	+	+

The maximum phenolic content was found in: MEMJC (Methanolic extract of mechanical cake of *Jatropha curcas*) which was 2.25±0.64 µg of GAE/mg. It was followed by, MEHJCAT (Alkali treated mechanical cake of *Jatropha curcas* seeds) which had 0.938±0.06µg of GAE/mg (Table 2, Figure.1). Total flavonoid was higher in, MEMJC 26.15±3.84 µg of QE/ mg, followed by 17.48.±1.05µg of QE/ mg in MEHJCAT. The tannin content was much in, MEMJC 76.96±5.13 µg of TAE/ mg and least in MEHJCAT 56.16±1.23 µg of TAE/ mg. According to HPLC result the concentration of phorbol ester in MEHJCAT 22.12% and in MEMJC is 77.45% (Figure.2 and 3). The group of phorbol ester peaks was detected at 254 nm and appeared at 2–2.3 min of chromatogram. The results were expressed as equivalent to an external standard, phorbol-12-myristate-13-acetate.

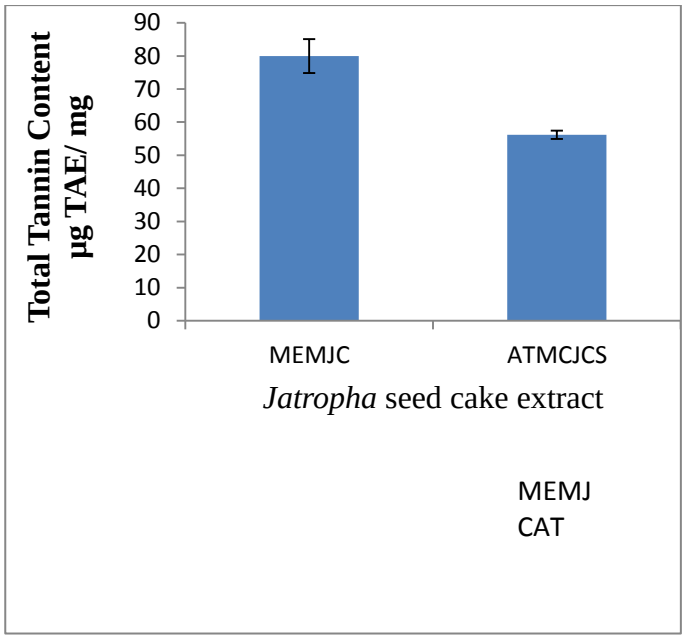
Table 2. Quantitative estimation of major secondary metabolites in of two cakes of *Jatropha curcas seeds*. (Mean, n=3)

Different cakes of *Jatropha curcas seeds*	Total phenolic Content (µg of GAE/mg)	Total Flavones content (µg of QE/ mg)	Total Tannin content (µg of TAE/ mg)
MEMJC	2.25±0.64	26.15±3.84	76.96±5.13
MEHJCAT	0.938±0.06	17.48.±1.05	56.16±1.23

*The values are measured in mean ± SEM



A



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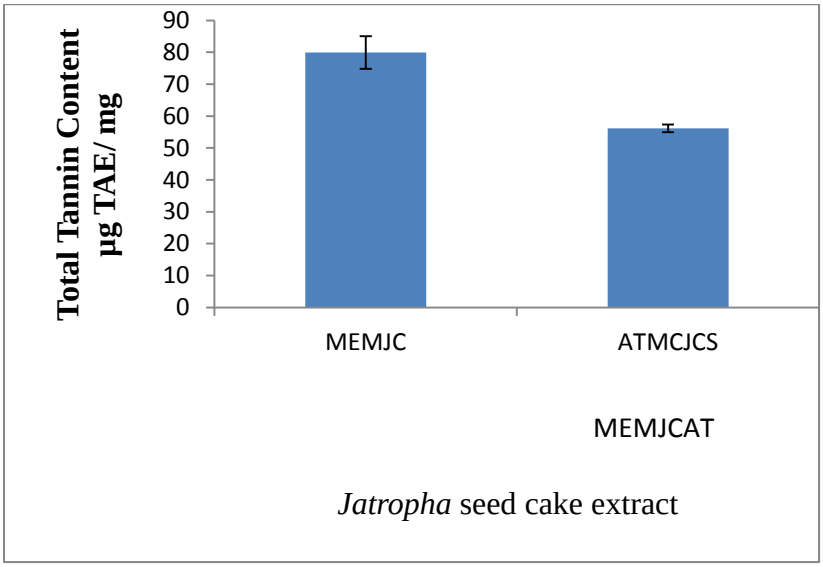

(C)

Figure 1: (A). Graphical representation of total phenolic content of *Jatropha* seed cake extract. (B). Graphical representation Total Flavonoids content of *Jatropha* seed cake extract. (C). Graphical representation Total tannin content of *Jatropha* seed cake extract.

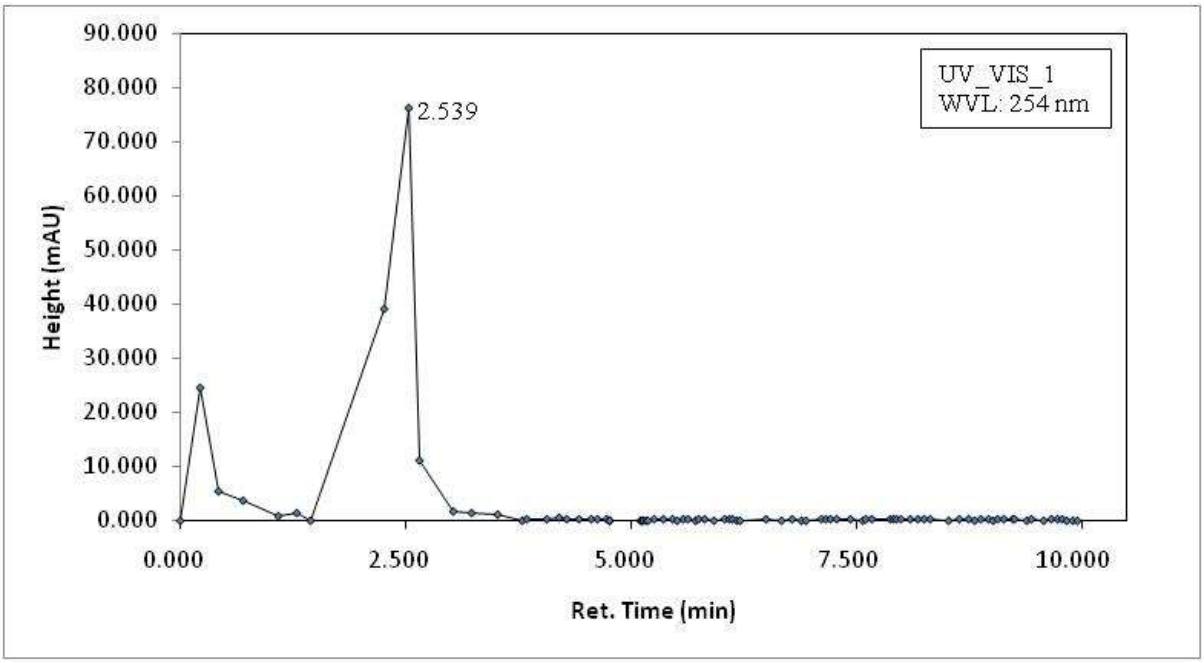

Figure 2: HPLC Chromatogram showing the phorbol ester of peak area decrease from (appears at RT= 2.539) for Methanolic extract of mechanical cake of *Jatropha curcas* (MEMJC) found 77.45% phorbol ester

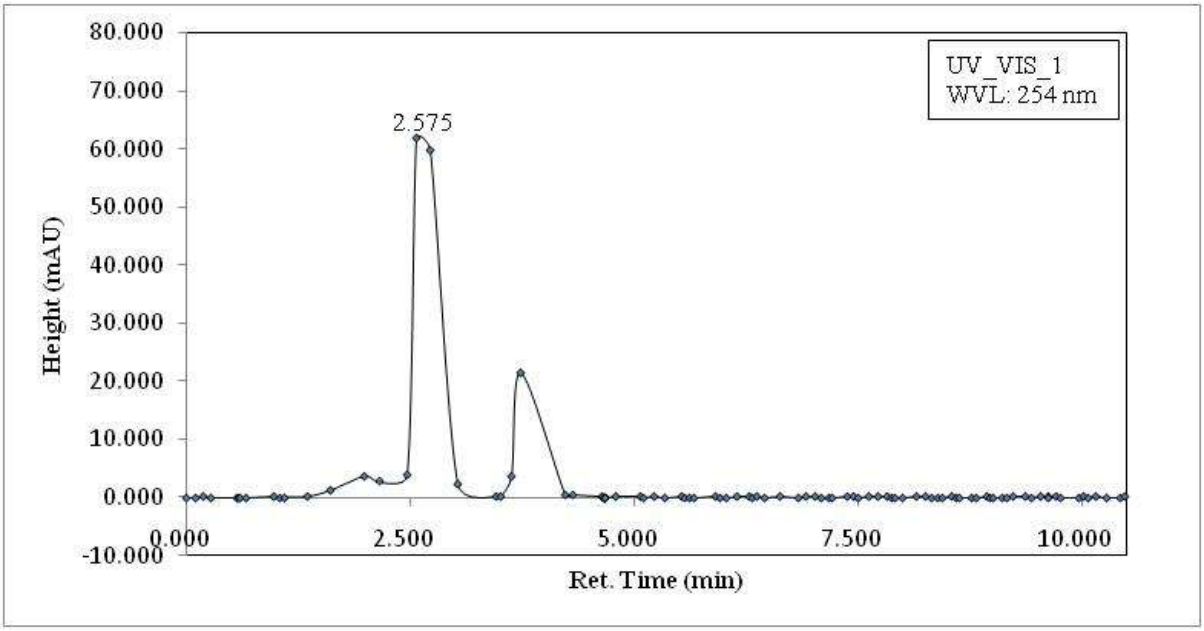

Figure 3: HPLC Chromatogram showing the phorbol ester of peak area decrease from (appears at RT=2.575) for Alkali treated mechanical cake of *Jatropha curcas* seeds (MEHJCAT) found 22.12% phorbol ester

Survival study

According to LD_{50} determination, both drugs MEMJC and MEHJCAT at the concentrations of 6.25 and 3.125 showed 100 % survival during 30 days treatment (Table 3, 4 and Figure 4).

Table 3: LD_{50} Of methanolic extract of mechanical cake of *Jatropha curcas* (MEMJC)

S.N.	Dose mg/100BW	% survival		
		10days	**20days**	**30days**
1.	6.25	100%	100%	100%
2.	3.125	100%	100%	100%
3	Drug vector	100%	100%	100%
	(LD_{50} at 30 days)	Not detected	Not detected	Not detected

Table 4: LD_{50} of alkali treated of mechanical cake of *Jatropha curcas seeds* (MEHJCAT)

S.N.	Dose mg/100BW	%survival		
		10days	**20days**	**30days**
1.	6.25	100%	100%	100%
2.	3.125	100%	100%	100%
3	Drug vector	100%	100%	100%
	(LD_{50} at 30 days)	Not detected	Not detected	Not detected

*The values are measured in mean ± SEM

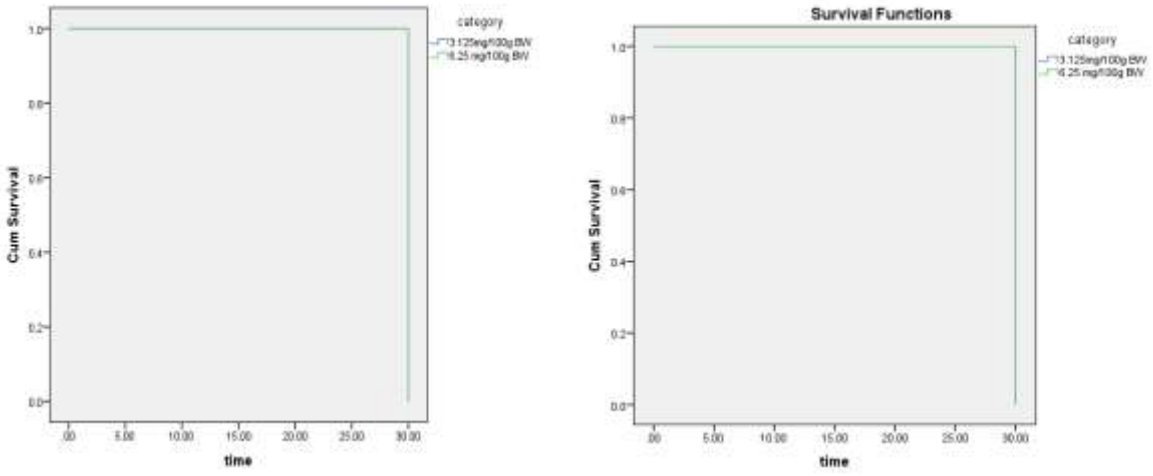

Figure 4: (A) LD_{50} Graphical representation of kaplen meier survival curve of Methanolic extract of mechanical cake of *Jatropha curcas* (MEMJC)., (B) LD_{50} Graphical representation of kaplen meier survival curve of alkali treated of mechanical cake of *Jatropha curcas seeds* (MEHJCAT).

Antioxidant Enzymatic Activity

In both drugs MEMJC and MEHJCAT the activity of SOD (Table.5, Figure. 5) increases 1st after 10 days treatment and then decreases further. At 10 days we found that the activity of SOD of MEMJC at both conc. i.e. 6.25 and 3.125 were higher than the activity of SOD found in alkali treated drug (MEHJCAT) at both concentration. At 20 days we found that the activity of Catalase (Table.6, Figure.6) of MEMJC at both conc. i.e. 6.25 and 3.125 were higher than the activity of Catalase found in alkali treated drug (ATMCJCS) at both concentration.

Table 5: Effect of methanolic press cake and alkali treated cake of Jatropha curcas on Superoxide dismutase activity (U/Hb Protein)

MEMJC				**MEHJCAT**			
Dose mg/ 100bw	**10 days**	**20days**	**30days**	**10 days**	**20 days**	**30 days**	**Normal**
6.25	0.69±0.001	0.32±0.003	0.24±0.005	0.58±0.002	0.46±0.006	0.37±0.008	0.42±0.002
3.125	0.67±0.007	0.17±0.006	0.13±0.001	0.46±0.006	0.38±0.001	0.12±0.007	0.42±0.007

*The values are measured in mean ± SEM

Table 6: Effect of methanolic press cake and alkali treated cake *Jatropha curcas* on Catalase (U /mgHb)

MEMJC				**MEHJCAT**			
Dose mg/100bw	**10 days**	**20days**	**30days**	**10 days**	**20 days**	**30 days**	**Normal**
6.25	0.25±0.001	6.09±0.03	1.91±0.005	0.92±0.002	4.29±0.06	0.89±0.008	1.6±0.002
3.125	0.17±0.007	5.38±0.06	1.36±0.01	0.51±0.006	3.29±0.09	0.68±0.007	1.6±0.007

*The values are measured in mean ± SEM

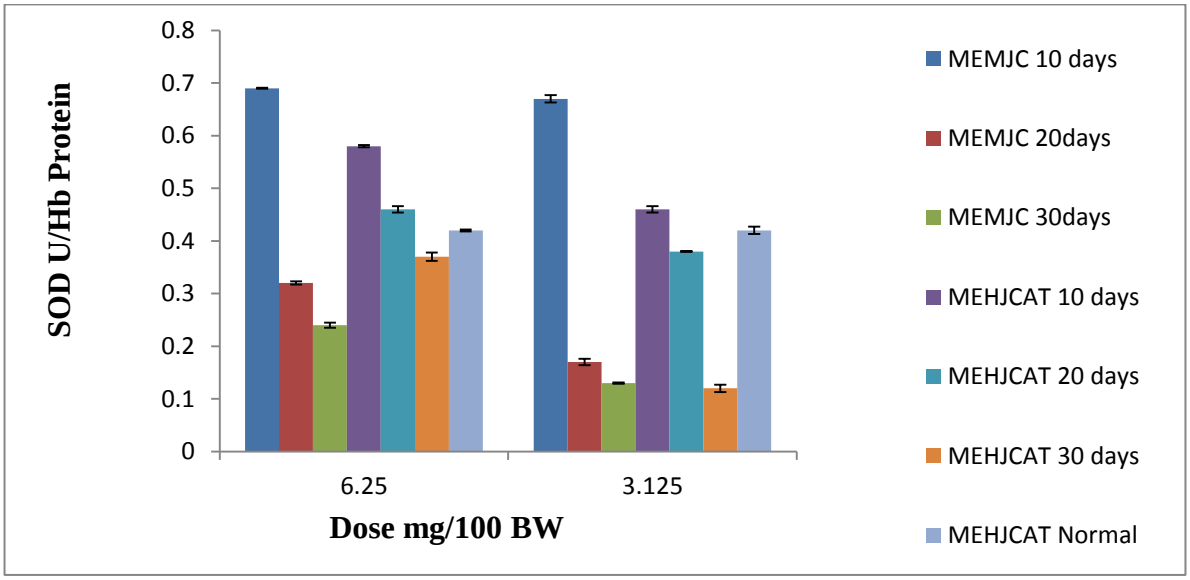

Figure 5: Graphical representation of methanolic press cake and alkali treated cake of *Jatropha curcas* on Superoxide dismutase activity (U/HbProtein)

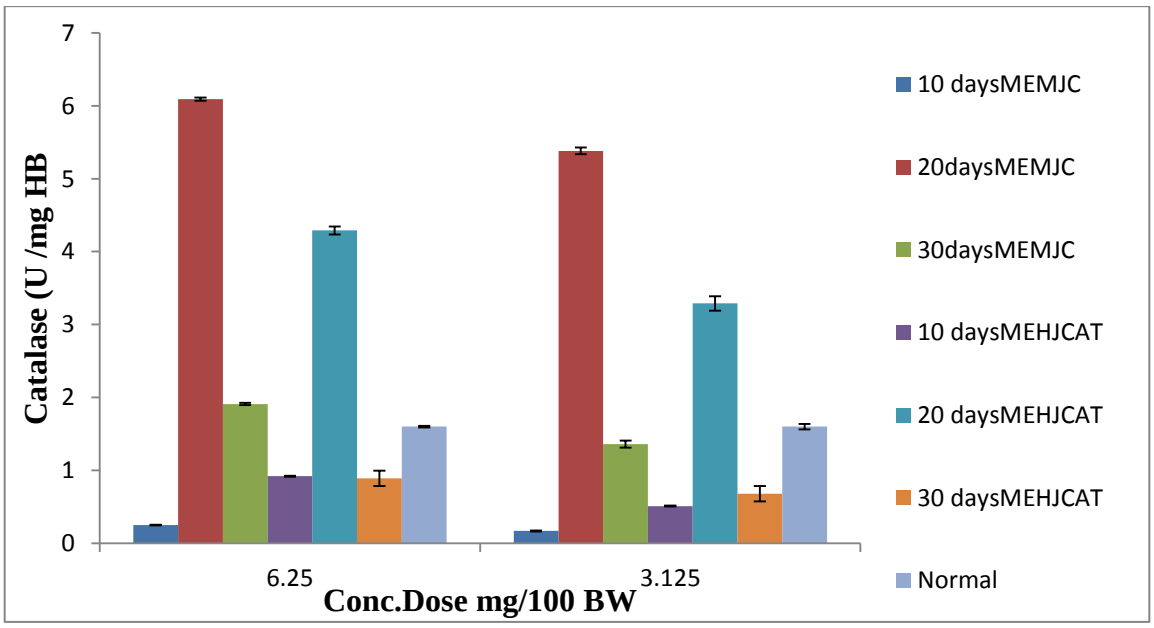

Figure 6: Graphical representation of methanolic press cake and alkali treated cake *Jatropha curcas* on catalase (U /mg Hb)

Urea

The drugs without alkali treatment (MEMJC) showed toxic effect on kidney by comparing their blood urea conc. with normal rat's urea conc. The urea conc. of (MEHJCAT) showed no toxic effect i.e., the urea level exist in normal conc (Table 7, Figure.7).

Table 7. Effect of Methanolic press cake and alkali treated cake *Jatropha curcas* on Urea mg%

MEMJC			MEHJCAT		
Dose mg/100bw	**10 days**	**20days**	**10 days**	**20 days**	**Normal**
6.25	77.99±3.45	44.91±4.46	28.27±3.56	29.74±3.12	36.93±2.01
3.125	62.47±5.45	43.85±5.21	25.82±5.12	28.78±5.12	36.92±3.09

*The values are measured in mean ± SEM

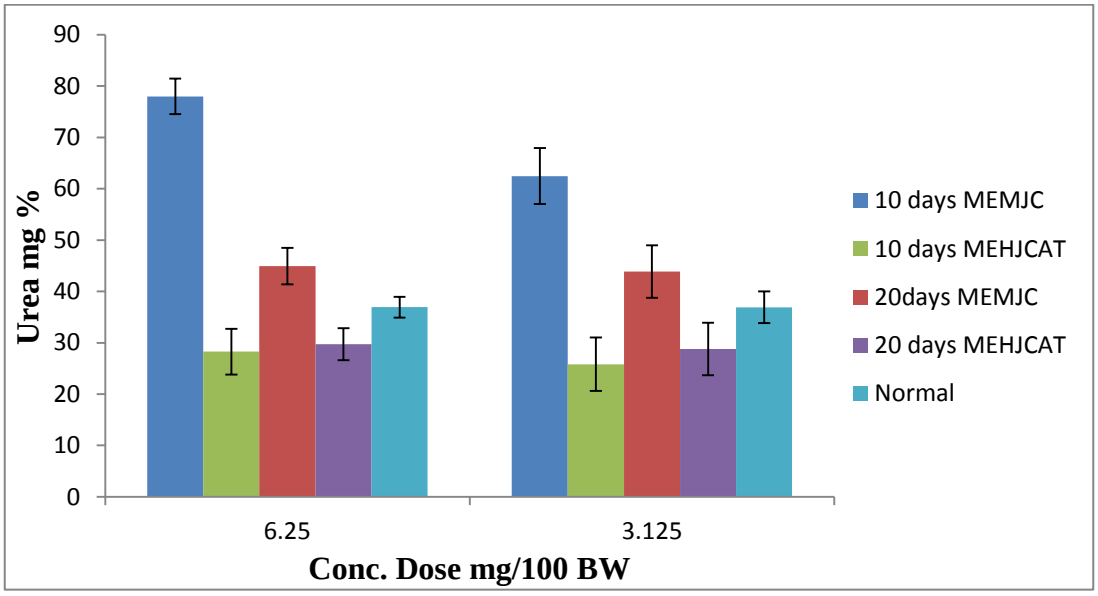

Figure 7: Graphical representation of Methanolic press cake and alkali treated cake *Jatropha curcas* on Urea

Creatinine

Only at the conc. of 6.25mg/ 100 BW (MEMJC) after 20 days treatment, we have found toxicity. In contrast to MEMJC, the concentration of creatinine was found to be decreased in MEHJCAT as compared to normal. (Table 8, Figure 8).

Table 8: Effect of Methanolic press cake (MEMJC) and alkali treated cake (MEHJCAT) *Jatropha curcas* on creatinine (gm/L)-

MEMJC			MEHJCAT		
Dose mg/100bw	**10 days**	**20days**	**10 days**	**20 days**	**Normal**
6.25	0.68±0.001	0.77±0.03	0.44±0.002	0.61±0.06	0.67±0.002
3.125	0.53±0.007	0.64±0.06	0.37±0.006	0.40±0.09	0.67±0.007

*The values are measured in mean ± SEM

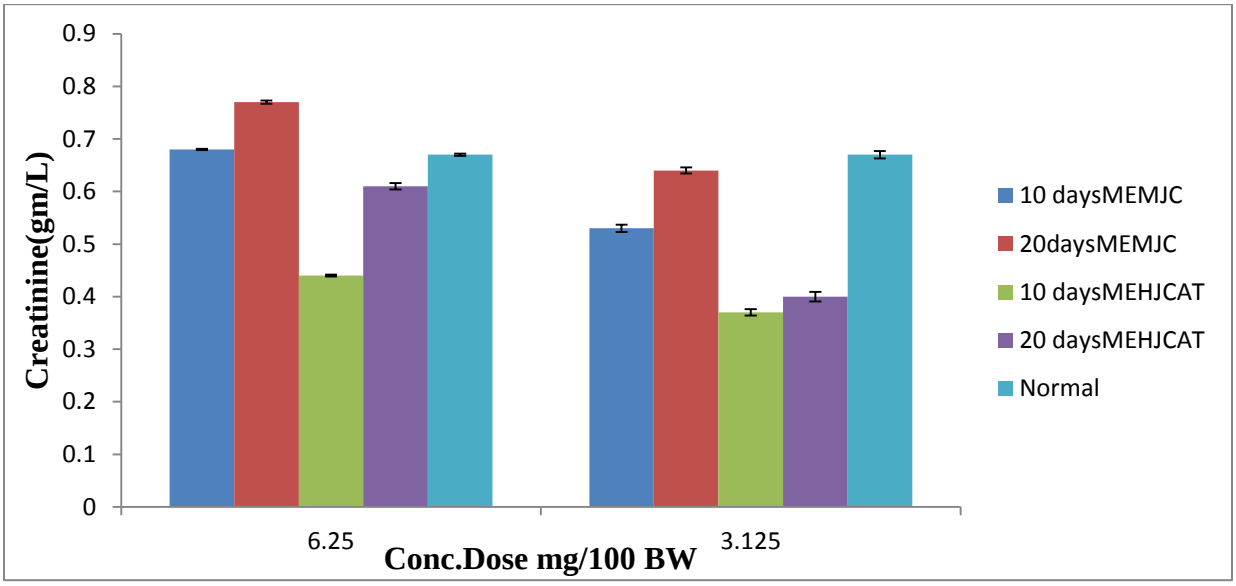

Figure 8: Graphical representation of Methanolic press cake (MEMJC) and alkali treated cake (MEHJCAT) *Jatropha curcas* on creatinine (gm/L)-

Weight Change

No significant weight change had been found throughout the whole experiment (Table 9).

Table 9: Analysis of weight change during drug treatment at 10, 20 and 30 days

Doses		1 day	10 days	20 days	30 days
MEMJC	6.25	120	130	130	140
	3.125	90	100	100	100
MEHJCAT	6.25	60	70	70	78
	3.125	140	150	150	158

*The values are measured in mean ± SEM

Oral Fat Tolerance Test

According to the results (Table 10, 11, 12 and 13) *Jatropha curcas* press mechanical cake enhance the absorption of triglyceride from the intestine when it was given 30 minute before feeding high fat diet. MEHJCAT showed lesser absorption of TG as compared to MEMJC. This suggest the phorbol ester content of the press cake could be responsible for increasing the fat absorption from intestine. The values of triglyceride of the comparative analysis of triglyceride absorption through OFTT at both concentration showed that sub-chronic treatment leads to higher toxicity because the difference of triglyceride concentration between basal plasma and plasma after 6 hrs of drug +HFD was found to be higher at 20 days treatment compare to 1 day treatment.

Table 10: Comparisons of MEMJC and MEHJCAT at fixed concentration of 6.25 mg/100 g BW on intestinal triglyceride absorption through OFTT after 20 days treatment

	Triglyceride mg%					
S.N.	**Group-1 Only water**	**Group-2 (Only Drug)**		**Group-3**	**Group-4 (Only Drug+ HFD)**	
		MEMJC 6.25 mg/100 BW	**MEHJCAT 6.25mg/100BW**	**Drug vector +HFD**	**MEMJC 6.25 mg/100 BW**	**MEHJCAT 6.25mg/100BW**
0	61.20±4.89	60.63±4.89	87.43±6.34	46.93±2.45	29.64±3.42	63.7±4.89
2	55.79±3.34	55.58±5.67	69.08±4.56	70.69±4.56	84.37±5.23	29.43±2.56
4	52.73±3.90	44.78±3.56	33.08±3.12	80.04±2.45	149.08±2.34	149.47±6.56
6	59.18±3.78	64.14±6.23	77.54±4.78	146.52±4.31	217.53±5.90	168.0±5.89
% Change (0 hrs. to 6 hrs.)	-3.41±0.23	13.39±1.21	12.72±1.01	67.97±5.67	86.37±5.32	62.08±4.12

*The values are measured in mean ± SEM

Table 11: Estimation of single doses 3.125 of MEMJC and MEHJCAT on absorption of triglyceride through OFTT after 20 days treatment

	Triglyceride mg%					
S.N.	**Group-1 Only water**	**Group-2 (Only Drug)**		**Group-3**	**Group-4 (Only Drug+ HFD)**	
		MEMJC 3.125 mg/100 BW	**MEHJCAT 3.125 mg/100BW**	**Drug vector +HFD**	**MEMJC 3.125 mg/100 BW**	**MEHJCAT 3.125 mg/100BW**
0	61.20±4.89	68.6±4.23	77.43±6.34	46.93±2.45	38.29±3.42	58.63±4.89
2	55.79±3.34	54.58±5.67	59.08±4.56	70.69±4.56	16.91±4.24	33.77±2.56
4	52.73±3.90	43.9±3.56	52.02±3.12	80.04±2.45	161.58±3.34	117.7±6.56
6	59.18±3.78	60.35±6.23	67.54±4.78	136.52±4.31	139.45±5.90	119.6±5.89
% Change (0 hrs. to 6 hrs.)	-3.41±0.23	-13.67±2.23	-14.64±3.21	65.62±5.67	72.54±6.23	50.97±4.12

*The values are measured in mean ± SEM

Table 12: Estimation of Change in triglyceride absorption in blood between MEMJC, 1 day and 20 days MEMJC +HFD through oral fat tolerance test

	MEMJC +HFD					
	6.25 mg/100 g bw			**3.125 mg/100 g bw**		
Time	**Control**	**1 day drug treatment +HFD**	**20 days drug treatment +HFD**	**Control**	**1 day drug treatment +HFD**	**20 days drug treatment +HFD**
0 hr	67.63±4.56	34.14±4.89	29.64±3.24	68.67±4.23	51.67±4.90	21.29±3.45
2 hrs	59.58±4.23	51.99±2.56	84.37±5.23	54.58±3.45	47.75±5.12	16.91±4.24
4 hrs	41.78±4.56	101.78±6.56	149.06±2.34	43.9±4.56	144.60±6.09	161.58±2.34
6 hrs	59.64±2.34	111.89±5.89	217.53±5.90	60.35±2.34	133.90±7.10	139.45±5.00
% Change(0 hrs. to 6 hrs.)	-13.39±1.11	69.48±4.56	86.37±5.31	11.78±1.19	61.41±5.67	84.73±7.32

*The values are measured in mean ± SEM

Table 13: Estimation of Change in triglyceride absorption in blood between MEHJCAT 1 day MEHJCAT +HFD and 20 days through oral fat tolerance test.

	MEHJCAT +HFD					
	6.25 mg/100 g bw			**3.125 mg/100 g bw**		
Time	**Control**	**1 day drug treatment +HFD**	**20 days drug treatment +HFD**	**Control**	**1 day drug treatment +HFD**	**20 days drug treatment +HFD**
0 hr	83.47±6.23	71.14±3.79	22.54±4.24	87.63±4.56	61.67±4.90	23.79±2.45
2 hrs	44.18±4.45	41.99±1.56	94.67±6.23	69.58±4.23	57.75±5.12	26.51±4.24
4 hrs	53.7±3.56	77.28±5.56	147.06±7.34	51.78±4.56	164.60±6.09	171.88±2.34
6 hrs	101.25±3.34	145.19±5.89	150.03±6.10	99.64±2.34	133.90±7.10	115.45±6.40
% Change (0 hrs. to 6 hrs.)	17.58±2.44	77.26±4.56	84.97±6.34	12.05±6.12	53.94±4.45	79.39±7.32

*The values are measured in mean ± SEM

According to our results MEMJC has been found to contain higher amount of phenols, flavonoids and tannins (Table 1) than MEHJCAT. Some of the member of these families were found to be health beneficial while others were found to be toxic in nature at higher concentration [7]. In MEHJCAT, the presence of all these compositions at lower concentration in addition with removed phorbol ester might be the probable reason why it was acting as better antioxidants and having less toxic effect compared to MEMC. The higher activity of SOD and Catalase in plasma of MEMJC treated rats compared to MEHJCAT treated leads to the conclusion that the removal of phorbol ester through alkali treatment makes the MEHJCAT less toxic or less vulnerable to generate free radical in rat. The results of renal function test also favored MEMJC as more toxic than MEHJCAT. Also from kaplen meier survival curve, it has been found that both drugs MEMJC and MEHJCAT has no mortality significance proved by the measurement LD_{50}. According to previous studies, *Jatropha curcas* has been found to have many medicinal aspect. It has antioxidant[7]. antimicrobial nature[8], hepatoprotective[9], wound healing[10], antimetastatic[11], anti-proliferative[11], antidiabetic[12], anti-inflammatory[13], pregnancy terminative[14], anti-ulcer[15], antihelmintic[16], antifungal effect[17]. But its toxic and side effects makes peoples to avoid its consumption as medicine. In this study we have found that the presence of phorbol ester plays the major toxic effect on rat's physiology. According to the previous study [18, 19, 20, 21, 22] PMK (phorbol ester) has found to have the higher potential to accumulate triglyceride[23,24]. MEHJCAT treated rats was found to be more beneficial medically with respect to weight, triglyceride absorption, plasma stress as compared to MEMJC in both acute and chronic doses. In Phytochemical test , both MEMJC and MEHJCAT both shows similar qualitative compound but quantitatively the amount of all Phytochemical was found to be less in MEHJCAT as compared to MEMJC. All the results were found to have significant changes dose dependently. Consistent weight throughout the experiment showed the non-toxicity effect of drugs.

CONCLUSION

Our results showed that the removal of phorbol ester (MEHJCAT) in *Jatropha curcas cake* have very significant change in healthy rats blood physiology. Its reduced toxicity could be proved to be beneficial for medicinal purposes.

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