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DETOXIFICATION OF TRYPSIN INHIBITORS IN THE KERNEL CAKE OF JATROPHA CURCAS BY REGULATING ITS INHIBITORY ACTIVITY AND ITS EFFECT ON COMMON CARP FISHES

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ABSTRACT

Biodiesel has attracted its considerable attention during the past decade as a renewable, biodegradable and non-toxic fuel source for alternative to fossil fuels. Biodiesel can be obtained from vegetable oils (both edible and non-edible) and also from animal fat. *Jatropha curcas Linnaeus*, a multipurpose plant, which contains high amount of oil in its seeds which can be converted to biodiesel. *J. curcas* is probably the most highly promoted oil seed producing crop at present in the world. The presence of oil in the seeds helps in the process of biodiesel production. After the oil extraction process, the kernel cakes were dumped as a waste product called bio-mass. The kernel cake generated as a by-product of oil production was rich in protein, which can be used as potential stock for animal feed. But the problem is the presence of toxic substances like Phorbol esters and trypsin inhibitors. The main objectives of the study are to detoxification of these toxins and to reuse the waste product as a live stock feed. SDSpage helps in isolating the bands of the trypsin inhibitors. Researchers universally have studied that these trypsin inhibitors and investigated ways of improving and detoxifying the kernels and applications of the seed meal on live stock. This review paper outlines the previous research done on the *Jatropha curcas* plant, the toxicity due to Trypsin inhibitors and the efforts made to detoxifying the seed. It also stated that these treated kernels can be used as a live stock feed. Here common carp fish was used in the toxicological studies which are closely related to the human as food. It also highlights the knowledge gaps concerning the chemistry of trypsin inhibitors toxicity and the probable areas for the future research.

KEY WORDS: Trypsin Inhibitors (TEs); Jatropha *curcas;* kernel cake; SDSPAGE; detoxification.

INTRODUCTION

Plants were used as a source of medicine in from the centuries ago and today the scientists and the general public recognize the value of plants as a source of new or complimentary medicinal products. Beyond this pharmaceutical approach to plants, there is a wide range of tendency to utilize herbal products to supplement the diet; mainly with the intention of improvising the quality of life and preventing the diseases of aged people (Priya *et al*., 2011). The Plants are the essential sources of medicines. The advancement in pharmacology and synthetic chemistry and organic chemistry, the dependence of natural products were remains unchanged. In India the majority of the populations were using traditional

natural preparation derived from the plant materials for the treatment of various diseases (Siddique *et al*., 2008). *Jatropha curcas* is a kind of flowering plant in the spurge family; this plant belongs to family Euphorbiaceae and is native to the American tropics. This plant is most likely Mexico and Central America (Janick, Jules and Robert E. Paul 2008). The oil extracted from the seeds contains four fatty acid-palmitic acid, stearic acid, oleic acid and linolic acid (Vaknin Yifatch *et al*., 2011). *Jatropha* meals made from defatted kernel contain high quantity of trypsin and phytate inhibitors (Aderibigbe *et al*., 1997). Crude *Jatropha oil* contains 78-80% of phorbol esters (Devappa *et al*., 2010). In addition to this *Jatropha curcas* seed has problem of anti- nutritional factors (anfs) like the soybean and they are thermo liable compounds like lecithin and trypsin inhibitors were present. Along with this a lipid soluble and thermo stable compound is present namely Phorbol esters (pes). The Phorbol esters (pes) are the naturally-occurring compounds which are widely distributed in plant species in the families of Euphorbiaceae and Thymelaeceae. They are tetracyclic diterpenoids of phorbol type and esters of tigliane diterpenes (Devappa, Makkar and Becker, 2010b, 2011a). Symptoms of Phorbol ester toxicity include dehydration, sunken eyes, skin irritation, loss of appetite, loss of condition and finally death (Belewu, 2008).

Detoxification is the only process for eradicating these toxic substances from the seed. The detoxification of the seed is done by chemically treating the seeds with solvents like hexane, benzene and petroleum ether along with heating process (Makkar, H.P.S. and K.Becker, 1997). Denaturing agent SDS play a vital role in this process. SDS (Sodium Dodecyl Sulfate) page is one of the technique which helps in pointing out of the detoxification process. Negatively charged SDS in the acrylamide gel guarantees that all proteins are completely denatured, with secondary structures destroyed. As a result, protein migration is relative to molecular mass, irrespective of structural morphology. This also dictates that any primary antibody used in Western blot analysis must recognize the protein that it was raised against in its denatured state. *Cyprinus carpio (*Common carp) is kind of fresh water fishes. The common carp fishes are native to Asia, and have been introduced all over the world with the exception of the Middle East and the poles. The original common carp was seen in the inland delta of the Danube River about 2000 years ago. This fish looks like a torpedo in a golden-yellow in color. It also has two pairs of barbells and a gauge mesh scaling pattern Balloon, E. K. This breed was used in this study (Makkar, H.P.S. and K.Becker, 1997). This breed can tolerate all physical stress and long tolerable to hunger. Because of the high protein content, high digestibility, relatively well-balanced amino acid profile, reasonable price and steady supply, SBM is widely used as a cost-effective feed ingredient for many aquaculture animals (Storebakken et al. 2000). Similar to soybean meal (SBM) the *Jatropha curcas* meal san also be used if it is detoxified. Hence my present research aims in investigating the toxic level of *Jatropha curcas* by inhibiting the trypsin inhibitors and to analysis the inhibition by using SDS PAGE and Assessment of the toxicity level by in vivo studies on *Cyprinus carpio fishes*.

MATERIALS AND METHODS

CHEMICALS REQUIRED

- **62mm sodium phosphate buffer:**
- **Soybean standard**
- **1N HCl**
- **Trypsin inhibitor solution**
- **BAEE extract**
- **Trypsin enzyme solution**

SAMPLE COLLECTION

Fresh *Jatropha carcus* seeds where collected from nearby areas of Madukkarai. The seeds were dried and the seed coat was removed mechanically.

"*Figure 1.0 Jatropha carcus plant*"

"Figure 1.1***Jatropha carcus*** **seeds**

SOLVENT TREATMENT

After removing the seed coat from the seeds and dried for a day, the kernels were collected from it. These kernels were treated with different solvents like hexane, methanol, petroleum ether and benzene. The kernels and solvents were taken in the ratio of 1:5. Then they were run in Soxhletion apparatus for a minimum of 35 cycles (3 hours). The oil was collected along with the kernel cake. The kernel cake was separated from oil by the filtration process.

KERNEL CAKE CRUSHING

Kernel cake was collected separately and dried to remove the excess of solvent in it. Then it was crushed with the help of mortar & pestle. Fine granules were collected from this process. Hence the Kernel cakes of Hexane, Methanol, and Pet. Ether and Benzene were extracted. Untreated Kernel cake was also prepared.

RUNNING SDS-PAGE

The kernel cake was dried to remove the excess of oil. Then the 1g of kernel was taken as sample (from all the 4 solvent extracts). Then it was crushed in a Mortar & Pestle along with 400µl (0.4ml) of Standard protein extracting buffer. All the 4 solvent extracts were treated in the same manner. Then along with standard protein marker the samples were loaded in SDS PAGE apparatus.

EVALUATING THE QUANTITY OF TRYPSIN INHIBITORS FROM THE TREATED AND UNTREATED KERNEL CAKES.

This process helps in finding the amount of trypsin inhibitors in the various Treated and Untreated Kernel cakes.

SOYBEAN STANDARD

Soy bean was used as a standard because of its natural trypsin inhibitor which will not affect more on human digestive system

INHIBITOR EXTRACTS PREPARATION

Taken 0.2 g powdered kernel cake (using mortar &pestle) and Added 9.9 ml of double distilled water + 0.1ml of 1 N NaoH and Stirred well by using a magnetic stirrer for minimum of 4 hours and then added 0.1ml of it was mixed with 9.9ml of Sodium phosphate Buffer at Ph 7.6

TRYPSIN INHIBITOR SOLUTION

Prepared a 0.1 V/V 1N HCl solution with all the inhibitor extracts. Then mixed thoroughly in magnetic stirrer apparatus.

BAEE EXTRACT

BAEE (α-N-Benzoyl –L- Arginine-Ethyl-Esterase Hydrochloride) was taken for the assay. 100mg of BAEE was taken and mixed with 100ml of 0.1 V/V 1N HCl.

TRYPSIN ENZYME SOLUTION

Taken 860mg of trypsin enzyme (**sigma product –TRYP T8003**) and mixed it with 100ml of 62mM sodium phosphate buffer

Invivo STUDIES ON THE EFFECT OF TREATED AND UNTREATED KERNEL CAKES ON Cyprinus carpio FISHES

This process helps in finding the amount of protease ensyme activity from the gut of the fishes .

PREPARATION OF FISH FEED FROM THE KERNEL CAKE:

Dried kernel cake was made as paste with double distilled water with a concentration of 1mg /ml. Feed was made according to the samplea as follows

- Control
- Treated &
- Un-treated

GROUPING THE FISHES

Common carp fishes were collected from Aazhiar Dam , Pollachi, Coimbatore. These fishes were divided into three groups . Each group consist of a minimum of 10 fishes.

ADMINISTRATION OF THE FEED TO THE FISHES

Based on the size and weight , 100mg of feed per fish was administered . normal fish feed from the local market was administred to the fishes in control group . treated kernel cake feed was administered for the fishes in treated group. Simillarly the untreated kernel cake feed was administered for the fishes in the un-treated group. This type administration is a random type administration

After 15 days of feed administration the fishes were slotted and the blood , liver , gut and muscles were collected from it . blood samples were collected in a microfuge tube containing 10mM EDTA salt solution for the preservation of the blood , then the muscles, gut and liver were collected in a air tight container for the preservation. Then it was stored in -20c temperature.

EXTRACTION OF THE ENZYME FROM THE GUT

Enzyme samples were prepared by the cohen(1993) method. The fishes were starved for nearly 24 hours before dissection to standardize them and to allowed the accumulation of digestive enzymes.The fishes were placed at -20^0C for 4 minutes and then dissected in ice-cold 0.9% NaCl solution under a dissection Microscope. The gut was removed carefully in order to keep the gut enzyme alive . The extracted gut was placed in ice-cold sodium phosphate buffer (p^H 7.1). Then the gut tissues were homogenized and centrifuged at 16000rpm for 10 minutes at 4^0C . then the supernatant was collected and stored at 4^0C.

ESTIMATION OF THE PROTESE ENZYME AND THERE BY ASSESSING THE QUANTITY OF TRYPSIN INHIBITORS PRESENT

Protenase activity was analysed by Morihara and Tsuzuki (1997) method. The reaction mixture consist of 1ml 1% (w/v) casein and 0.5 ml of the enzyme prepared . Then it was incubeted in a water bath for 35^0C for 30 minutes. Then the reaction was terminated by the addition of 3ml cold 10% (w/v) Trichloroacetic acid (TCA). Then the mixture was allowed to stand at 4^0C for nearly 30 minutes. Then the mixture was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected for the determination of non-precipitated TCA protein. This was followed by the sddition of Folin-Ciocalteu's phenol reagent method of Lowry *et al.*(1951). 1.0ml of the TCA protein was mixed with 5 .0ml of lowry's reagent C (sodium potassium tartrate in distilled water), mixed thoroughly and then incubated at room temperature for 10 minutes . then 3 fold diluted Folin-Ciocalteu's phenol reagent (0.5ml) was added to the mixture with shaking and incubated at room temperature for 30 minutes. The optical density was measured at 670nm in a spectrophotometer. The amount of Non- precipitated TCA protein was analysed and estimated as tyrosine standard curve of known concentrations of tyrosine as protein standard . 1 unit of protease activity is defined as the quantity which is required to produce 100µg of tyrosine in 1ml of TCA filtrate under the above conditions.

RESULTS

The Seed cakes or kernel cakes are the cakes which have been derived after the oil extraction process. It has been used as fertilizer, solid fuel, or in biogas production. Non toxic varieties or detoxified press cake has been used as feed for animal (Heller. J, 1996). Despite all various purposes, the application as fuel is probably the most interesting one for both economical and ecological point of view (Bredeson, D. K, 1982).

Figure 4.1.2 Untreated kernel cake

Figure 4.1.2 Hexane kernel cake

Figure 4.1.3 Benzene kernel cake

Figure 4.1.4Petroleum ether kernel cake

Figure 4.1.5 Methanol kernel cake

The oil extracted from the *Jatropha curcas* by Soxhletion process shows the oil content and the purity of the oil content. From these various oil extracts, Hexane oil extract shows high purity and less impurities (Salimon and Abdullah, 2008).

Figure 4.1.6 The Oil extracted from Jatropha carcus by using different solvents

The cake which was obtained after the extraction of the oil contains a crude protein content of between 58 and 64 percent. Hence, it has high potential to complement and substitute to soybean meal as a protein source in

livestock diets [Makkar and Becher, 1997b]. The SDS PAGE results shows some missing bands in the treated samples whereas the band was seen in untreated samples . From this, the missing band should be a protein group and this is simillar to the missing band in *Crotalaria paulina* [Luiza A. Pando, *et al.* 1999].

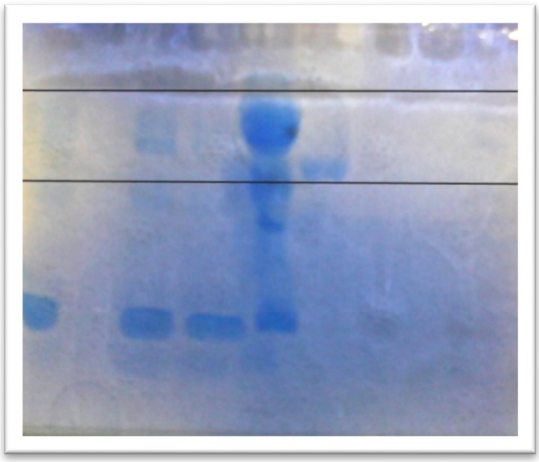


Figure 4.1.6 The* SDS PAGE *results shows some missing bands

The Hexane samples show highest inhibiting activity on trypsin inhibitors. Hence from this study, the Hexane can be used for the extraction of bio-diesel extraction process with less trypsin inhibitors. The Untreated shows higher inhibitory activity against the trypsin, whereas the treated samples show their own inhibitory activity against the trypsin inhibitors. From these treated samples Hexane has the highest inhibitory activity against the trypsin inhibitors.

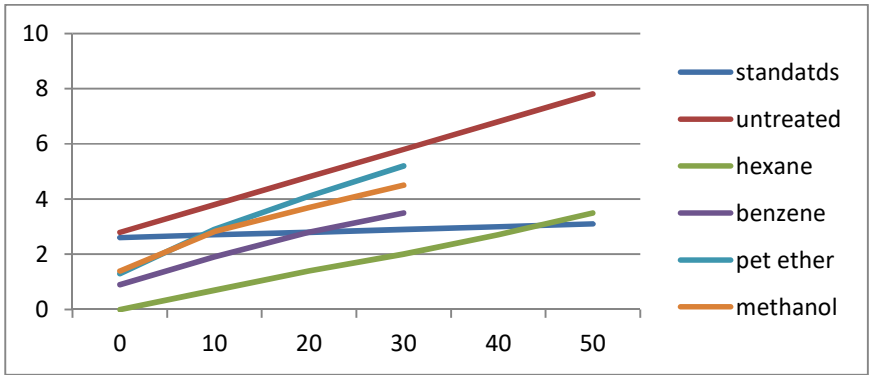


Figure 4.1.6 The graph shows the inhibiting activity the different solvents on trypsin inhibitors.

The Hexane samples show high protein release units whereas there is no protein release value in untreated samples. Hence hexane samples can be used for the live stock feed.

Contents	Hexane	Pet. Ether	Benzene	Methanol	Untreated
Protein release value units/ ml	0.0592	0.0360	0.0284	0.0591	0

Table 4.2 the table shows the protein release value of the different solvents.

During the feed administration the Control and Hexane group fishes survived well whereas the untreated group fishes shows a wide range of physiological changes and inflammation in the abdominal cavity [Partoens *et al.*, 1996].

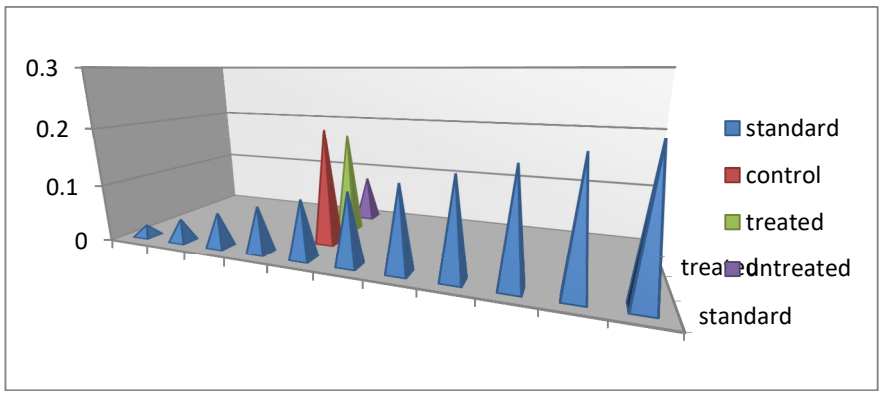
Day	Control	Un-treated	Treated
1	Normal	Normal	Normal
2	Normal	Normal	Normal
3	Normal	Normal	Normal
4	Normal	Normal	Normal
5	Normal	Normal	Normal
6	Normal	Abnormal Physical activities	Normal
7	Normal	Strange lesions in some fishes	Normal
8	Normal	Yellow color Inflammation in abdominal cavity	Normal
9	Normal	Fish dies with sudden inflammation in abdominal cavity	Normal
10	Normal	Fish dies with inflammation in abdominal cavity	Normal
11	Normal	Swellings were occurred in some fishes	Normal
12	Normal	Depletion of pigment in eyes	Normal
13	Normal	Fishes dies with inflammation in abdominal cavity	Normal
14	Normal	Lesions in abdominal cavity	Normal
15	Normal	All fish dies with swellings in abdominal cavity	Normal

Table 4.2.1 the table shows the physical changes in fishes during the feed administration

From the protease enzyme activity in the gut during the gut assay. From this the control and the treated samples shows more likely similar to each other with high protease enzyme synthesis whereas the untreated samples show less protease enzyme synthesis. Hence the hexane treated samples can be given as a feed to the live stock (Makkar *et al*., 2008).

Sn.o	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Control	Untreated	treated
O.D Values 670nm	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18	0.20	0.22	0.20	0.08	0.18

Table 4.2.2 the table shows the synthesis of protease enzyme in the fish gut during the feed administration



Graph 4.2.3 shows the synthesis of protease enzyme in the fish gut during the feed administration

This study the *Jatropha curcas* kernels were extracted from the seed by removing the seed coat. The kernels were used for the oil extraction. In this study the extraction of oil was done by a chemical treatment process. Solvent extraction process helps in extraction of oil and also removes the toxic components from it. The quantity of trypsin inhibitors after the oil extraction by solvent treatment were analyzed from the kernel cake with the soy bean as standard. The reduction of toxicity of the trypsin inhibitors were quantified and administered to the common carp fishes (*Cyprinus carpio)* for the in vivo toxicology analysis. This study reveals the proper extraction of oil from *Jatropha curcas* and enhances the protein diet supplement for the live stock. This study enhances the production of biodiesel as an economically cheaper and the wastage of the biomass can be reduced.

DISCUSSION

Jatropha carcus is a novel oil plant which is rich in oil. During the biodiesel extraction, the kernel cakes were dumped as a waste product named as biomass. These dumped kernel cake / press cake were rich in protein. This kernel cake also has the toxic substances namely Phorbol esters and Trypsin inhibitors. Because of the toxicity the kernel cakes were deposited as the waste product (biomass) These toxic substances were the anti nutritional components which cause death to the live stock and also harmful to humans. These toxic substances can be detoxified by the solvent extraction method. This study reveals the treatment and detoxification of the kernel cakes which elucidates toxic free protein rich diet feed for the live stock and enhance the usage of the waste product as the live stock feed. This study also reveals the trypsin inhibitor in-activation in the *Jatropha carcus* kernel and increasing the biodiesel production which endures the recycling capacity of the biomass from the bio diesel production.

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