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Phorbol Ester Degradation Using Biological Treatment in Jatropha Kernel Meal

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A B S T R A C T

The presence of anti-nutritional factors in jatropha kernel meal such as, phorbol esters, lectins, trypsin inhibitor, phytate and saponins is of great concern. Toxicity of meal is mainly due to the presence of phorbol esters which limits its use. Several methods have been tried for detoxifying kernel meal that includes physical, chemical, biological and radiation methods. In the study, four different samples, i.e., raw, defatted, one-time mechanically oil expressed and two-times mechanically oil expressed samples were prepared from jatropha kernels. These samples were subjected to biological treatment for phorbol ester degradation. For biological treatment, strain *Pseudomonas aeruginosa* was used. Cell-free extract obtained from growing strains in a specific media was mixed with kernel meal samples to carry out detoxification. In biologically treated kernel meal, phorbol esters were found to be in range of 0.051-0.102 mg/g which was considered acceptable and hence, the treatment was found to be effective in phorbol ester degradation.

Introduction

Jatropha is an oilseed crop belonging to *Euphorbiaceae* family which has gained remarkable interest as a raw material for biodiesel industries. Jatropha seed contains approximately 30-35% oil that can be converted into high-quality biodiesel upon trans-esterification which can be used as a substitute for diesel fuel (Makkar and Becker, 2009). Presence of various anti-nutritional factors in the jatropha kernel meal (prepared using mechanical oil expellers) prevents its use as highly nutritiousprotein supplement in animal feed. The anti-nutritional factors are phorbol esters, lectin, trypsin inhibitor, phytate and saponins (Table 1) (Makkar *et al.*, 1998; Makkar and Becker, 1997a).

Jatropha kernel meal contains lignocelluloses and proteins in abundance and thus can be used advantageously as bio-fertilizer (Makkar *et al.*, 1998) for production of biogas and as well as animal feed (Gubitz *et al.*, 1998). But, toxic phorbol esters that are naturally-occurring compounds are also present. Phorbol esters 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. Animal consumption of these phorbol esters can cause diarrhoea, inflammation of the gastrointestinal tract and death. If jatropha kernel meal is detoxified, it could be an excellent protein source. Several methods have been tried for detoxifying kernel meal that includes physical, chemical and biological methods (Ahluwalia *et al*., 2017a).

Some researchers (Azhar *et al*., 2014; Chang *et al*., 2014; Xing *et al*., 2013) employed biological detoxification methods by the use of fungi and bacteria for detoxifying jatropha seed cake by solid state fermentation process. Bacterialcultures may reduce the detoxification time which can make the process rapid and economical. Also, bio-detoxification does not involve the application of any chemicals or mixtures and taking into consideration the safety and energy concerns; the biological methods are more advantageous than the others. But, at the same time, bio-detoxification may be inconvenient and time-consuming (de Barros *et al*., 2011; Belewu and Sam, 2010).

This study was carried out to find the effects of biological treatment on phorbol ester degradation in jatropha kernel meal. This kind of research is of great importance so as to incorporate jatropha kernel meal in commercially produced aqua-feeds after evaluating the toxicity levels of treated jatropha kernel meal. Keeping in view, the present work was conducted with the following specific objectives include to study the effect of biological treatment on phorbol ester degradation in jatropha kernel meal and to estimate phorbol ester contentin the kernel meal after treatment

Materials and Methods

Jatropha seeds were obtained from Samarlakota, East Godavari District, Andhra Pradesh. Seed coat was removed mechanically using castor sheller adjusted suitably to obtain kernels. Four different jatropha kernel meal samples were prepared, namely, a) raw, b) defatted (solvent extracted), c) one-time mechanically oil expressed and d) two-times mechanically oil expressed. Jatropha kernels were size reduced in Wiley mill (Ultra Lab Instruments, New Delhi) using 20 mesh screen (850 µm) for preparing raw sample. This 20 mesh raw sample was oil extracted with automatic Soxhlet apparatus using petroleum ether (boiling point: 65 ºC) as solvent to produce defatted sample. Mechanical oil expression of jatropha kernels was done using mechanical mini oil expeller (Rajkumar Agro Engineers Pvt. Ltd., Nagpur) to obtain one-time mechanically oil expressed sample and two-times mechanically oil expressed sample. It is to be noted that the defatted sample was without oil in it. All the prepared samples were stored at 4 ºC in a refrigerator till treatment was carried out (Fig. 1).

Biological treatment

Biological treatment was done as reported by Ahluwalia *et al.,* (2017b) using submerged fermentation method. This method was adopted because it reported treatment time of 15 h which was very short treatment time as compared to other methods. Also, submerged fermentation method results in more toxins degradation than solid-state fermentation

(Phengnuam and Suntornsuk, 2013).

Culture

Culture media were procured from Department of Microbiology, University of Pondicherry, Pondicherry. Strain *Pseudomonas aeruginosa* obtained from soil samples was used for detoxification.

Preparation of inoculum

Culture was first grown in nutrient broth. Nutrient broth (1.3 g) was dissolved in 100 mL of distilled water in a conical flask which was autoclaved at 15 psi (103.4 kN/m^2) for 15 min. Flask was cooled and transferred to laminar flow chamber. Exactly 0.1 mL of the *Pseudomonas aeruginosa* strain was added to the broth and kept in incubator shaker at 37 ºC and 100 rpm for 24 h.

Strain was allowed to grow in petri-dishes to obtain inoculum. For this, 2.8 g of agar was added to 100 mL distilled water and autoclaved for 15 min. Three petri-dishes were also autoclaved. After cooling, petri-dishes and agar solution were transferred to laminar flow chamber, where approximately 10 mL of agar solution was poured into each petri dish and allowed to solidify. Approximately 0.1 mL of strain which was grown in nutrient broth was spread over solidified agar in petri-dishes which were kept in incubator at 37ºC for 24 h for strains to grow.

About 1% of prepared culture stated as above was used to inoculate the media containing starch (2%), KH_2PO_4 (0.5%), KNO_3 (1.01%), NH_4Cl (0.535%), $MgSO_4.7H_2O$ (0.001%), $CaCl_2.2H_2O$ (0.01%) and $Na_2HPO.12H_2O$ (0.8%). Incubation was done in a rotary shaker at 37 ºC for 24 h at 100rpm and was centrifuged at 10000g rpm for 20 min at 4 ºC using refrigerated centrifuge (Model: C-24 Plus; Remi Laboratory Instruments, Mumbai) for obtaining a cell-free supernatant. Enzymes present in the cell-free supernatant were responsible for detoxification of jatropha kernel meal samples.

Submerged fermentation of jatropha kernel meal

Exactly 5 g of kernel meal was added to the cell-free supernatant and incubated in a rotary shaker at 37 ºC, pH 7, 100 rpm for 15 h to undergo submerged fermentation. Experiment was replicated thrice. Mixture of kernel meal and cell-free supernatant was filtered and kept in hot air oven at 37 ºC for 48 h to obtain dried kernel meal. Microbiologically treated jatropha kernel meal samples are shown in Fig.2.

Estimation of phorbol esters

Phorbol esters were determined according to the modified method of Haas and Mittelbach (2000) proposed by Saetae and Suntornsuk (2010).

Jatropha curcas kernel meal samples (5 g) were ground by using a blender and poured into flasks containing 20 mL of methanol. Mixture of kernel meal and methanol was stirred by using a shaker operated at 250 rpm for 5 min. It was then filtered using a Whatman No. 4 filter paper and vacuum pump. Residue on the filter paper and the extract were collected. This process was repeated and the residue was extracted four additional times. The extract fractions from all five extractions were combined and dried under vacuum at 40 ºC using a vacuum oven. The dried extract was dissolved in 5 mL of methanol and passed through a 0.2-µm membrane filter (ChroMex, U.K.). Exactly 20 µL of extract solution was analyzed for phorbol esters by HPLC (Model 1100; Agilent, USA).

HPLC analytical column used was a 150×3.9 mm ID, 4-µm particle size, Nova-Pak C18 (Waters, Ireland), with a SBC18 guard column (12.5×4.6 mm ID), 5-µm particle size (Agilent, USA). The column was thermally controlled at 25^{o}C. A mixture of acetonitrile (HPLC grade; Fisher Scientific, U.K.) and deionized water in the ratio of 80:20 (v:v) was used as the mobile phase at a flow rate of 1 mL/min. The detector wavelength was set at 254 nm. Results were expressed as equivalent to phorbol-12-myristate-13-acetate (PMA) (Sigma, U.K.) used as an external standard. The PMA was dissolved in methanol (Fisher Scientific, U.K.) for preparation of standard curve.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA in Microsoft Excel. Statistical significance of phorbol ester content in raw, defatted, one-time and two-times mechanically oil expressed samples before and after treatment was analyzed at $p < 0.05$.

Results and Discussion

Phorbol esters content before treatment

Phorbol ester content was analyzed for raw, defatted, one-time and two-times mechanically oil expressed kernel meal before treatment. The phorbol ester content was 0.901 mg/g of kernel meal for raw sample, whereas it reduced to 0.250 mg/g of kernel meal for defatted sample (Fig. 3). One-time and two-times mechanically oil expressed samples showed phorbol ester content of 0.458 and 0.350 mg/g of kernel meal.

Phorbol esters reduced in mechanically expressed and defatted samples because of extraction of oil. During mechanical extraction of oil from seeds, 70-75% of PE comes out with oil, but the rest are still retained in the kernel meal, thus making both the meal and oil inedible (Devappa *et al*., 2012). Thus, with decrease in oil content PE content also decreased (Fig. 4). Statistical analysis by ANOVA showed no significant difference in PE content ($p < 0.05$).

Phorbol ester content after biological treatment

Effect of biological treatment on PE content of raw, defatted, one-time and two-times mechanically oil expressed sample was analyzed. PE content reduced for all the cases (Fig. 5) when compared with the untreated samples (Fig. 3).

PE content reduced to 0.102 and 0.051 mg/g of kernel meal for raw and defatted samples, respectively. One-time and two-times mechanically expressed samples showed PE content of 0.072 and 0.055 mg/g of kernel meal, respectively. PE content of biologically treated raw, defatted, one-time and two-times mechanically oil expressed samples decreased by 88.68%, 79.60%, 84.28% and 84.29%, respectively, compared to untreated samples (Table 2).

Acceptable limit of PE content for food and feed purposes is 0.11 mg/g (Makkar and Becker, 1997). PE content in biologically treated samples was less than the acceptable limit. Hence, biologically treated jatropha kernel meal can be used in food or feed.

In conclusion, study on phorbol ester degradation using biological treatment in jatropha kernel meal was done to find the effectiveness of the treatment to detoxify jatropha kernel meal. Toxin content was also determined to ensure its suitability in food or feed purposes. Biological treatment involved fermentation with the strain *Pseudomonas aeruginosa*. Treatment was done for 15 h at 37 ºC, pH 7 and 100 rpm in an incubator shaker. Phorbol ester content was found to be

high in untreated samples and was not within acceptable limits. Biological treatment was found to be effective in reduction of phorbol esters. Phorbol esters in biologically treated kernel meal was lower than untreated samples and was observed to be 0.102 mg/g for raw sample and 0.051 mg/g for defatted sample whereas, 0.072 and 0.055 mg/g for one-time and two-times mechanically expressed samples and they reduced by 88.68%, 79.60%, 84.28% and 84.29%, respectively.

Table.1 Anti-nutritional components in jatropha kernel meal

Toxic and Anti-Nutritional Compound	Seed Variety		
	Cape Verde (Highly Toxic)	Nicaragua (Highly Toxic)	Mexican (Non-Toxic)
Phorbol ester (mg/g kernel)	2.70	2.17	0.11
Lectin (mg/g kernel)	102.00	102.00	51.00
Trypsin inhibitor activity(mg inhibition/ g kernel meal)	21.30	21.10	26.50
Phytate (% in kernel meal)	9.40	10.10	8.90
Saponin (% diosgenin eqv. in kernel meal)	2.60	2.00	3.40

Table.2 Per cent reduction in phorbol esters due to biological treatment over untreated samples

Sample	Per cent Reduction
Raw	88.68
Defatted	79.60
One-time mechanically oil expressed	84.28
Two-times mechanically oil expressed	84.29

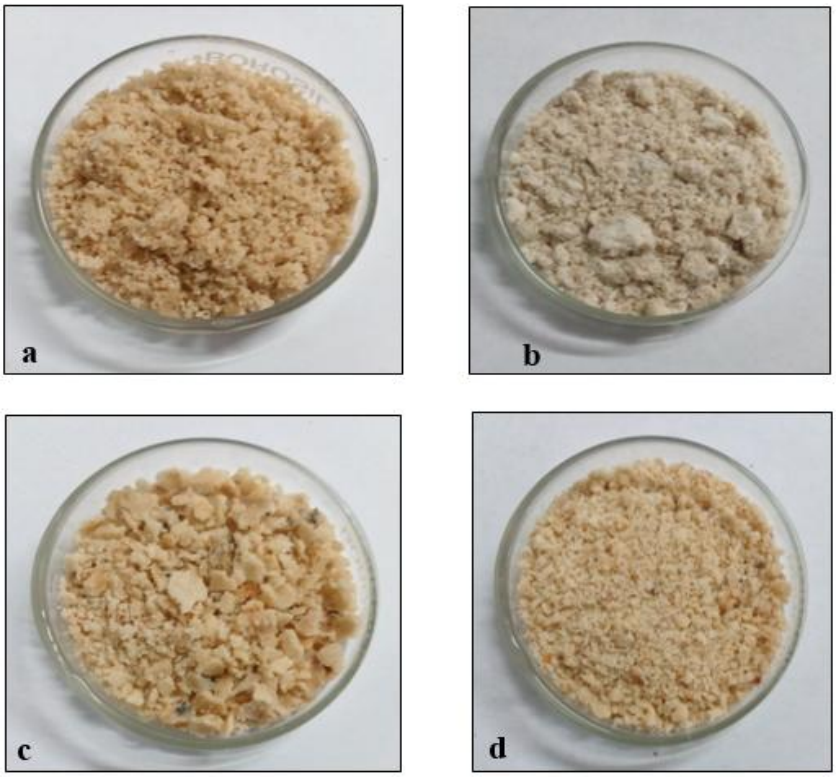

Fig.1 Jatropha kernel meal samples before treatment a) Raw, b) Defatted, c) One-time mechanically oil expressed and d)Two-times mechanically oil expressed

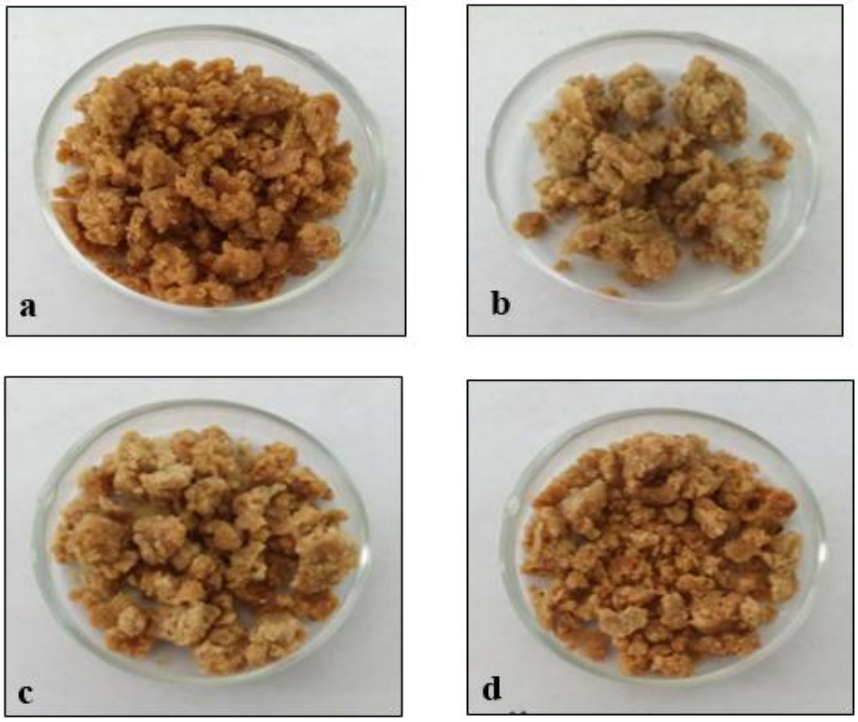

Fig.2 Microbiologically treated jatropha kernel meal samples a) Raw, b) Defatted, c) One-time mechanically oil expressed and d) Two-times mechanically oil expressed

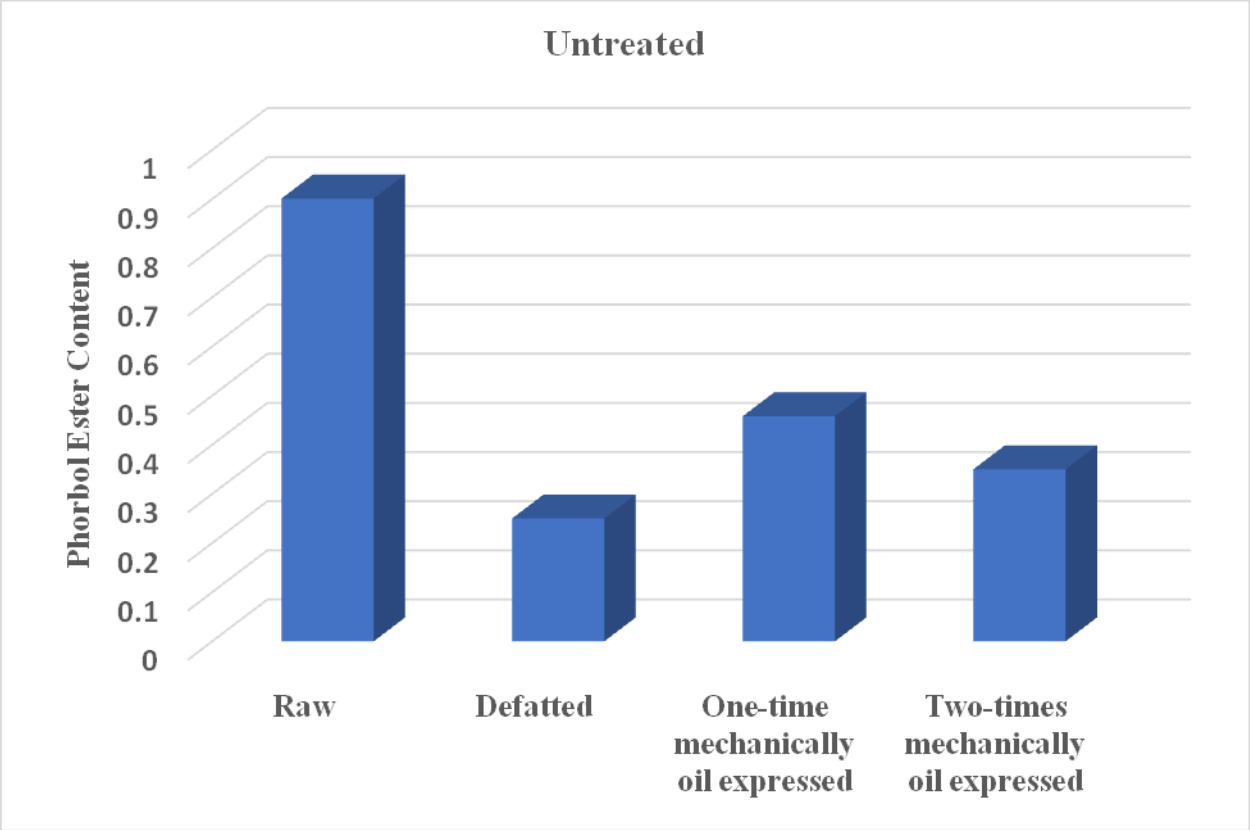

Fig.3 Phorbol ester content before biological treatment

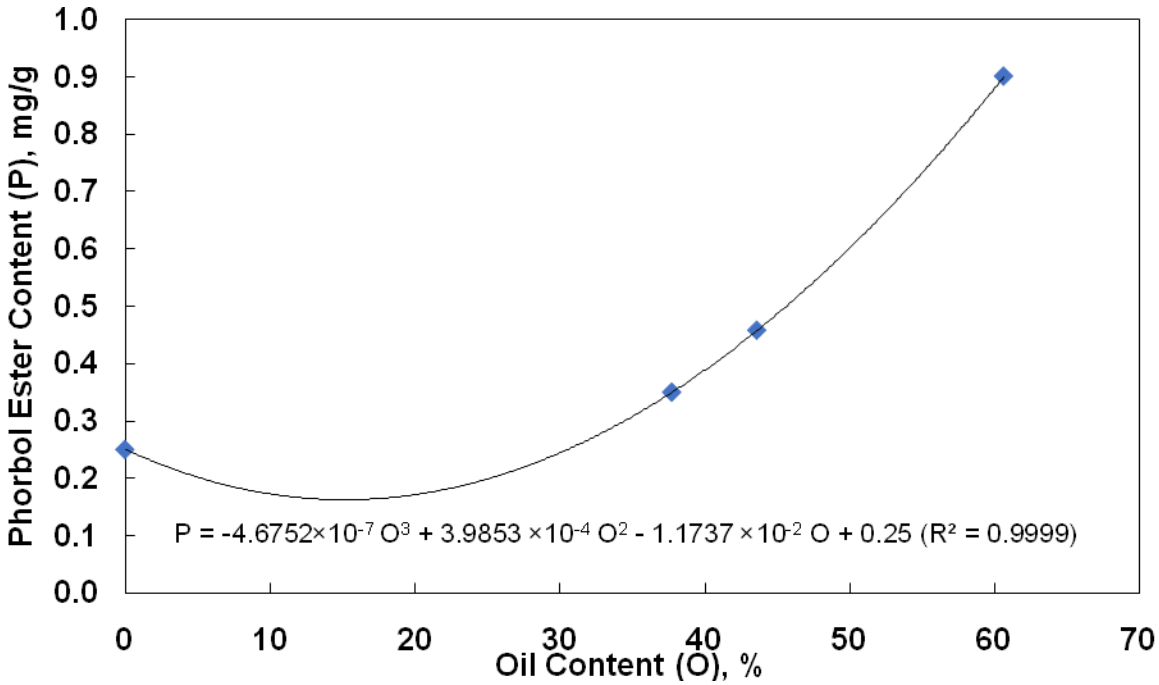

Fig.4 Variation of phorbol ester content with oil content

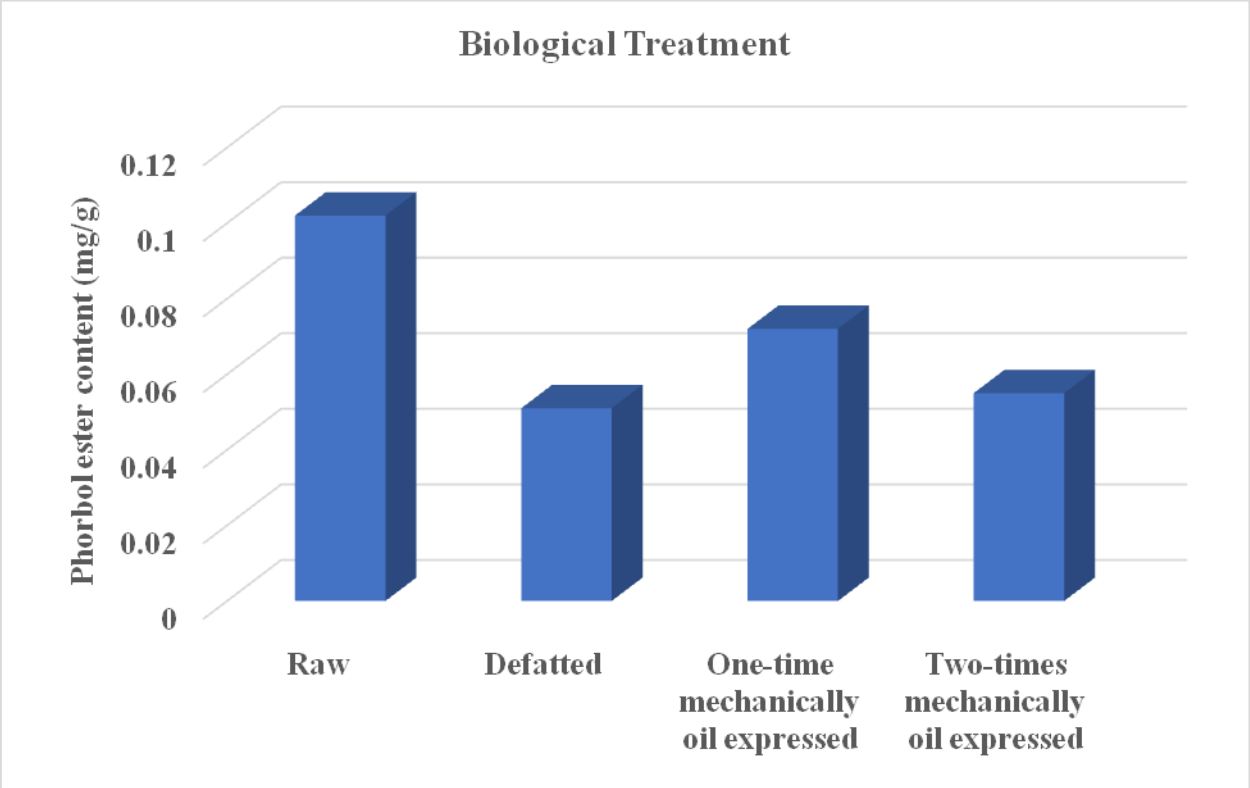

Fig.5 Phorbol ester content after biological treatment

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