QUANTIFICATION OF PHORBOL ESTERS IN JATROPHA SEEDS, OILCAKE, BIODIESEL AND METHODS TO AMELIORATE TOXICITY LEVELS

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Dedicated to
my sweet mom and
my guide

DEPARTMENT OF FORESTRY AND ENVIRONMENTAL SCIENCE
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU

CERTIFICATE

This is to certify that the thesis entitled **"Quantification of phorbol esters in Jatropha seeds, oilcake, biodiesel and methods to ameliorate toxicity levels"** submitted by Ms**. Sonia, B. S. ID No. PAK 9200,** for the award of degree of **MASTER OF SCIENCE (Agriculture)** in **Environmental Science** of the University of Agricultural Sciences, Bengaluru, is a record of the research work carried out by her during the period of her study in this university, under my guidance and supervision and no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
July 2011

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(Sharanappa)

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JULY, 2011 ***(Sonia. B.S)***

Quantification Of Phorbol Esters In Jatropha Seeds, Oilcake, Biodiesel And Methods To Ameliorate Toxicity Levels

THESIS ABSTRACT

The seed samples were collected from germplasm collection present in GKVK, Bengaluru. Seed yield of 20 accessions ranged from 51 to 457.5 g, oil content ranged from 21 to 40 per cent and phorbol esters were found using HPLC which ranged from 0.02-3.97mg/g. Out of 20 accessions, 6 contained phorbol esters less than 0.1mg/g, which is considered as non-toxic accessions. Both toxic and non-toxic accession of *Jatropha curcas* were found in southern Karnataka. Different treatments were given to detoxify the oil which contained the highest amount of phorbol ester (3.08 mg/g) out of which methanol treated sample gave the best result (1.44 mg/g). The untreated oilcake contained highest amount of phorbol esters (3.07 mg/g). This was reduced by different treatments and among them oilcake treated with hydrochloric acid was found to be the best (0.09 mg/g) treatment along with sodium hydroxide followed by 92 per cent methanol wash in reducing phorbol ester levels. Biodiesel also contained highest amount of phorbol esters which was higher than oil and oilcake (5.38 mg/g) which was reduced by bleaching (2.39 mg/g). The chemical composition of the oilcake after different treatments was analysed to know the nutritive value. Detoxification of oilcake was done to see that it could be used as a supplementary feed stock for animals and also as protein supplement in human diet. Though there was success in detoxification of cake to a great extent, further refinement is needed.

SONIA, B. S
July 2011

K. T. PRASANNA
(Major advisor)

ಜಟ್ರೋಫ ಬೀಜ, ಹಿಂಡಿ, ಜೈವಿಕ ಇಂಧನಗಳಲ್ಲಿರುವ ಫರ್ ಬೊಲ್ ಎಸ್ಟರ್ಸ್‌ಗಳ ಮಾಪನ ಹಾಗೂ ಅವುಗಳ ವಿಷಯುಕ್ತತೆಯ ಸುಧಾರಣ ಕ್ರಮಗಳು

ಸಾರಾಂಶ

ಕಾಡುಹರಳು ಬೀಜದ ನಮೂನೆಗಳನ್ನು ಗಾ.ಕೃ.ವಿ.ಕೇಂದ್ರ, ಕೃ.ವಿ.ವಿ ಬೆಂಗಳೂರಿನಲ್ಲಿ ಬೆಳೆಸಿರುವ ಸಸ್ಯ ಸಂಕೀರ್ಣದಿಂದ ತೆಗೆದುಕೊಳ್ಳಲಾಯ್ತು. ಇಪ್ಪತ್ತು ರೀತಿಯ ಸಂಕೀರ್ಣ ಸಸ್ಯಗಳ ಬೀಜದ ಸರಾಸರಿ ಇಳುವರಿ ಗಿಡ ಒಂದಕ್ಕೆ ೫೨–೪೫೭.೫ ಗ್ರಾಂ ಇದ್ದು, ಬೀಜದಲ್ಲಿನ ಎಣ್ಣೆ ಶೇ.೨೧–೪೦ ರವರೆಗೆ ಇದ್ದಿತು. ಬೀಜಗಳಿಂದ ಎಣ್ಣೆ ತೆಗೆದ ಹಿಂಡಿಯಲ್ಲಿನ ಫರ್‌ಬೊಲ್ ಎಸ್ಟರ್ಸ್ ಪ್ರಮಾಣವನ್ನು ಹೆಚ್.ಪಿ.ಎಲ್.ಸಿ ಮೂಲಕ ಪರೀಕ್ಷಿಸಿದಾಗ ಅದು ಪ್ರತಿ ಗ್ರಾಂ ಹಿಂಡಿಯಲ್ಲಿ ೦.೦೨–೩–೯೭ ಮಿ.ಗ್ರಾಂ/ಗ್ರಾಂ ಇರುವುದು ತಿಳಿಯಿತು. ಇಪ್ಪತ್ತು ಸಂಕೀರ್ಣ ಸಸ್ಯ ಮಾದರಿಗಳಲ್ಲಿ ಆರು ನಮೂನೆಯಲ್ಲಿ ಮಾತ್ರ ಈ ವಿಷಕಾರಕ ಫರ್‌ಬೊಲ್ ಎಸ್ಟರ್ಸ್‌ಗಳು ೦.೧ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ ಗಿಂತ ಕಡಿಮೆ ಇದ್ದು, ಇವುಗಳನ್ನು ವಿಷಕಾರಕಗಳಲ್ಲವೆಂದು ನಿರ್ಧರಿಸಲಾಯ್ತು. ಕರ್ನಾಟಕದಲ್ಲಿ ವಿಷಯುಕ್ತ ಮತ್ತು ವಿಷರಹಿತ ಸಸ್ಯ ಮಾದರಿಗಳು ಇರುವುದರಿಂದ ವಿಷನಿವಾರದ ಪ್ರಯೋಗಗಳನ್ನು ಕೈಗೊಳ್ಳಲಾಯ್ತು. ಜೈವಿಕ ಇಂಧನ ಘಟಕದಿಂದ ಎಣ್ಣೆ, ಹಿಂಡಿ ಮತ್ತು ಜೈವಿಕ ಇಂಧನವನ್ನು ತೆಗೆದುಕೊಂಡು, ಅದರಲ್ಲಿ ಇರುವ ಫರ್‌ಬೊಲ್ ಎಸ್ಟರ್ಸ್‌ಗಳ ಪ್ರಮಾಣವನ್ನು ಪರೀಕ್ಷಿಸಲಾಯಿತು. ಅತ್ಯಂತ ಹೆಚ್ಚಿನ ಫರ್‌ಬೊಲ್ ಎಸ್ಟರ್ಸ್‌ಗಳ (೩೦೮ ಮಿ.ಗ್ರಾಂ/ಗ್ರಾಂ) ಇರುವ ಕಾಡುಹರಳು ಎಣ್ಣೆಯನ್ನು ವಿವಿಧ ರೀತಿಯ ಸಂಸ್ಕರಣೆಗೆ ಒಳಪಡಿಸಲಾಯ್ತು. ಇವುಗಳಲ್ಲಿ ಮೆಥನಾಲ್ ಮಧ್ಯಸಾರದ ಉಪಚಾರ ಪರಿಣಾಮಕಾರಿಯಾಗಿದ್ದು, ೩೦೮ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ ಇಂದ ೧.೪೪ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂಗೆ ಕಡಿಮೆಯಾಯಿತು. ಹಿಂಡಿಯಲ್ಲೂ ಕೂಡ ಅತಿ ಹೆಚ್ಚು (೩.೦೭ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ) ಇದ್ದಿತು. ವಿವಿಧ ರೀತಿಯ ಸಂಸ್ಕರಣೆಗಳಲ್ಲಿ ಹೈಡ್ರೊಕ್ಲೋರಿಕ್ ಆಮ್ಲ (೦.೦೯ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ) ಮತ್ತು ಸೋಡಿಯಂ ಹೈಡ್ರೋಆಕ್ಸೈಡ್ ಉಪಚಾರ ನಂತರ ಮಿತೈಲ್ ಮಧ್ಯಸಾರದ ನೆನೆಸುವಿಕೆ (೦.೧೪ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ) ಅತ್ಯಂತ ಪರಿಣಾಮಕಾರಿಯಾದ್ದಿತು. ಎಣ್ಣೆ ಮತ್ತು ಹಿಂಡಿಗೆ ಹೋಲಿಸಿದಾಗ ಜೈವಿಕ ಇಂಧನದಲ್ಲಿ ಕೂಡ ಹೆಚ್ಚಿನ ಅಂಶ (೫೦.೩೪ ಮಿ.ಗ್ರಾಂ/ ಗ್ರಾಂ) ಇದ್ದಿತು. ಬ್ಲೀಚಿಂಗ್ ಗೊಳಿಸುವ ಉಪಚಾರದಿಂದ ಇದು ಕಡಿಮೆಯಾಯ್ತು. ಹಿಂಡಿಯನ್ನು ಸಂಪೂರ್ಣವಾಗಿ ವಿಷರಹಿತ ಮಾಡಿ ಅದನ್ನು ದನಗಳಿಗೆ ಪೂರಕ ಪೋಷಕವಾಗಿ ಹಾಗೂ ಮಾನವನ ಆಹಾರದಲ್ಲಿ ಪ್ರೋಟಿನ್ ಕೊರತೆಯನ್ನು ನಿವಾರಿಸಲು ಇನ್ನು ಹೆಚ್ಚಿನ ಪ್ರಯೋಗಗಳ ಅವಶ್ಯಕತೆ ಇದೆ. ಆದುದರಿಂದ ಈ ನಿಟ್ಟಿನಲ್ಲಿ ಇನ್ನೂ ಹೆಚ್ಚಿನ ಸಂಸ್ಕರಣೆ ವಿಧಾನಗಳನ್ನು ಹೆಚ್ಚು ನಿಖರವಾಗುವಂತೆ ಮಾಡಲು ಪ್ರಯೋಗಗಳನ್ನು ಮುಂದುವರಿಸಬೇಕು.

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ಜುಲೈ, ೨೦೧೧

ಪ್ರಸನ್ನ, ಕೆ. ಟಿ.
ಪ್ರಧಾನ ಮಾರ್ಗದರ್ಶಕರು

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

I. INTRODUCTION

Fast exploitation of world petroleum reserves for consumption as fuel and also industry has lead to alarming levels of environmental pollution. This is due to increasing exhaust emissions and accumulating non degradable solid wastes. The fast depleting resources calls for finding the alternative fuels to meet the needs of variety of applications, especially for Internal Combustion (IC) engines. In view of this, vegetable oil is a promising alternative because it is renewable, environmental friendly and produced easily in rural areas. Therefore, systematic efforts have been made around the world to use vegetable oils as fuel in IC engines and also for other industrial needs. Obviously, the use of non-edible vegetable oils compared to edible oils is very significant because of the tremendous demand for edible oils as food and they are far too expensive to be used as fuel at present.

Current consumption of fossil fuel i.e. diesel is around 40 million tonnes annually and only 20-30 per cent is produced in our country. Biodiesel is an alternative biofuel, prepared from renewable biological sources such as vegetable oils and animal fat. It is biodegradable, nontoxic, has low emission problems and so is environmentally beneficial (Shay, 1993 and Krawczyk, 1996). Biodiesel can be used in diesel engines with little or no modification. Biodiesel has a higher cetane number than diesel fuel, no aromatics, no sulfur, and contains 10–11 per cent oxygen by weight. According to an estimate, even 5 per cent replacement of fossil fuel by biodiesel will help to save foreign exchange of over Rs 4000 crores annually (Bhattacharya and Joshi, 2006). The present day estimates of Rs. 3, 60,000 crores are spent on import bills while 5% accounts for almost Rs. 18,000 crores.

The worldwide production of biofuels (bio-ethanol and biodiesel) exceeded 33 billion litres in 2004, of which 31 billion liters per year was ethanol and 2.2 billion litres of biodiesel. The biodiesel sector grew by 25 per cent per annum between 2000 and 2004. The production of biofuels is highest in Germany, with 50 per cent growth in 2004. France and Italy come second and third respectively (Renewable Energy Policy Network, 2005).

Of late, Jatropha received a lot of encouragement by the government of India as a medium term alternative to energy security scheme in the country through the biodiesel (Muktha and Sreevalli, 2007).

The word Jatropha is derived from two greek words 'Jatros' meaning a 'Doctor' and 'trophe' meaning 'nutrition'. *Jatropha* is a genus of 176 species distributed throughout the World. Among them, 12 species are recorded in India. Two species of *Jatropha* that are cultivated commercially include *Jatropha curcas* and *J. glandulifera*. *Jatropha curcas* is a shrub or small tree, belonging to the family Euphorbiaceae which also includes rubber, castor, etc. It is cultivated in Central and South America, South-East Asia, India and Africa (Herrera *et al.*, 2006). It is commonly known as Kachi, Kaduharalu, Dravanti, Janglirandi, Rratanjyot and Jamalghota in different states of India. It is known as physic nut and purging nut in English. Plant reaches a height of 3-8 m with stem up to 20cm diameter. It is a fast growing and easy to propagate. Life cycle is around 45-50 years (Takeda, 1982). It can grow and survive in any agro climatic conditions even in non productive soils of arid, semi arid and dry zones. It is anti-herbivorous. Jatropha can be used to alleviate soil degradation, desertification and deforestation. Also, it can be used for climatic amelioration and carbon capture, and increasing the income for agro-industries. Time taken for nut yield is

between 2 to 5 years. Seed yield varies from 0.5kg/plant to 1.5kg/plant and oil per cent from 21 to 40 per cent (Gaydou *et al.,* 1982). *Jatropha glandulifera* is known for its beautiful flowers but oil content is lower.

Jatropha curcas is valued for obtaining good amount of petro-fuel/biodiesel from its seeds (Bhattacharya *et al.*, 2005). It has become the major focus species owing to its high non-edible oil source and can also be used in soap and candle industries and its by-product glycerine can be used in the pharmaceutical industry. The seed cake has also been reported as animal feeds for its high protein and essential amino acid contents (Makkar *et al.*, 1998).

The Jatropha oil is slow-drying oil which is odorless and colorless when fresh but becomes yellow on standing. The seed oil is a renewable and safe source of energy. It is a viable alternative to diesel, kerosene, LPG, furnace oil, coal and fuel wood. Its oil is also used in resin, polish, paint, soap and candle industries. Biodiesel prepared has higher cetane number, no sulphur and flash point comparable to diesel. The fatty acid composition of Jatropha is classified as a linoleic or oleic acid type, which is unsaturated fatty acids, composed of myristic, palmitic, stearic, arachidic, oleic and linoleic acids. However, phorbol esters remain the principle of the toxicity that averts utilization of such cake and oil as food ingredients.

The byproduct of oil extraction, the oilcake is good organic manure and a viable alternative for chemical fertilizers. Other Jatropha products from fruits, flesh, seed coat and seed cake are rich source in nitrogen, phosphorus and potassium and are nutrients that improve soil. (Jones and Miller, 1992)

Phorbol esters, the toxic compounds in Jatropha (Makkar *et al.*, 1998) remain as the major barrier in using the oilcake for further

exploitation either as cattle feed or in human diet supplements. It is also reported that its presence and concentration vary widely throughout the world based on environmental conditions. The term 'Phorbol esters' are naturally occurring compounds widely distributed in plant species of the families Euphorbiaceae and Thymelaeceae. Haas *et al.*, (2002) reported six Phorbol esters from *J. curcas s*eed oil, where all compounds possess the same diterpene moiety, namely, 12-deoxy-16-hydroxyphorbol. They are highly toxic natural compounds, cathartic and skin irritant (Adolf *et al.*, 1984). The ester groups of these compounds are recognized as being essential; besides, their undesirable features. Accidental ingestion of *Jatropha* seeds caused marked giddiness, vomiting, and diarrhea, nausea, gastro-intestinal irritation, abdominal pain in humans (Joubert *et al.*, 1984). Toxicity to sheep, goats, calves, chicks, and fish by consumption of *Jatropha* seed or seed meal has been reported (Makkar and Becker, 1999). It has also the highest molluscicidal activity (Liu *et al.*, 1997). Such undesirable features of this compound can also be used as pharmacological probe in cancer cell signal transduction process studies. It has strong affinity towards Protein kinase C (PKC) which is involved in several cell signaling pathways. Phorbol esters are co-carcinogens which intensify cancer cell growth following exposure to sub-carcinogenic dose of carcinogen (Goel *et al.*, 2007).

The toxicity of the compounds indicates the potential for their use in pharmaceutical (animal and human) sectors and also for plant protection in agriculture field. A few studies aimed at detoxification of the Jatropha oil and meals by reducing phorbol esters concentration have been reported. Traditional oil refining methods like degumming, de-acidification, bleaching and deodorization can decrease the phorbol ester content of the seed by about 50 per cent (Haas and Mittelbach, 2000). The seed, oil and oilcake requires detoxification even if it is used for industrial purpose because there is possibility of direct contact with

people handling. A method for detoxification can be extracting phorbol esters using ethanol. (Gross *et al.*, 1997).

Therefore with these milieus, a comprehensive study entitled **"Quantification of Phorbol Esters in Jatropha Seeds, Oilcake, Biodiesel and Methods to Ameliorate Toxicity Levels"** was undertaken with the following objectives,

- To estimate the toxicity levels in selected *Jatropha* accessions.
- To find out the relationship of toxin with oil and yield parameters.
- To develop methods to reduce the toxic level in oil, oilcake and biodiesel.

Review of Literature

II. REVIEW OF LITERATURE

Annual crude petroleum requirement of India is 105 million. Only about 28-30 per cent is produced locally and about 70 per cent is imported (Kumar *et al.*, 2004). This demand is increasing from year to year putting pressure on foreign exchange reserves. The only alternative is to produce our own fuel from other sources and meet the growing energy demand. Biodiesel is the substitute for diesel.

Bio-diesel is a potential renewable alternative for non-renewable fossil fuels. It can be mixed with ordinary diesel; it improves cetane rating and engine lubrication (Raju and Rao, 2005). The Karnataka state government has instituted a State Bio-diesel Board to promote bio-diesel production.

2.1 *Jatropha curcas*

2.1.1 Distribution

Baker (1877) reported that *Jatropha curcas* as a native species of Mexico and Central America cultivated throughout the tropics and is sub-spontaneous in Mauritius and Seychelles.

Srivastava (1999) reported that Jatropha plant is able to thrive in a number of climatic zones with rainfall of 250-1200mm. In India, it is believed to have been introduced by Portuguese navigators in the 16th century. It is now found growing in all the parts of India and is commonly grown as a hedge around farm land.

2.1.2 Description

Dehgan (1984) stated that the name *Jatropha* refers to its medicinal value, in Greek, *Jatropha* means doctor and *trophe* means nutrition. *Jatropha curcas* is a perennial large shrub/small tree

belonging to the family Euphorbiaceae that grows up to a height of 4 to 5 m. *Jatropha* is a morphologically diverse genus comprising 160–175 species of trees, shrubs, rhizomatous sub shrubs and suffrulescent herbs.

Raju and Ezradanam (2002) stated that *J. curcas* is a perennial shrub or tree which flowers during the rainy season mainly late July to late October. It varies from state to state under different agro climatic conditions. Each inflorescence, once it begins flowering, flowers daily, and the flowering lasts for 11 days. Bhattacharya *et al.* (2005) reported that *J. curcas* flowers during July to September in the National Botanic Garden, Lucknow.

2.1.3 A multipurpose tree

The Jatropha oil is used as purgative, emetic and applied in rheumatism, herpes, and pruritus typically in Guinea. “Curcas oil” and latex are also believed to have anticancer property (Horiuchi *et al.*, 1987).

Paroda and Mal, (1989) reported that *Jatropha* grows quickly, survives in poor stony soil, resistant to drought. It can be grown on waste lands or barren and marginal agricultural lands where no irrigation facility is available and an ideal choice to make use of wasteland resources that are presently under or unutilized in tropical countries.

Seeds, leaves and bark are used in traditional medicine and for veterinary purposes (Heller, 1996). The oil has a strong purgative action and is also widely used for skin diseases and to cure the rheumatic pain. A decoction of leaves is used against cough and as an antiseptic after birth.

Matsuse *et al.*, (1999) reported that the root bark is used externally for rheumatism in Goa. It is mixed with asafoetida and butter-milk and used in cases of dyspepsia and diorrhoea in Konkan. The green tender leaves are used for stopping bleeding. Aqueous extract of *Jatropha curcas* exhibited anti-HIV activity.

Jatropha curcas is a tropical plant that can be grown in low to high rainfall areas and can be used to reclaim land, as a hedge and/or as a commercial crop (Openshaw, 2000). *Jatropha curcas* oil has similar properties to palm oil. It can be used in place of kerosene and diesel and as a substitute for fuel wood for cooking, lighting and locomotive power. Oil for soap making is the most profitable use (Openshaw, 2000).

Jatropha curcas is a potentially valuable source of germplasm, possessing rare and beneficial characters such as drought resistance, photoperiod insensitivity, resistance to major insect pests and diseases, non-palatability to livestock and desired oil quality (Sujatha, 2004). Traditionally *Jatropha curcas* has been used as medicine (Rajendran *et al.*, 2008).

Use of *Jatropha curcas* as a feedstock for the production of bio-diesel is rapidly growing and project developers are considering Jatropha as a substitute for fossil fuels to reduce greenhouse gas emissions (Achtens *et al.,* 2008).

Gressel, (2008) studied and inferred that the biofuel crops will be cost-effective in the long run only when domesticated, toxin factors and environment pollutants are removed by genetic engineering and made more productive.

Singh, (2008) reported that *J. curcas* seed oil and its components possess significant inhibitory effect on growth of the fungus,

Schizophyllum commune after 7 days of inoculation and also nematicidal activity against *Meloidogyne incognita*.

2.1.4 Curcas oil as biodiesel

Foidl *et al.,* (1996) developed technical process for the processing of the seeds and the production of the methyl esters from the seed oil. Methyl and ethyl esters from the oil of the seeds were also prepared and the fuel properties of both ester fuels were studied.

High viscosity of the *Jatropha curcas* oil was considered as a potential alternative fuel for the compression ignition engine. The performance of the engine using blends and Jatropha oil was evaluated in a single cylinder C.I. engine. Significant improvement in engine performance was observed in blends compared to vegetable oil, fuel consumption and the exhaust gas temperature were reduced due to decrease in viscosity. Thermal efficiencies of the engine were high with 50 per cent blending. Based on the properties of Jatropha oil and engine test results, it has been seen that 40–50 per cent of Jatropha oil can be mixed with diesel requiring no engine modification and preheating of the blends (Pramanik, 2003).

Bangawal and Krishnaswamy, (2004) reported that biodiesel reduces carbon dioxide exhaust emission by up to 80 per cent. Agarwal and Agarwal, (2007) reported that fuels derived from triglycerides present promising “greener” substitutes for fossil fuels.

The fuel properties of Jatropha biodiesel was found to be comparable to those of diesel and confirming to the American and European standards (Tiwari *et al.,* 2007) showing its potential as alternative to conventional diesel and confirmed by many studies (Banapurmath *et al.*, 2008, Berchmans and Hirata, 2008, Surendra *et al.,* 2008, Vyas *et al.*, 2009, Diwani *et al.*, 2009, Parawira, 2010).

2.2 Oil extraction

Terminalia bellerica seed oil was extracted by soxhlet extraction using petroleum ether (60-80^{0}C).After 16-18hr refluxing, the solvent was distilled off at 80^0 C. The oil content is the ratio of mass of oil to the mass of the crushed seeds used for extraction. The oil content was 31 per cent by weight of dry crushed seeds (Ajiwe and Obika, 2000).

Pongamia seed oil was extracted using soxhlet method. Yield was found to be in the range of 26.0-33.0 per cent by using different solvents. The oil was dark in colour with a disagreeable odour. Soxhlet extraction yielded best results with maximum yield being closer to 33 per cent using n-Hexane. The advantage of using organic solvents in extraction technique is that they can be recovered during the process and is easy for implementation and has been employed in many potential biodiesel crops (Akintayo, 2004).

Ultrasonication as a pretreatment before aqueous oil extraction and aqueous enzymatic oil extraction was found to be useful in the case of extraction of oil from the seeds of *Jatropha curcas* L. The use of ultrasonication for 10 min at pH 9.0 followed by aqueous oil extraction gave a yield of 67 per cent. The maximum yield of 74 per cent was obtained by ultrasonication for 5 min followed by aqueous enzymatic oil extraction using an alkaline protease at pH 9.0. Use of ultrasonication also resulted in reducing the process time from 18 to 6 h (Shah *et al.*, 2005).

Nzikou (2009) extracted oil from *Jatropha curcas* seeds variety "Congo-Brazzaville" using two oils extraction methods with petroleum ether (soxlhet) and extraction with a mixture of chloroform: methanol (1:1). The concentration of oil ranged from 50 per cent to 47 per cent. *Jatropha curcas* seeds have ash content of 4.2 per cent with the presence

of Ca, Mg, K and Na minerals. The oil was found to contain high levels of unsaturated fatty acids i.e oleic (40.10%) and linoleic (37.60%). *Jatropha curcas* oil was classified in the oleiclinoleic acid group. The dominant saturated acids were palmitic (15.63%) and stearic (5.78%). *Jatropha curcas* seeds were founded to contain high levels of crude protein (25%). The content of insaponifiables is 0.89 per cent.

2.3 Nutritive potential of *Jatropha curcas* L.

Bautista *et al.,* (1990) reported that rape seed cake and canola oilcake have protein content of 33 per cent and good source of amino acids but are deficient in lysine. Cotton seed cake has protein content of about 40 per cent and fiber content of 11–13 per cent, is deficient in lysine, methionine, threonine and tryptophan. Coconut oilcake rich in residual oil composed of short chain saturated fatty acids and deficient in amino acids such as lysine, methionine, threonine and histidine but high in arginine.

Sunflower oilcake was fractionated into three main components, a lignocellulosic fraction (LCF), a proteinaceous fraction (PF) and a soluble fraction (SF), which represented 23.2–25.3 per cent, 55.4–57.6 per cent and 17.1–21.4 per cent of the dry weight, respectively. After removal of the PF, the remaining sub products (LCF and SF) have a high potential for use as feed in fermentation. It is high in methionine, but low in lysine and threonine (Bautista *et al.,* 1990).

Doijode, (1990) stated that seed moisture plays an important role in germination. Seed moisture is to be satisfactory and at safe level before put for storage.

Sesame oilcake is a relatively good source of crude protein which can replace part of basic ingredients in diets such as soybean. The chemical composition of sesame oilcake varies according to the method

processing such as mechanical or solvent extraction. It has been reported that the dry matter (DM) content ranges from 83 - 96 per cent. Also it has been reported that the CP, ash, ether extract, nitrogen free extract (NFE) and crude fiber (CF) are 23-46 per cent 7.5-17.5 per cent, 1.4-27 per cent, 25-31 per cent and 5- 12 per cent on dry weight basis, respectively (FAO., 1990).

Makkar *et al.,* (1997) characterized eighteen different provenances of Jatropha for nutrient and anti-nutritional factors in Mexico. There was a large variation in the contents of crude protein (19-31%), lipid (43-59%), neutral detergent fibre (3.5-6.1%) and ash (3.4-5%) in kernels. The gross energy of kernels was relatively similar (28.5-31.2MJ/kg). A wide variation was observed for saponins, phytates and lectin activity. Phorbol esters were not detected in the seeds collected from Papantla, whereas in other seventeen provenances it ranged from 0.87-3.32mg/g.

The seeds collected from Quintana Roo state seemed to be of better quality as levels of protein (27-30%), lipid (55-62%) and ash (3.7-5.2%) were higher and anti-nutritional and toxic factors lower in most samples. Phorbol esters in kernels from raw seeds were not detected in four samples and in other three samples it ranged from 0.01 to 0.02mg/g. Wide variation exists in levels of nutrients and anti-nutrients in seeds of edible provenances. Thus food security in various tropical countries could be improved by providing edible oil, roasted nut and seed cake which potentially could be good sources of protein supplementation for both humans and animals (Makkar *et al.,* 1998).

The Simarouba proteins showed maximum solubility (92%) at pH 9.0. Typical protein solubility profiles with similar solubility values reported by Wang *et al.*, (1999) for rice bran, Jatropha and Karanja proteins.

Herrera *et al.*, (2006) analyzed four provenances of Jatropha with different morphological characters in Mexico. The seed kernels were rich in crude protein (31-34%), lipid (55-58%) and detergent fibre (3.9%).The gross energy of kernels ranged from 31.1-31.6MJ/kg dry matter. The contents of starch and total soluble sugars were below 6 per cent. The levels of essential amino acids, except lysine were higher than that of the FAO. The major fatty acids found in the oil samples oleic (41.5-48.8%), linoleic (34.6-44.4%), palmitic (10.5-13%) and stearic (2.3-2.8%) acids. Trypsin inhibitors, phytates, saponins and lectins were the other major anti-nutrients present in the seed meals.

Kabeh and Jalingo (2007) observed that alkali treated neem cake performed significantly better when incorporated into poultry feeds. Several animals and plant pathogenic fungi, bacteria, viral, protozoan and Helminths are sensitive to neem preparations with antiseptic properties. Neem seed oil and leaves extract significantly inhibited fertility in males, but not anti-ovulatory, hence “sensal” a contraceptive.

Sirisomboon *et al.,* (2007) reported that the hull of the Jatropha fruit had very high moisture content compared to nut shell and kernel. The whole fruit contained 77.03 per cent moisture content. Bulk densities of fruits, nuts and kernels were 0.47, 0.45 and 0.42 g/cm^3, solid densities were 0.95, 1.04 and 1.02 g/cm^3 and the porosities were 50.53 per cent, 56.73 per cent and 58.82 per cent, respectively. The surface area of fruit was larger than those of nut and kernel, by 5.88 per cent and 10.24 per cent respectively.

Moisture content in Jatropha seeds ranged from 4.75–19.57 per cent. The average length, width, thickness and 1000 seed mass were 18.65 mm, 11.34 mm, 8.91 mm and 741.1 g at moisture content of 4.75 per cent. As the moisture content increased from 4.75 - 19.57 per cent, the angle of repose and surface area were found to increase from 28.15^0

to 39.95^0 and 476.78 to 521.99 mm^2. The static coefficient of friction of Jatropha seed increased linearly against the surfaces of three structural materials, namely plywood (44.12%), mild steel sheet (64.15%) and aluminum (68.63%) as the moisture content increased from 4.75 -19.57 per cent (Garnayak *et al.,* 2008).

Makkar *et al.,* (2008) reported that the recovery of protein concentrate was highest when the proteins from the seed cakes were solubilised at pH 11 for 1 h at 60^0 C and the precipitation of these proteins was done by lowering the pH to 4 and over 53 per cent was from cakes. Phorbol esters in the protein concentrates ranged from0.86–1.48mg/g. Trypsin inhibitor was present at an tenfold higher level in the protein concentrates than in the seed cakes. Lectin and phytate were also present at high levels, but their levels were lower than in the seed cakes.

Sirisomboon and Kitchaiya (2009) reported that the kernels contained moisture 3.78, 4.01 and 2.82 per cent wet basis at 40, 60 and 80^0 C respectively. The sphericity of dried kernels was 0.65–0.66 and 0.53 for steamed kernels. The bulk densities of dried kernels and steamed kernels were 403–513, and 509 kgm^3. Drying at 80^0 C gave the highest oil yield at 47.06 per cent and had highest acid value. Drying at 40^0 C gave a lower oil yield at 36.83 per cent but had the lowest acid value. The temperature of the drying process had a minor effect on viscosity and ash content but had a significant effect on free fatty acid content and acid value. The viscosity of the kernel oil was 33.91–34.53 cSt at 40^0 C.

Arab and Salem, (2010) reported that *J. curcas* seeds are a good source of protein (32.88%), oil (27.36%) and carbohydrates (30.11%). Seeds were found to be rich in micro nutrients such as Mn, Fe and Zn were 28.37, 0.38 and 47.13 mg/kg, and macro-elements such as

contents of K, Ca, Na, Mg and P were 103.13, 34.21, 8.44, 109.89 and 185.17 mg/kg .

Defatted cake after the extraction of non-edible oil from *Jatropha* seeds contains 5.73 per cent nitrogen, 1.5 per cent phosphorus and about 1 per cent potassium. On the basis of its chemical composition, its application as substrate to the biogas plant can be a sustainable alternative as compared to the other applications of *Jatropha* press cake. It cannot be used directly as animal feed due to presence of toxic substance called 'phorbol esters'. This toxin renders it unsafe for the animal feed and other purposes (Ali, 2010).

The Simarouba oilcake being rich in nitrogen (8%), phosphorus (1.1%) and potash (1.2%), is good organic manure. The shells can be used in the manufacture of particleboard, activated charcoal or as fuel (Lele, 2010).

2.4 Phorbol esters in *Jatropha curcas* L.

Castagna *et al.,* (1982) showed that tumor-promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) directly activate *in vitro* Ca^{2+} activated, phospholipid-dependent protein kinase, which normally requires unsaturated diacylglycerol. Various phorbol derivatives which have been shown to be active in tumor promotion are also capable of activating this protein kinase in *in vitro* system.

Wink *et al.,* (1997) reported that toxicity of Jatropha is caused by several components which include phorbol esters. Phorbol esters activate protein kinase C (PKC) and hence must be removed from oil and oilcake which are being used as animal and for human nutrition. Phorbol esters exhibit insecticidal and molluscicidal property over a wide range of organisms and hence have potential for agriculture use. Phorbol esters are not mutagenic or carcinogenic.

The seed/ meal from the non-toxic Mexico variety is of as good quality as those from the toxic varieties. The meal contained significant levels of trypsin inhibitor, lectin and phytate, and their levels did not differ much between the non-toxic and the toxic varieties. Very low level of phorbol esters were observed in the seeds of non-toxic varieties. But in toxic varieties it was 20 times higher and the toxic compound in seeds is the phorbol esters. The non-toxic variety of Jatropha from Mexico was a suitable alternative source of oil for human consumption, and the seed cake can be a good protein source for humans as well as livestock. (Makkar *et al.*, 1998).

Goel *et al.,* (2007) stated that Phorbol esters are the tetracyclic diterpenoids generally known for their tumor promoting activity. The phorbol esters mimic the action of diacyl glycerol (DAG), activator of protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities. The biological activities of the phorbol esters are highly structure specific and even at very low concentrations, show toxicological manifestations in animal feeds.

Ahmed and Salimon, (2009) determined that phorbol ester content of three different provenances of tropical *Jatropha curcas seed* from Malaysia, Indonesia and India. There were significant variations in the oil content and phorbol ester content of the seed from three provenances. Phorbol esters level was low in Malaysian seed oil (0.23%), whereas the level of phorbol esters on Indonesian and Indian seed oil were 1.58 per cent and 0.58 per cent respectively.

The seed oil obtained from the *Jatropha* plants is converted to biodiesel after transesterification reaction and was used as a fuel for automobiles instead of fossil fuels. It is known that there are toxic components present in the seed cake and oil of *Jatropha*. Toxic compounds present in the cake are the phorbol esters. Isolation and

characterization was done for the removal of phorbol esters. During the analytical HPLC, peaks of phorbol esters were detected between retention time 20 to 25 minutes (Gaudani *et al.,* 2009).

The extent of reduction in phorbol esters was more than 99.4 per cent in methanol. Solvent: oil ratio, time and temperature were optimized using a magnetic stirrer to get phorbol esters rich fraction and virtually phorbol esters free oil. Phorbol esters were fourteen folds higher in this fraction than the control oil. Phorbol esters rich fraction was used in agricultural, medicinal and pharmaceutical applications and the remaining oil can be used for biodiesel preparation (Devappa *et al.,* 2009).

Demissie and Lele, (2010) reported bio-assay assisted isolation of phorbol ester in optimized solid and liquid system tissue culture. The effect of the compound on human leukemic cell line was confirmed using Tetrazolium assay and Sulforhodamine assay. Phorbol ester composition in seed oil was found to be 1.78 per cent. The composition from callus and suspension cultures sources was 0.145 per cent and 0.173 per cent. The HPLC profile of isolated PMA found to be Rf = 0.38. The biological activity of phorbol ester showed significant proliferation of the human leukemia cell line even at 5µg/ml. This result provides the evidence that phorbol ester can be produced through plant cell *in vitro*.

Jatropha is a non edible oil seed plant. *Jatropha curcas* kernel meal contains negligible amounts of total phenols (0.2−0.4%) and tannins (0.02−0.04%). The saponin content in *J. curcas* kernel meal ranged between 1.8 and 3.4%. Trypsin inhibitor (TI) activity in the *J. curcas* kernel meal of both toxic and nontoxic genotypes was 18.4 to 27.3 mg/g. The toxic extracts possess moluscicidal, pesticidal, insecticidal, rodenticidal, antimicrobial, and cytotoxic properties, and exert adverse

effects on animals including rats, poultry, and ruminants (Devappa *et al.*, 2010).

2.5 Detoxification

Aregheore *et al.,* (1997) defatted and ground seeds from toxic variety of *Jatropha curcas* were processed to obtain the meal. The meal was subjected to 14 different chemical treatments to detoxify the meal of lectin and phorbol esters. Heat treatment inactivated lectin, but not phorbol esters. Phorbol esters were reduced to a tolerable level of 0.09mg/g when it was heat-treated and washed 4 times with 92 per cent methanol. The meal derived from it had a crude protein content of 68 per cent, which was higher than the crude protein content of most oilseed meals.

Staubmann *et al.*, (1997) reported that *Jatropha* seed cake contains curcin, a highly toxic protein similar to ricin in castor, which makes it unsuitable for animal feed but it has been potentially used as a fertilizer and biogas production.

Makkar and Becker, (1999) reported that the protein efficiency ratio(PER) for untreated and treated Jatropha meal containing diets was 37 per cent and 86 per cent of casein diet which was highest in rats which increased the weight. The body weight, PER and feed conversion ratio of fish were statistically similar for unheated and heated Jatropha meal. Trypsin inhibitor and lectin activities decreased drastically (>83 and 99 per cent) after 30 and 45 minutes of heat treatment. Jatropha trypsin inhibitors and lectins do not have adverse effects on carp at least up to 35days of feeding. The nutritional value of Jatropha meal of non-toxic provenance is high and potential exists for its incorporation into the diets of monogastrics, fish and possibly humans.

Haas and Mittelbach, (2000) determined that oil extracted from *J. curcas* seed was treated by traditional oil refining process examining the effect on the content of phorbol esters. Parameters of several refining steps were varied to optimize the grade of detoxification. Almost no effect was observed with degumming and deodorization, whereas the steps of de-acidification and bleaching could reduce the content of phorbol esters up to 55 per cent.

Laboratory scale petroleum ether extraction reduced the phorbol ester content in the *Jatropha curcas* seeds by 67.69 per cent [6.5 mg/g in the raw kernels to 2.10 mg/g], double solvent extraction followed by moist heat treatment reduced phorbol esters by 70.77 per cent to 1.90 mg/g. Double solvent extraction accompanied with wet extrusion, re-extraction with hexane and moist-heat treatment reduced phorbol ester content to 0.80 mg/g, an 87.69 per cent decrease (Chivandi *et al.,* 2004).

Panda *et al.*, (2005) studied the performance of broiler chickens feed mixed with processed Karanja cake. It is concluded that 1.5 per cent Sodium hydroxide (w/w) treated Karanja cake could be incorporated up to 6.43 per cent replacing 12.5 per cent of soybean meal nitrogen of reference diet in broilers chickens without affecting growth, nutrient utilization & tibia mineralization during 0 to 4 weeks of age.

The detoxification of karanja seed meal for minor constituents like saponins, tannins, phytates and protease inhibitors were approached by different methods such as leaching with boiling water, boiling with 0.1 per cent and 0.5 per cent calcium hydroxide and 2 per cent hydrochloric acid. There was a reduction in phytate content up to 79 per cent and 81 per cent in treatment with 2 per cent hydrochloric acid and 0.5 per cent Ca(OH)2. 2 per cent hydrochloric acid treatment was effective in reducing tannins up to 69 per cent and trypsin inhibitor activity was reduced drastically (NOVOD., 2007).

Different physical, chemical and physico chemical methods were tried to detoxify the simaruba oilcake. *Simarouba glauca* oilcake contains the bitter toxic constituents known as quassinoides. It was found that ammonia treatment was a suitable method of detoxification of the oilcake without much adverse effect on its nutrient composition (Behura *et al.,* 2008).

Makkar *et al.,* (2008) showed that *Jatropha curcas* seeds are highly toxic to livestock. The presence of phorbol esters and anti-nutrients such as trypsin inhibitor, lectin and phytate and the high level of shells in the seed cake prevent its use in animal diets. The recovery of protein concentrate was highest by solubilising at pH 11 for 1 h at 60^0 C and the precipitation of these proteins was done by lowering the pH to 4.53 per cent of the total proteins present in the seed cakes were recovered. The protein contents in the oil-containing seed cake and defatted seed cake were 760 and 820 g/kg. Substantial amounts of phorbol esters were present in the protein concentrates (0.86–1.48mg/g).

Jatropha curcas, a tropical plant introduced in many Asian and African countries is presently used as a source of biodiesel. The cake after oil extraction is rich in protein and is a potential source of livestock feed. The high toxic nature of whole as well as dehulled seed meal due to the presence of toxic phorbol esters and lectins. The meal was subjected to alkali and heat treatments to deactivate the phorbol ester as well as lectin content, where the phorbol ester content was reduced up to 89 per cent in whole and dehulled seed meal (Rakshit *et al.,* 2008).

The defatted seed cake of karanja (control sample) was found to contain about 0.386 per cent w/w of karanjin and 0.652 per cent of pongamol. Various methods were used to detoxify the de-fatted karanja seed cake, but method of reflux with methanol (100%) and Cold maceration with agitation using methanol were found to have no

karanjin and pongamol content with respect to reference standard of karanjin and pongamol (Singh, 2008).

Vinay and Sindhu, (2008) studied the effect of detoxification on the functional and nutritional quality of proteins of Pongamia seed meal treatments such as water leaching, mild acid & mild alkali were found to bring down the levels of anti-nutrients components. 2 per cent hydrochloric acid improved the nutritional value by reducing the content of physate (81%), tannin (69%) and trypsin inhibitor activity (84%). Solubility of proteins at various PH value varied from 2 -11. The available lysine content (3.46%) in acid treated meal was comparable with the control (3.6%).

Detoxification of Karanja (*Pongamia glabra*) oilcake by liquid-liquid extraction was done and found, cake that was treated with hydrochloric acid in the ratio of 1:0.35 (W/V), followed by repeated extractions by alkaline methanol in the ratio of 1:2(W/V) completely detoxified oil. Physico chemical characteristics of refined oils are acceptable. Overall oil yield (75-80% w/w) is obtained when alkaline MeoH, Isopropanol (IPA) & Dimethylformamide (DMF) are used but higher yield (85-88% w/w) is obtained when acetonitrale was used (De and Patel, 2009).

Jatropha curcas seeds are rich in oil (28-32%), which can be converted to high quality biodiesel. Phorbol esters (PE) are the main toxins present in the oil and seedcake. Phorbol esters (PE) present in oil (3.15 mg/g) were used as bio-pesticides and antimicrobial agents. Different methods yielded 77.8-80 per cent PE in the isolated fraction. This also reduced oil free fatty acid content from 2.9 to 1.3 per cent, and phosphorus content from 133 to less than 40 ppm. Biodiesel prepared from PE-isolated oils, reduced the oxidative stability with increased time of the treatment (Devappa *et al.*, 2009).

Phorbol esters that are present in *Jatropha curcas* oil are toxic when consumed and are co-carcinogens. Phorbol esters were reduced during degumming. Silica treatment did not decrease the phorbol esters, but deodorization at 260^0C at 3 mbar pressure with 1per cent steam injection completely degraded phorbol esters. Phorbol esters were not detected in stripped oil, fatty acid distillate, biodiesel and glycerine (Makkar *et al.,* 2009).

Annongu *et al.,* (2010) conducted an experiment to investigate the utilization of Jatropha seed cake by Albino rats. Jatropha Seed Cake (JSC) treated by boiling, fermentation followed by extraction with equal volumes of hexane and ethanol was included in diets at graded levels of 5, 10, 15, 20 and 25 per cent. Data obtained on performance and body organ indices showed that rats tolerated up to 15 per cent dietary JSC without adverse effects on the measured parameters in relation to the corn-soy reference diet ($p>0.05$). However, 20 and 25 per cent inclusion levels elicited mortality in all the animal subjects receiving the diets within one week in the course of the experimental trial.

The Seeds of *Jatropha curcas* contain 52.59 mg/100 g, 25.58 mg/g, 39.95 mg/100 g and 1.51 g/100 g of phytic acid, trypsin inhibitor activity, total phenols and phorbol esters. Jatropha is nutritionally promising because of its high nutrient content and low anti-nutrient level. Some physical treatments like soaking, germination and roasting and some chemical treatments like NaHCO3, ethanol extraction and Sodium hydroxide was successful in inactivating the anti-nutrients (Arab and Salem, 2010).

Washing with 2 per cent hydrochloric acid followed by neutralization was found effective in reducing the anti-nutrients such as tannins, phytates and trypsin inhibitors. The detoxified meal that was prepared by washing with 2 per cent hydrochloric acid followed by

neutralization was fed to weaning rats at 5 per cent and 10 per cent level. Though the rats were safe, the food intake was not satisfactory, which was due to the presence of saponins. Hence, methods were looked at to decrease saponin content such as (i) cold water washing (ii) hot water washing (iii) 2 per cent hydrochloric acid wash (iv) 2 per cent hydrochloric acid wash and boiled with water (v) Ethanol wash (vi) 2 per cent hydrochloric acid wash and ethanol wash. Up to 70 per cent saponins were removed by 2 per cent hydrochloric acid wash and ethanol wash (Basu, 2010).

Jatropha curcas seed cake which was a main by-product from biodiesel production had high protein levels. The detoxified seed cake after ethanol extraction was incorporated in starter broiler diet. The proteins isolated from the seed cake and from the detoxified seed cake were applied to diets. The phorbol ester content of each diet was reduced by approximately 50 per cent or higher after an extrusion process which was therefore considered as an effective method to remove phorbol ester (Saetae and Suntornsuk, 2010).

The untreated simarouba meal had 3.7 per cent, 0.88 per cent and 0.96 per cent of saponins, phenolics and alkaloids respectively. Different detoxification treatments such as boiling in water for 20 minutes reduced saponins and phenolics (75.8% and 88.2% reduction). Treatment with 2 per cent acetic acid and 2 per cent ethanolic acetic acid reduced the saponins and alkaloids content by 58.6 per cent and 89.6 per cent and 84.3 per cent and 81.5 per cent. Treatment with 2 per cent sodium hydroxide brought 74.8 per cent reduction in saponins but in alkali treatment there was no reduction in alkaloids. Boiling of simarouba meal in acetic acid medium was found to be promising in detoxification (Basu, 2010).

Department of Microbiology at University of Technology Thonburi, Bangkok, Thailand (2010), cultivated *Jatropha curcas* in Thailand and classified into toxic and non toxic genotypes depending upon the phorbol ester content. Seed parts contained different concentrations of phorbol esters. *J.curcas* seed cake was a main product from biodiesel production and had a high protein level. The detoxified seed cake after ethanol extraction was formulated to be the starter broiler diet. The proteins isolated from the seed cake and the detoxified seed cakes were applied to different diet. The phorbol ester contents of each diet were reduced by approximately 50 per cent higher after an extrusion process. The extrusion was therefore an effective method to remove phorbol esters.

Material and Methods

III. MATERIAL AND METHODS

With growing International and National concern regarding "clean fuel" and sky rocketing prices of crude oil, plantation of non-edible tree born oil seeds, particularly *Jatropha curcas* in India has received a fillip in the last five to six years. *Jatropha curcas* is identified as potential feed stock as the source of biodiesel, which has brought industry and policy makers to harness the potential of Jatropha as commercial plantations.

The oilcake is good organic manure and a viable alternative for chemical fertilizers. Other Jatropha products from fruits flesh, seed coat and seed cake are rich source in nitrogen, phosphorus and potassium are nutrients that improve soil. The present study was carried out to know the toxic level and methods to reduce the toxic compounds in Jatropha. The details of the materials collected and the methods that were adopted during the course of study are described below.

3.1. Plant material and seed collection

Seeds were collected from germplasm collections of different agro climatic zones of Karnataka and maintained by Dept. of Genetics and Plant Breeding, UAS, Bengaluru. Mature, dry seeds of different accessions were collected for further processing. Seeds were further dried in an oven at 40^0 C. Dried seeds were ground into fine powder using mixer grinder. The ground material of seeds was then used for the oil extraction process using soxtherm apparatus.

3.2. Extraction of oil in seeds by soxtherm extraction method

The oil content in the seed was estimated using Gerhardt soxtherm apparatus which work on the principle of solvent extraction process.

Plate 1 : *Jatropha curcas L.* plant, flowers, fruits and seeds

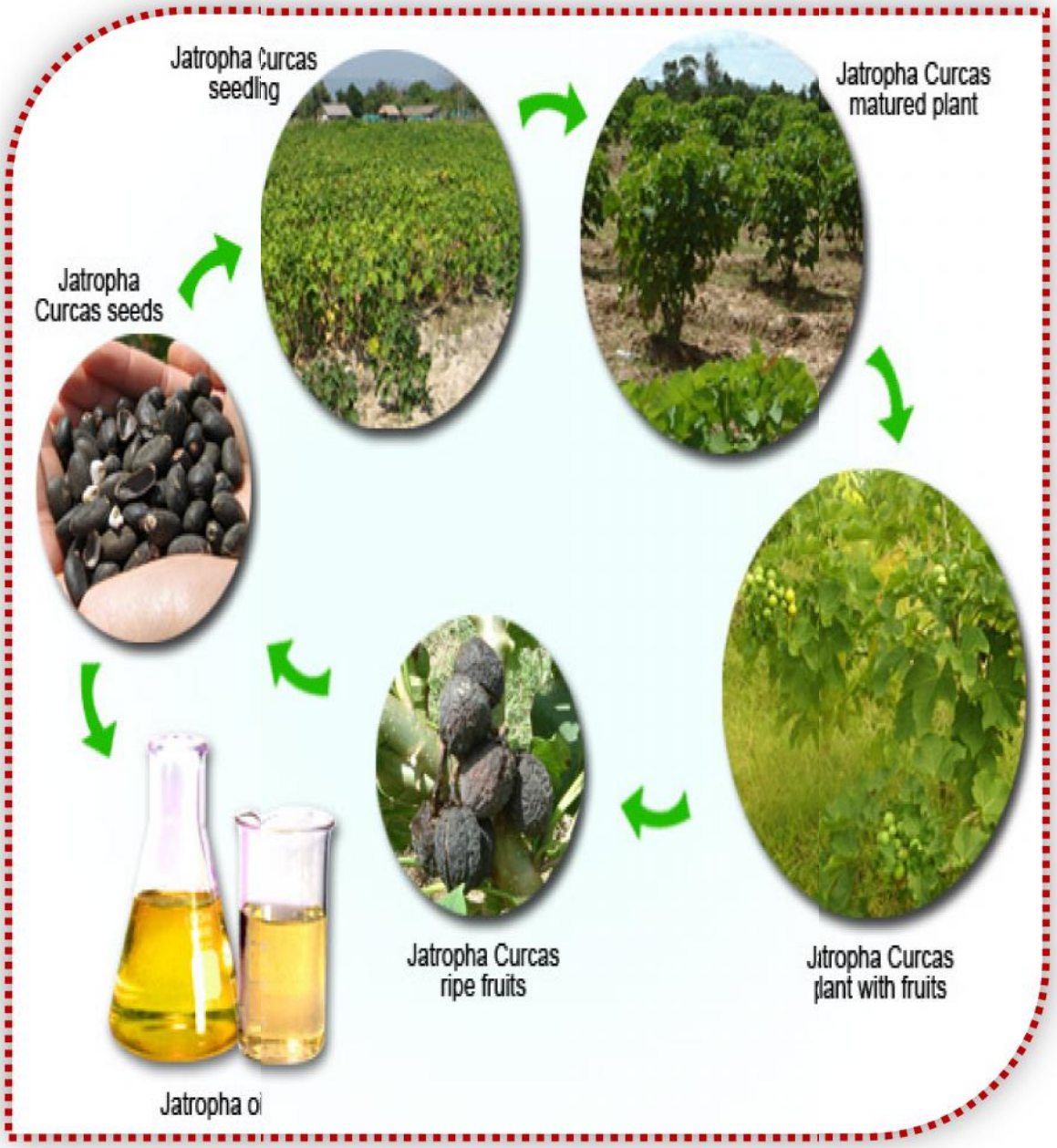

Plate 2 : Life cycle of *Jatropha curcas*. L.

Five grams finely ground seed material was placed in cleaned extraction thimble and covered with cotton. Weight of extraction jar along with boiling stones was recorded. Thimbles containing weighed seed material were placed inside extraction jars. Known volume (100ml) of solvent petroleum ether was introduced into each of extraction jars. These jars were placed in pre programmed oil extraction apparatus that was run for 4 hours 35 minutes. Jars were removed and dried in a thermostatic oven at 110^0C for 1 hour to remove the residual solvent and moisture, if any. Then jars were transferred to desiccator. After 1 hour they were weighed. Oil content in percentage basis were calculated based on the following equation.

$$\text{Oil content} = \frac{W_2 - W_1}{W} \times 100$$

Where,

W- Weight of seed (g)

W_1- Weight of jar with boiling stones (g)

W_2- Weight of jar, boiling stones and oil (g).

3.3. Extraction of phorbol esters from Jatropha seeds

Three grams of seed from each accession was finely ground in 10 ml of methanol (HPLC grade) and subjected to ultrasonication in an ultrasonic bath for 3 minutes. Supernatant was removed after centrifugation. Process was repeated three times and the supernatants were pooled and used for the assay of phorbol esters.

3.3.1. Assay of phorbol esters

The Shimadzu HPLC system equipped with Prominence column oven of CTO-20A, pump LC-20T and UV-VIS detector SPD -20 A was to set up for a good base line with a suitable solvent system i.e. acetonitrile and water in 80:20 ratios. The experimental studies were performed with

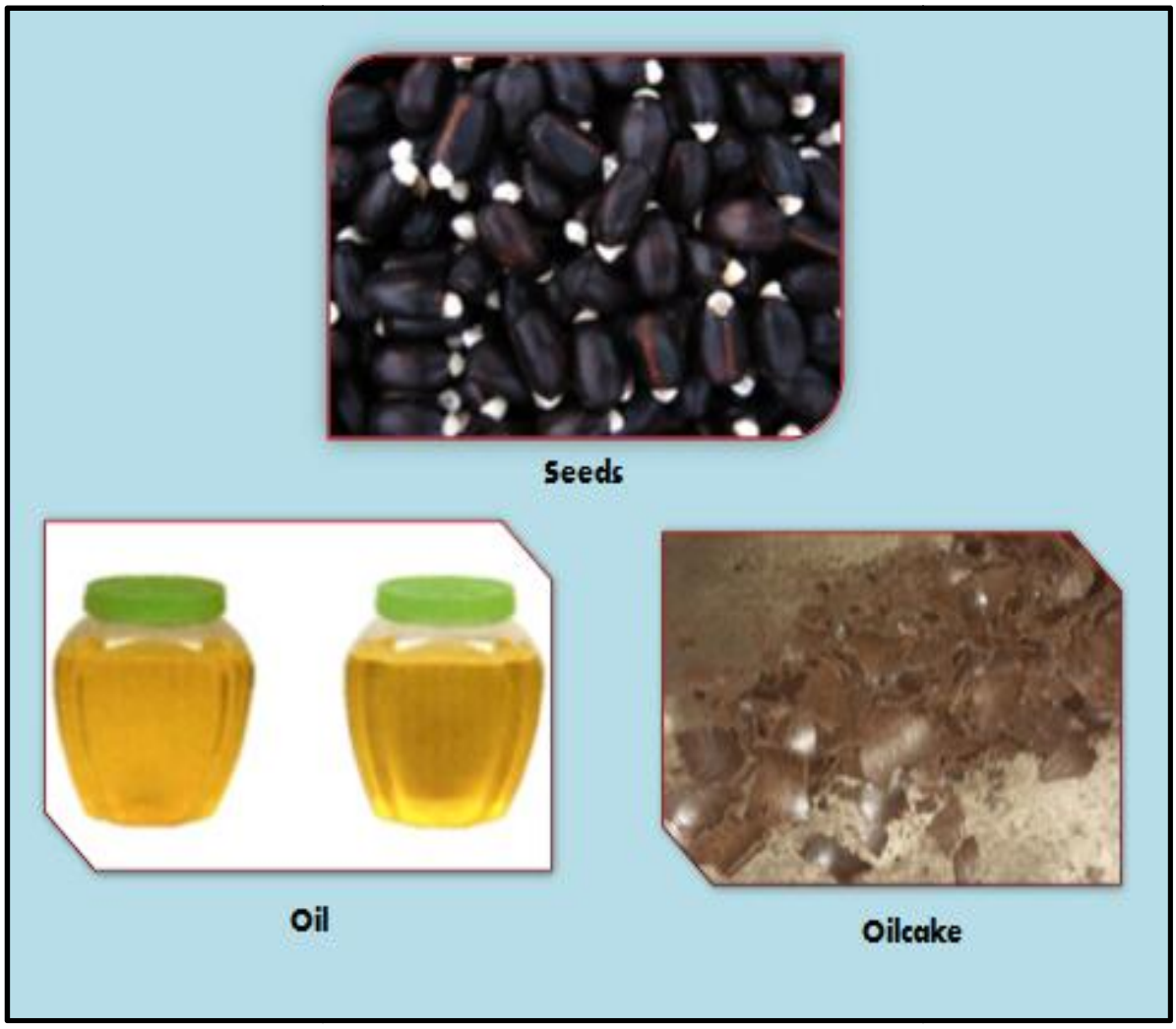

Plate 3 : Overview of Jatropha seed, oil and oil cake

Plate 4 : Soxtherm apparatus used for extraction of oil

RP-18, 5µ, 250 x 4.6 mm analytical HPLC column. The study was conducted at 280 nm using UV detector. After obtaining the straight & stable base line with a suitable gradient system, extracted material which was filtered through 0.22µ syringe filter before was used to analyze on HPLC System. 20µl of sample solution of the extracted material was injected into flow system with 1 ml/min flow rate.

3.3.2. General parameters for HPLC

- Flow rate: 1ml/min
- Column temperature: 40^0C
- Pressure of the column: 70-160 bar
- Wavelength: 280nm
- Phorbol ester peak observed between: 6-11 min.

3.4. DETOXIFICATION

Bulk *Jatropha curcas* oil, oilcake and biodiesel available at Biofuel unit of Department of Forestry and Environmental science were used for the study. Different detoxification methods that was carried out to remove or reduce the phorbol esters in Jatropha oil, oilcake and biodiesel is listed below.

3.4.1. DETOXIFICATION OF OIL

The bulk Jatropha oil that was collected was used for further detoxification study. Acid degumming, neutralizing, bleaching and methanol treatments was carried out in replications to reduce the phorbol esters content. The methodology carried out is described below.

Plate 5 : High Performance Liquid Chromatography which is used to determine phorbol esters

3.4.1.1. Acid degumming

Jatropha curcas seed oil (100ml) was heated to 80^0 C under constant stirring in a beaker using magnetic stirrer. 3 ml distilled water which was first heated to 90^0 C was added followed by 0.2 per cent ortho-phosphoric acid. The mixture was stirred for 1 hour and cooled and centrifuged for 15 minutes at 4000 rpm. The treated oil was kept in a hot air oven at 100^0 C for 30 minutes and dried to remove the remaining water.

3.4.1.2. Bleaching

50 ml of the degummed oil was stirred at 100rpm in a beaker using magnetic stirrer at 80^0 C for 15 minutes. 2 per cent activated charcoal which is a bleaching agent was added to the oil. Again the mixture was stirred for 15 minutes. The mixture was separated by centrifugation at 4000 rpm. The remaining activated charcoal was separated by filtration. The bleached oil was collected and used for further analysis.

3.4.1.3. Methanol treatment

50 ml of the oil was extracted using 50 ml of methanol and it was stirred at 1000 rpm for 15 minutes. Later the mixture was separated by filtration and the oil was collected. The collected oil was used for further analysis.

3.4.1.4. Neutralization by caustic treatment

The weight (W_1) of the empty specific gravity bottle was recorded. The bottle was filled with *J. curcas* oil and weight (W_2) was recorded. Then the bottle was again filled with distilled water and the weight (W_3) was recorded. The value was calculated as follows.

$$d = \frac{W_2 - W_1}{W_3 - W_1} \times 1000$$

Where,

d = density

W_1=Weight of empty specific gravity bottle

W_2=Weight of oil with bottle

W_3=Weight of water with bottle

Three grams of the oil along with 20 ml of isopropyl alcohol and 2-3 drops of phenopthalein indicator was titrated against 0.1N potassium hydroxide which was taken in buirette. The appropriate amount of potassium hydroxide (KOH) solution required to neutralize 1gm of the oil was calculated using the following equation.

$$\text{KOH required} = \frac{\text{Titre value X 0.1N KOH X 56.1}}{\text{Weight of sample}}$$

The amount of KOH required to neutralize was divided by 2 to give the FFA per cent. The appropriate amount of alkaline solution to neutralize the free fatty acids was calculated using the following equation.

$$L = \frac{D \text{ X } FFA \text{ X } 1000}{M \text{ X } N}$$

Where,

L= volume of N-molar aqueous sodium hydroxide solution

d= density of oil

FFA=Free Fatty Acid

M= average molecular weight of the fatty acids (M=278)

N=concentration of the aqueous sodium hydroxide solution.

The oil was heated to 70^0 C under constant stirring at 1000 rpm using magnetic stirrer. Known volume of sodium hydroxide that was required to neutralize the oil was added. The mixture was stirred for 60 minutes at 70^0 C. After cooling, the oil was centrifuged at 4000 rpm for 15 minutes to separate the formed soap.

3.4.1.5. Control

The Jatropha oil was kept as control sample and no treatment was given to it.

3.4.2. DETOXIFICATION OF OILCAKE

Oilcake taken was finely ground by using mixer grinder. Oilcake also contains high amount of phorbol esters and hence the powdered oilcake sample was subjected to different methods of detoxification as discussed below.

3.4.2.1. Autoclaving (Moist heat method)

About 100g of the oilcake was weighed accurately. This was put in the autoclavable covers and autoclaved at 121^0 C and 15 psi for 30 minutes. The cake was then dried at 60^0 C to get free flowing powder. The powder was collected and used for further analysis.

3.4.2.2. Soaking with water

Twenty grams of defatted oilcake powder was mixed with water (1:10). After 24 hours it was boiled at 90^0 C for an hour. The excess of water decanted. Powder was dried at 60^0 C to a moisture level of 6-8 per cent w/w to get a free flowing powder.

3.4.2.3. Treatment with sodium bicarbonate

The defatted seed cake (20 g) was thoroughly mixed with 2 per cent sodium bicarbonate solution in 1:2 ratio. The paste was dried at 60^0 C to a moisture level of 6-8 per cent w/w and used for further analysis.

3.4.2.4. Treatment with calcium oxide

Twenty grams of the oilcake was mixed with 20 ml of 2 per cent calcium oxide solution to form a paste using a glass rod. The mixture was then dried at 60^0 C.

3.4.2.5. Hydrochloric acid treatment

Ten grams of finely powdered sample was soaked in 1 per cent hydrochloric acid in 1:10 w/v ratio with intermittent stirring. After 24hours the supernatant was carefully decanted without disturbing the sediments. Then the sample was washed two times with distilled water and dried under the sun.

3.4.2.6. Roasting

Twenty grams of powdered sample was uniformly spread in petri plates and incubated in hot air oven at 200^0 C for 15 minutes, sample was removed from the oven and cooled and used for further analysis.

3.4.2.7. Treatment with sodium hydroxide and sodium hypochlorite

Paste of 20 g of defatted meal was prepared by mixing with 4 per cent sodium hydroxide and 30 ml of sodium hypochlorite. Moisture content of paste was maintained at 66 per cent. The beaker containing the paste was covered with aluminum foil and placed inside autoclavable bags and autoclaved at 121^0 C at 15 psi for 30 minutes. The sample was then cooled and dried. Later the sample was used for further analysis which includes Estimation of phorbol esters, chemical compositon and NPK analysis.

3.4.2.8. Sodium hydroxide treatment followed by washing with distilled water

Twenty grams of defatted powdered meal was taken in a beaker and mixed with 4 per cent sodium hydroxide in a beaker to form a paste. The beaker was covered with aluminum foil and autoclaved at 121^0 C at 15 psi for 30 minutes. The sample was washed with distilled water for three times and dried at 60^0 C to get fine powder.

3.4.2.9. Sodium hydroxide treatment followed by washing with 92 per cent methanol

Twenty grams of defatted powder was taken in a beaker and mixed with 4per cent sodium hydroxide in a beaker to form a paste. The beaker was covered with aluminum foil and autoclaved at 121^0 C at 15 psi for 30 minutes. The sample was washed with 92 per cent methanol four times and the sample was dried at 60^0 C to get fine powder.

3.4.2.10. Control

The untreated defatted cake (20 g) was the control sample for all the treatment.

3.5. Estimation of chemical composition in oilcake

3.5.1. Estimation of residual oil per cent in oilcake (AOAC, 1980)

The amount of oil content in the cake was estimated by using Gerhardt soxtherm apparatus. This works on the principle of solvent extraction process.

Oilcake was finely powdered using mixer grinder and five grams of the sample was taken in the thimble and closed by using cotton. These thimbles were placed in the glass jar which was weighed before along with the boiling stones. Oil was extracted using petroleum ether as

solvent in the soxtherm apparatus. After extraction, the remaining petroleum ether and moisture was removed by keeping it in hot air oven at 110^0 C for 1hour and later kept in the desiccators. The oil content was measured on weight basis and expressed as per cent oil.

$$\text{Oil content} = \frac{W_2 - W_1}{W} \times 100$$

Where,

W- Weight of oilcake (g)

W_1- Weight of jar with boiling stones (g)

W_2- Weight of jar, boiling stones and oil (g).

3.5.2. Estimation of protein in oilcake (AOAC, 1980)

The protein content of the oilcake was estimated as per cent total nitrogen by the Micro-kjeldahl method and then computed by multiplying the per cent nitrogen using conversion factor 6.25. For the digestion of samples the samples Gerhardt Turbotherm digestion unit was used. The distillation was carried out in Gerhardt Vapodest automatically.

$$\text{Protein content} = \text{Nitrogen} \times 6.25.$$

3.5.3. Estimation of ash content in oilcake (AOAC, 1980)

Known weight (1g) of oilcake was taken in a silica crucible, charred at low flame followed by ignition in muffle furnace at 600^0 C for 4 hours. This was followed by desiccation and weighing.

$$\text{Ash content} = \frac{\text{Weight of the ash}}{\text{Weight of sample taken}} \times 100$$

3.5.4. Estimation of crude fiber in oilcake (AOAC, 1980)

Moisture and fat free samples weighing four grams each were taken in glass beakers. Two hundred ml of dilute sulphuric acid of 0.255N was added to the beaker and boiled for 30 minutes. Volume was maintained constant by addition of distilled water. This was followed by filtration. Residue was repeatedly washed with hot distill water and boiled with 0.313N sodium hydroxide for 30 minutes followed by repeated hot water wash. Alcohol and ether washes were given followed by oven drying at 100^0 C for 16 hours and weighed (W_1). Crucible containing fibre was heated in a muffle furnace at 600^0 C for 3 hours, cooled and weighed (W_2).

$$\text{Crude fiber} = \frac{100 - (\text{moisture} + \text{fat}) \times \text{wt of fiber}}{\text{Weight of sample}}$$

3.5.5. Estimation of moisture content in oilcake (AOAC, 1980)

Samples of oilcake weighing ten grams were dried in a thermostatic oven in a closed container at 103^0 C for 17 hours and cooled in desiccators. Dry weight of each sample was recorded and per cent moisture calculated.

$$\text{Moisture content} = \frac{M_2 - M_3}{M_2 - M_1} \times 100$$

Where,

M_1 = Weight of moisture bottle (g)

M_2= Weight of moisture bottle and oilcake before drying (g)

M_3= Weight of moisture bottle and oilcake after drying (g)

3.5.6. Estimation of carbohydrates in oilcake (AOAC, 1980)

Carbohydrates content was calculated by differential method.

Carbohydrate =100- [Ash+ Fat+ Moisture+ Crude fibre+ Protein]

3.6. Estimation of nitrogen, phosphorus & potassium contents in oilcake (Jackson, 1973)

3.6.1. Estimation of nitrogen

Oilcake samples were digested using sulphuric acid and nitrogen was estimated using Gerhardt digestion and distillation units. Samples weighing 0.5g were digested in 25 ml of concentrated sulphuric acid, in presence of 4g each of sodium sulphate and copper sulphate using digestion tubes and digestion system, till colour turned marine green. Volume was made upto 100ml in volumetric flasks with distill water. Ten ml of digested samples was pipette into distillation tubes of distillation unit. Sodium hydroxide (40%) solution and distill water were fed into auto injection system and distilled. Distillate was collected in flasks containing 20 ml of 2 per cent boric acid along with drops of indicator (methyl red and bromocresol green). Distillation was carried out for 5 minutes or there is no effect of distillate on ph paper. Colour of distillate in EM flasks turned from reddish pink to green. Estimation of nitrogen in the distillate was done by titrating against 0.1N hydrochloric acid, along with the blank.

$$\text{Per cent nitrogen (per cent)} = (V_a - V_b) \times 0.0014 \times \frac{V_1}{V_2} \times \frac{100}{W}$$

Where:

Va = Titre value of sample

Vb = Titre value of blank

V_1 = Volume to which digested sample was made up (100 ml)

V_2 = Volume to aliquot used in distillation

W = Weight of samples taken for digestion

3.6.2. Sample digestion for estimation of phosphorus and potassium

Powdered oilcake samples weighing 0.5g were partially digestion using concentrated nitric acid followed by di acid mixture digestion of

concentrated nitric acid and hydrochloric acid 9:4(v/v). Volume was made up to 100ml.

3.6.2.1. Estimation of phosphorous

5 ml of digested sample was mixed with vanadamolybdate reagent and the volume was made up to 50 ml. Later the colour intensity was read at 450 nanometer after 30 minutes in spectrophotometer. Unknown sample absorbance was compared with standard curve to know the total phosphorus present in the oilcake (Jackson, 1973).

3.6.2.2. Estimation of potassium

The digested sample solution was feed to the flame photometer and the readings were recorded. Unknown sample absorbance was compared with standard curve to know the total potassium present in the oilcake (Jackson, 1973).

$$K = \frac{\text{Graph ppm} \times \text{volume of digested sample} \times \text{volume made up}}{10^6 \times \text{weight of sample} \times \text{aliquot taken for dilution}}$$

3.7. DETOXIFICATION OF BIODIESEL

The bulk Jatropha biodiesel that was collected was used for further detoxification study. Neutralizing, bleaching, methanol and hot water treatments was carried out in replications to reduce the phorbol esters content. The methodology carried out is described below.

3.7.1. Neutralization by caustic treatment

Density of biodiesel was calculated followed by determination of free fatty acid. Considering these two factors the amount of sodium hydroxide solution required to neutralize the free fatty acids was calculated.

3.7.2. Bleaching

10ml of biodiesel was stirred at 100rpm in a beaker using magnetic stirrer at 80^0 C for 15 minutes. 2 per cent of activated charcoal which is a bleaching agent was added to the biodiesel. Again the mixture was stirred for 15 minutes. The mixture was separated by centrifugation. The remaining activated charcoal was separated by filtration. The bleached biodiesel was collected and used for further analysis.

3.7.3. Methanol treatment

50ml of the biodiesel was extracted using 50 ml of methanol and it was stirred at 1000 rpm for 15 minutes. Later the mixture was separated by filtration and the biodiesel was collected and used for further analysis.

3.7.4. Hot water treatment

20ml of biodiesel was mixed with water in 1:10 ratio for 24 hours. After 24 hours it was heated for an hour. The excess of water was decanted. Water treated biodiesel was dried at 100^0 C to a remove the remaining moisture level.

3.7.5. Control

The Jatropha biodiesel was taken as control sample and no treatment was given.

3.8. Statistical analysis

The following statistical tools were used in the analysis and interpretation of data. The results were analyzed using simple Completely Randomized Design (CRD) ANOVA. Correlation co-efficient was done to oil, yield and phorbol esters.

Experimental Results

IV. EXPERIMENTAL RESULTS

Results of the experiments conducted to know the effects of the detoxification treatments on phorbol ester contents in oil, oilcake and biodiesel of J*atropha curcas* maintained at GKVK, UAS, Bengaluru are presented in this chapter. The details of the different places from where the accessions were collected are presented in Table 1.

The survey conducted previously indicated that the plant population is fairly high along the roadside as a hedge plant and also in the waste or degraded lands which are near to the river bank. Most of the plants found are the natural ones which are used in this study.

4.1. Seed Yield in *Jatropha curcas* L.

The yield projection was the mean values of each accessions for one season. The above study was carried out to the samples collected from twenty different places and grown in GKVK farm (accessions). The results of the study are presented in the Table 2 and Fig. 1.

The yield ranged from 51-458 grams. The highest yield was recorded from the sample that was collected from Arasikere, Hassan district with 457.5g per plant and the lowest yield was obtained from the *J.curcas* sample that was collected from TERI-I with 51.1g per plant. Large variation was observed in the yield among the accessions.

4.2. Oil content in *Jatropha curcas* L. seeds

The oil content of *Jatropha curcas* L. seeds was determined using soxhlet extraction method. Various seed samples were collected from Southern Karnataka and also from TERI was analyzed for oil content using petroleum ether as solvent. The data obtained are presented in Table 3 and Fig. 2.

Table 1 : Collection locations of different accessions of *Jatropha curcas* L.

Sl. No.	Accessions
1.	Doddaballapur, Bangalore (R_2 L_2).
2.	GVKV-I, Bangalore (R_2 C_{14}).
3.	TERI-1 (R_3 V_1).
4.	GKVK –II, Bangalore (R_2 C_3).
5.	Kollegal, Chamrajnagar (R_1 B_2 L_{19}).
6.	Sakkarayapatna, Chikkamangalore (R_2 B_7 L_{26}).
7.	GKVK-III, Bangalore (R_1 C_{13}).
8.	Lakkihalliform, Tiptur (R_2 B_5 L_{11}).
9.	TERI-II (V_2).
10.	Arasikere, Hassan (R_2 B_5 L_{14}).
11.	Chikkamangalure (R_2 B_6 L_{18}).
12.	Gandasi, Hassan (R_2 B_8 L_5).
13.	Gijihali, Hassan (R_2 B_7 L_{17}).
14.	Mallavalli, Mandya (R_1 B_2 L_3).
15.	K.B cross, Tumkur (R_2 B_7 L_1).
16.	Nonavinakere, Tiptur (R_2 B_5 L_{25}).
17.	GKVK-IV, Bangalore (R_2 C_{17}).
18.	Shivarampura , Maddur (R_2 B_8 L_{14}).
19.	BAIF, Tumkur (R_1 B_1 L_{13}).
20.	Magadikaimara, Chikkamangalore (R_1 B_4 L_{16}).

Table 2 : Seed yield/plant in *Jatropha curcas* L.

Sl. No.	Accessions	Seed yield/plant (g)
1.	Doddaballapur, Bangalore.	98.00
2.	GVKV-I, Bangalore.	78.00
3.	TERI-1.	51.10
4.	GKVK –II, Bangalore.	56.00
5.	Kollegal, Chamrajnagar.	299.5
6.	Sakkarayapatna, Chikkamangalore	165.0
7.	GKVK-III, Bangalore.	157.0
8.	Lakkihalliform, Tiptur.	318.3
9.	TERI-II.	55.10
10.	Arasikere, Hassan.	457.5
11.	Chikkamangalure.	297.5
12.	Gandasi, Hassan.	228.0
13.	Gijihali, Hassan.	335.0
14.	Mallavalli, Mandya	218.0
15.	K.B cross, Tumkur.	85.00
16.	Nonavinakere, Tiptur.	148.0
17.	GKVK-IV, Bangalore.	90.00
18.	Shivarampura , Maddur.	312.5
19.	BAIF, Tumkur.	215.0
20.	Magadikaimara, Chikkamangalore.	260.0

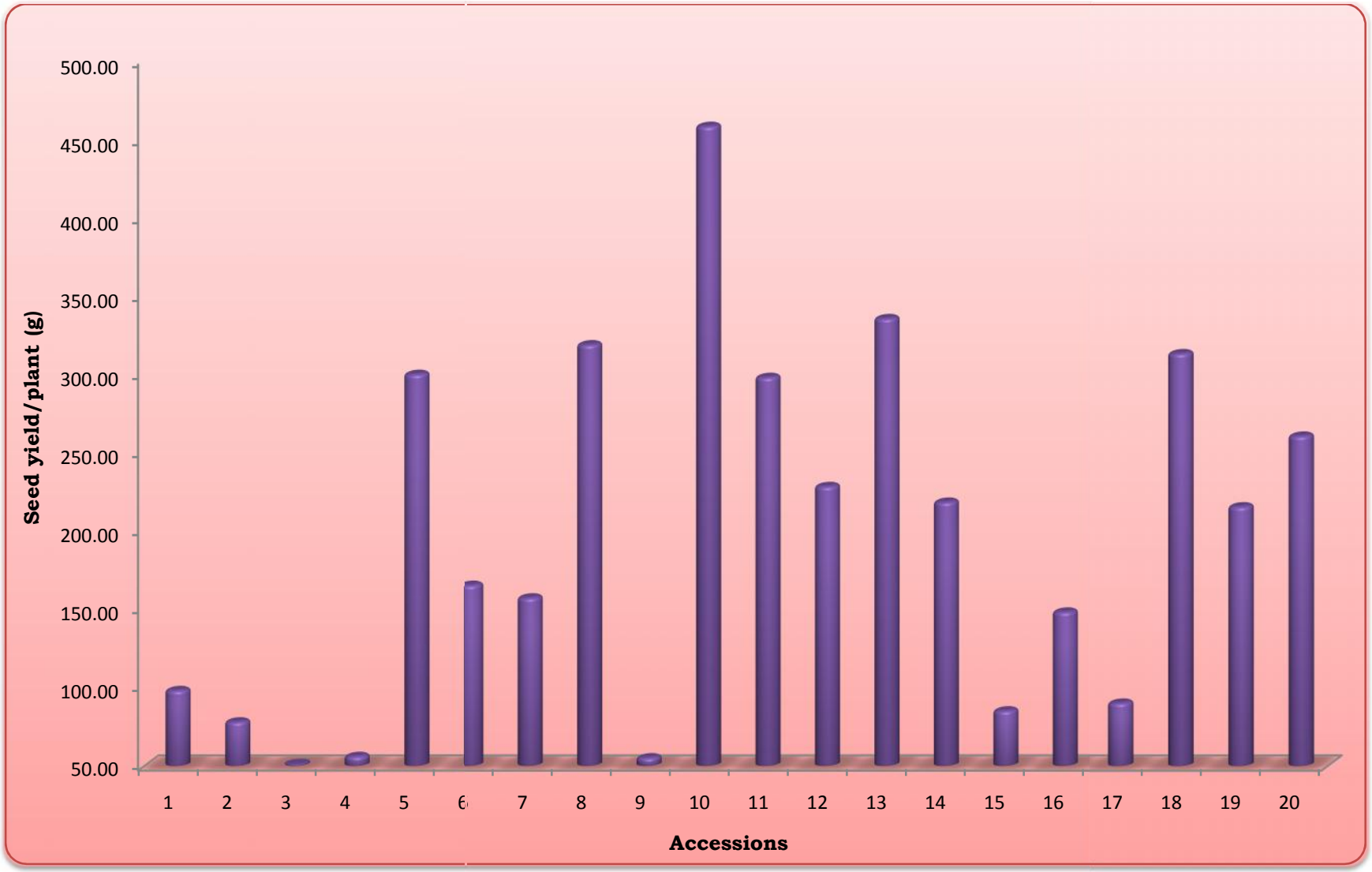

Fig. 1 : Seed yield/plant in selected *Jatropha curcas* L. accessions

The percentage of oil was found to be in the range of 21- 40 with an average value of 33.18. The maximum oil yield was recorded in the seeds collected from Magadikaimara from Chikkamangalore district which is 39.99 per cent. The least oil yield 21 per cent was recorded from the seeds collected from Doddaballapur. Three accessions were collected from GKVK had comparatively lower oil per cent than other places (27.3%, 29.8% and 31.1%). There was a significant variation in the oil contents in the seeds that was collected from different places at 5 per cent.

4.3. Concentration of phorbol esters in the seeds of *Jatropha curcas* L.

The main objective of the present study was to extract the phorbol esters from the seeds of *Jatropha curcas* L. Therefore the seeds were subjected to extract the phorbol esters using methanol. The methanol has greater affinity towards phorbol esters and is a polar solvent that was used for the extraction where the amount presents was quantified using High Performance Liquid Chromatography (HPLC). The results obtained are mentioned in the Table 4 and Fig. 3.

The results indicated that the phorbol esters in seed collected from different places were found in the range of 0.02-3.97 mg/g with an average value 0.91mg/g. The seeds of accession collected from GKVK-IV was found to have the lowest phorbol esters which was 0.02 mg/g whereas the accession collected from GKVK-II had the highest phorbol esters which was 3.97 mg/g. Chromatograms are presented in Fig.4. Statistical analysis showed that there was a significant difference among estimated values of phorbol esters of different accessions.

Table 3 : Oil content in the seeds of *Jatropha curcas* L.

Sl. No.	Accessions	Oil content (%)
1.	Doddaballapur, Bangalore.	21.00
2.	GVKV-I, Bangalore.	27.30
3.	TERI-1.	28.60
4.	GKVK –II, Bangalore.	29.80
5.	Kollegal, Chamrajnagar.	30.40
6.	Sakkarayapatna, Chikkamangalore .	30.97
7.	GKVK-III, Bangalore.	31.10
8.	Lakkihalliform, Tiptur.	31.30
9.	TERI-II	32.00
10.	Arasikere, Hassan.	32.39
11.	Chikkamangalure.	34.29
12.	Gandasi, Hassan.	35.70
13.	Gijihali, Hassan.	36.14
14.	Mallavalli, Mandya	36.40
15.	K.B cross, Tumkur.	36.60
16.	Nonavinakere, Tiptur.	36.80
17.	GKVK-IV, Bangalore.	37.49
18.	Shivarampura , Maddur.	37.55
19.	BAIF, Tumkur.	38.00
20.	Magadikaimara, Chikkamangalore.	39.98
	F	*
	CV(%)	2.53
	S.Em $\pm$	0.48
	CD at 5%	1.38

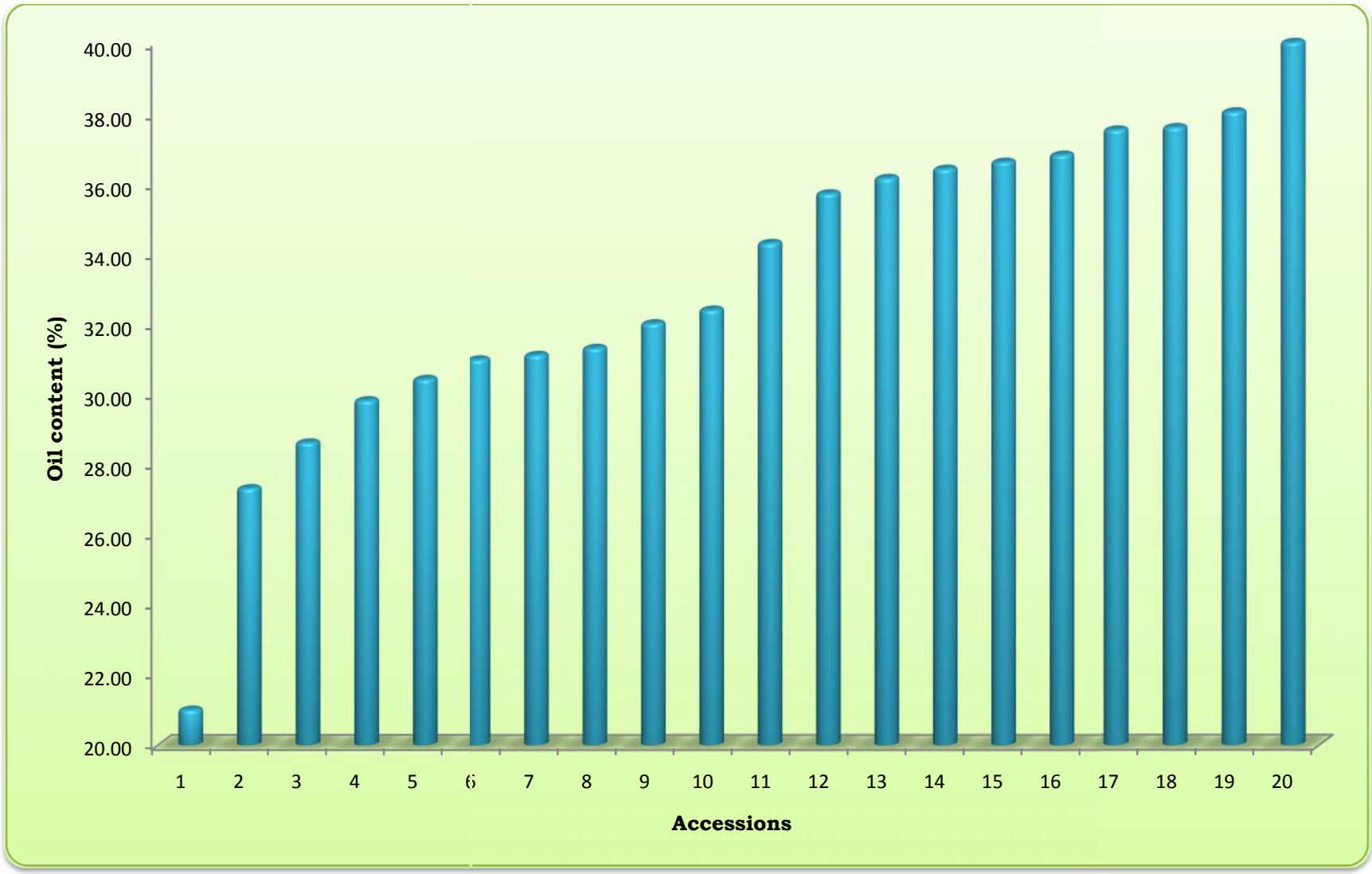

Fig. 2 : Oil content in selected *Jatropha curcas* L. accessions

Table 4 : Phorbol esters in the seeds of *Jatropha curcas* L.

Sl. No.	Accessions	Phorbol esters (mg/g)
1.	Doddaballapur, Bangalore.	0.94
2.	GVKV-I, Bangalore.	0.89
3.	TERI-1.	0.06
4.	GKVK –II, Bangalore.	3.97
5.	Kollegal, Chamrajnagar.	0.06
6.	Sakkarayapatna, Chikkamangalore	1.22
7.	GKVK-III,Bangalore.	2.88
8.	Lakkihalliform, Tiptur.	1.23
9.	GKVK-III, Bangalore.	2.19
10.	Arasikere, Hassan.	0.03
11.	Chikkamangalure.	0.26
12.	Gandasi, Hassan.	0.10
13.	Gijihali, Hassan.	1.39
14.	Mallavalli, Mandya	0.30
15.	K.B cross, Tumkur.	0.78
16.	Nonavinakere, Tiptur.	0.23
17.	GKVK-IV, Bangalore.	0.02
18.	Shivarampura , Maddur.	0.91
19.	BAIF, Tumkur.	0.81
20.	Magadikaimara, Chikkamangalore.	0.05
	F	*
	CV(%)	1.18
	S.Em $\pm$	0.01
	CD at 5%	0.02

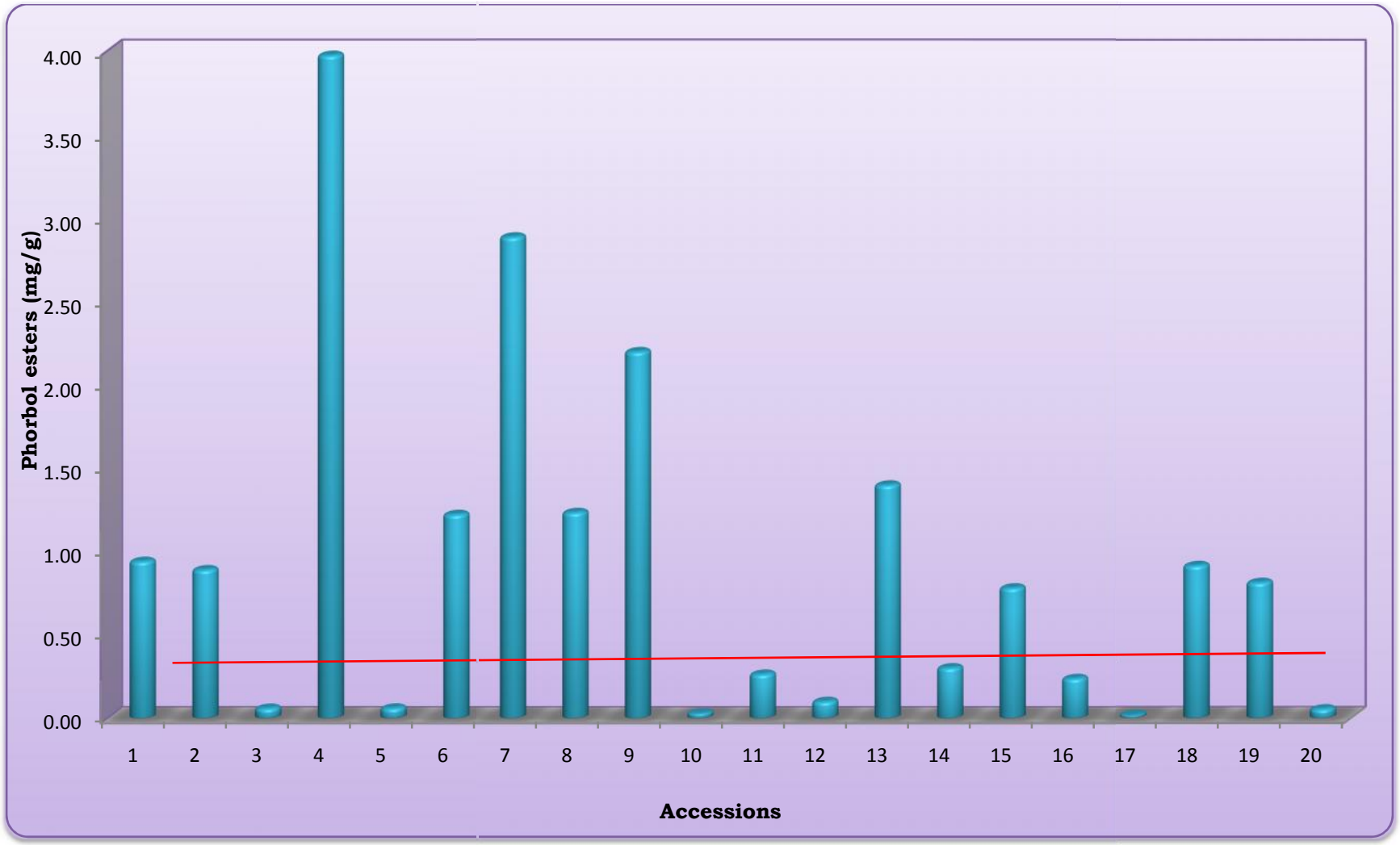

Fig. 3 : Phorbol esters in selected *Jatropha curcas* L. accessions

Note: The values that are below the red line are the non-toxic accessions

Fig. 4 : Chromatogram of phorbol esters extracted from seed samples of selected *Jatropha curcas* L. accessions

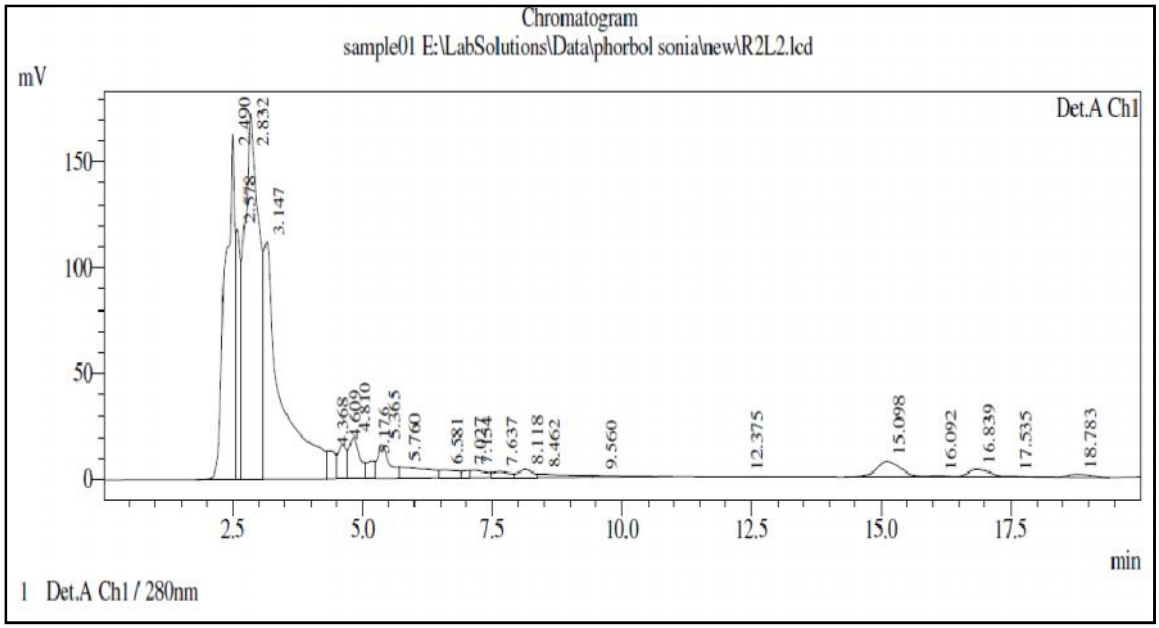

Fig. 4a : Chromatogram of phorbol esters extracted from R_2L_2 accession

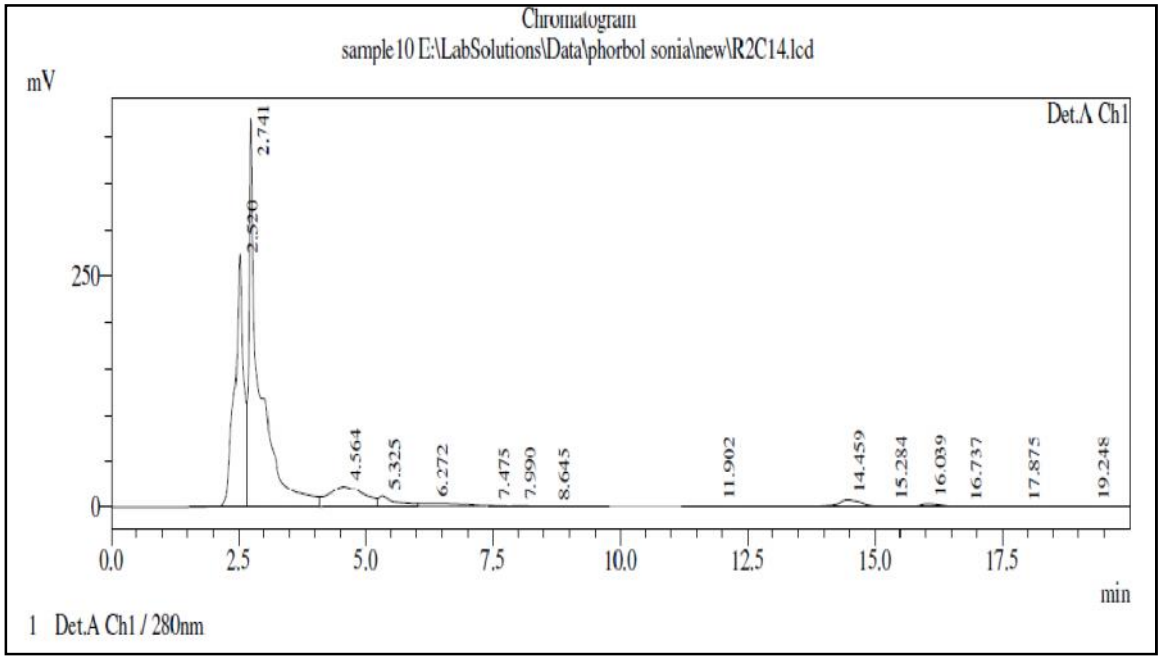

Fig. 4b : Chromatogram of phorbol esters extracted from R_2C_{14} accession

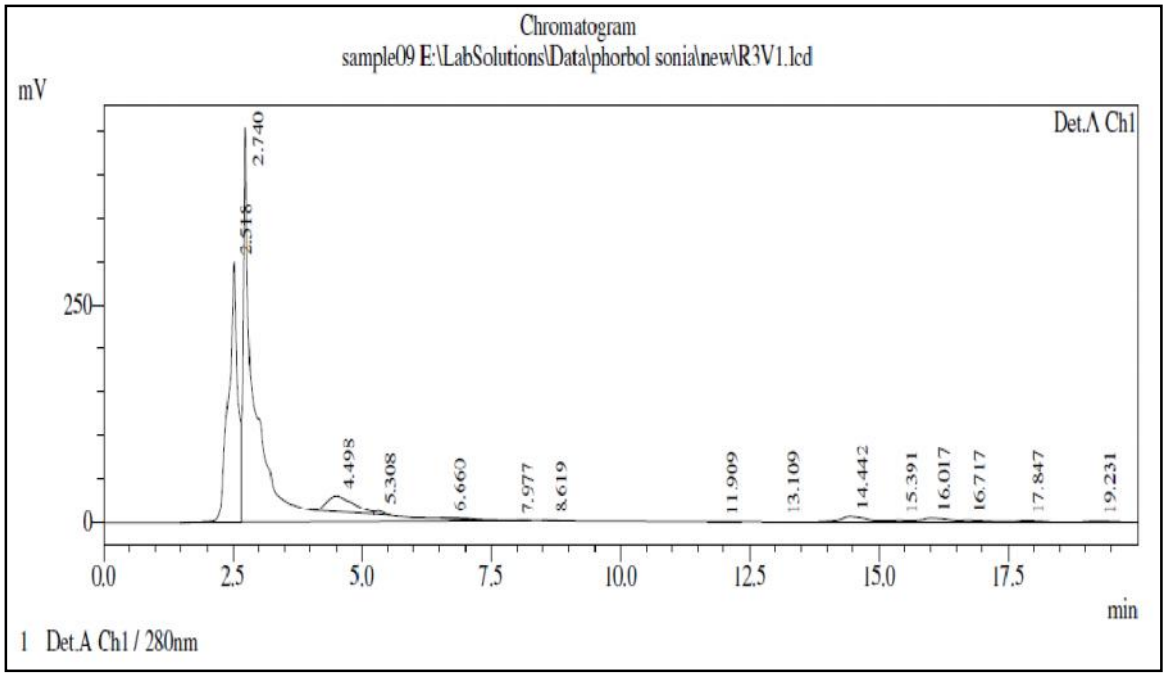

Fig. 4c : Chromatogram of phorbol esters extracted from R_3V_1 accession

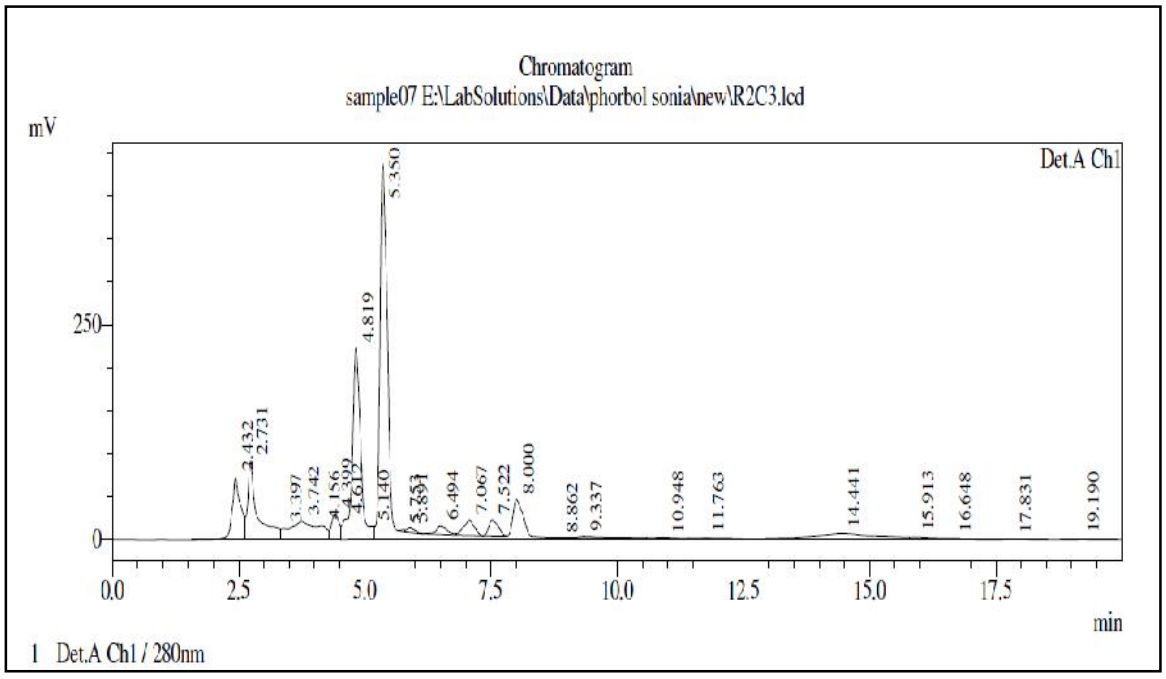

Fig. 4d : Chromatogram of phorbol esters extracted from R_2C_3 accession

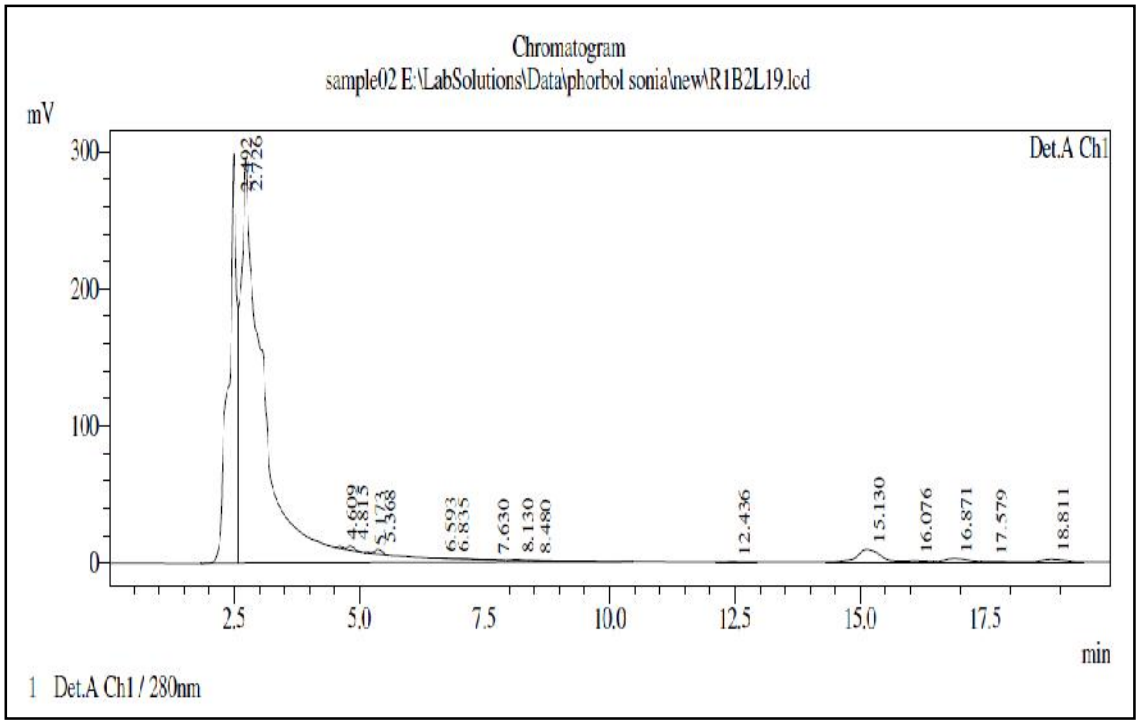

Fig. 4e : Chromatogram of phorbol esters extracted from $R_1B_2L_{19}$ accession

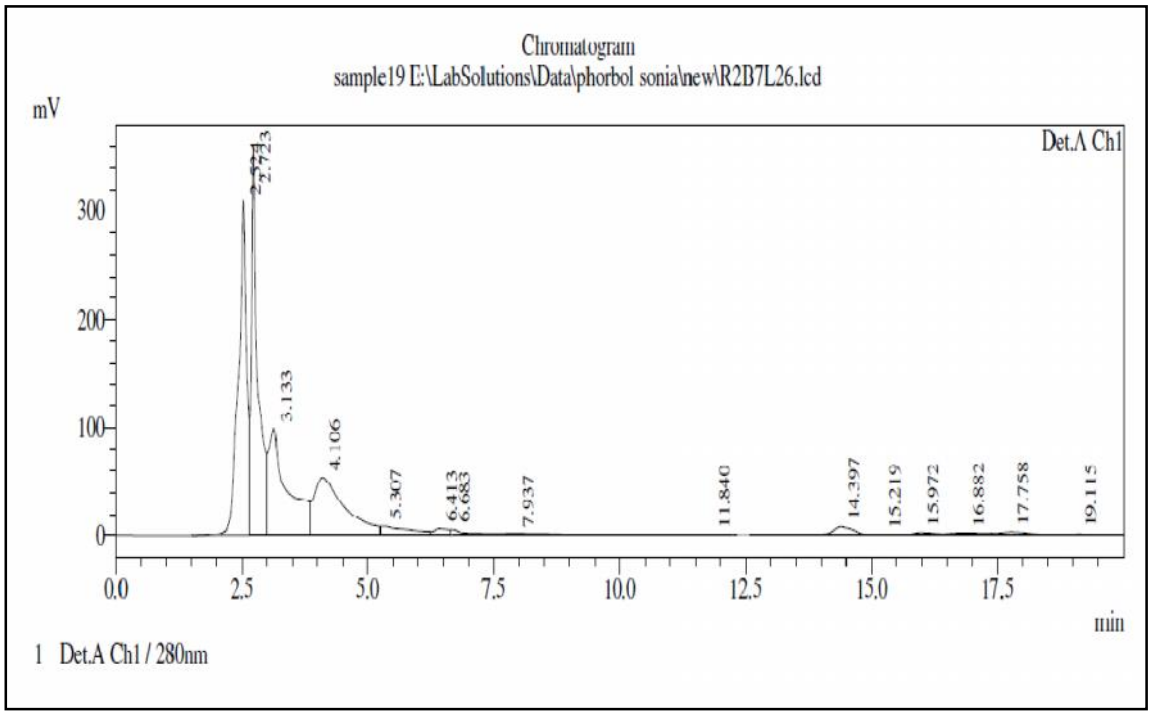

Fig. 4f : Chromatogram of phorbol esters extracted from $R_1B_7L_{26}$ accession

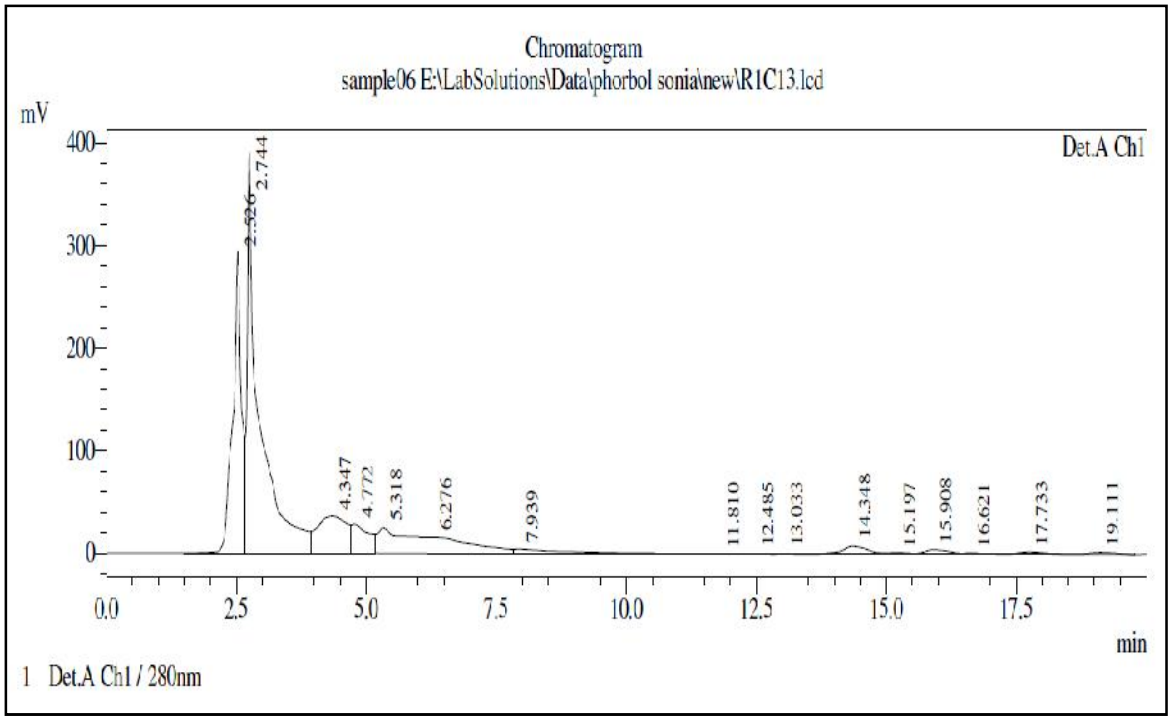

Fig. 4g: Chromatogram of phorbol esters extracted from R_1C_{13} accession

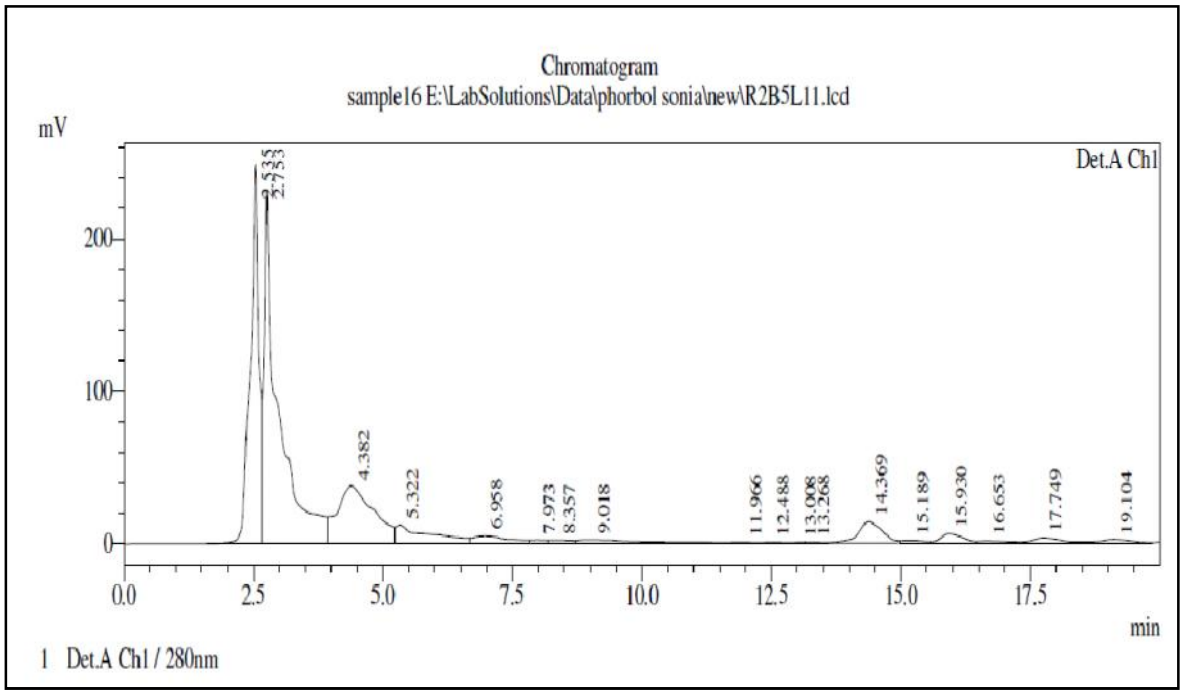

Fig. 4h: Chromatogram of phorbol esters extracted from $R_2B_5L_{11}$ accession

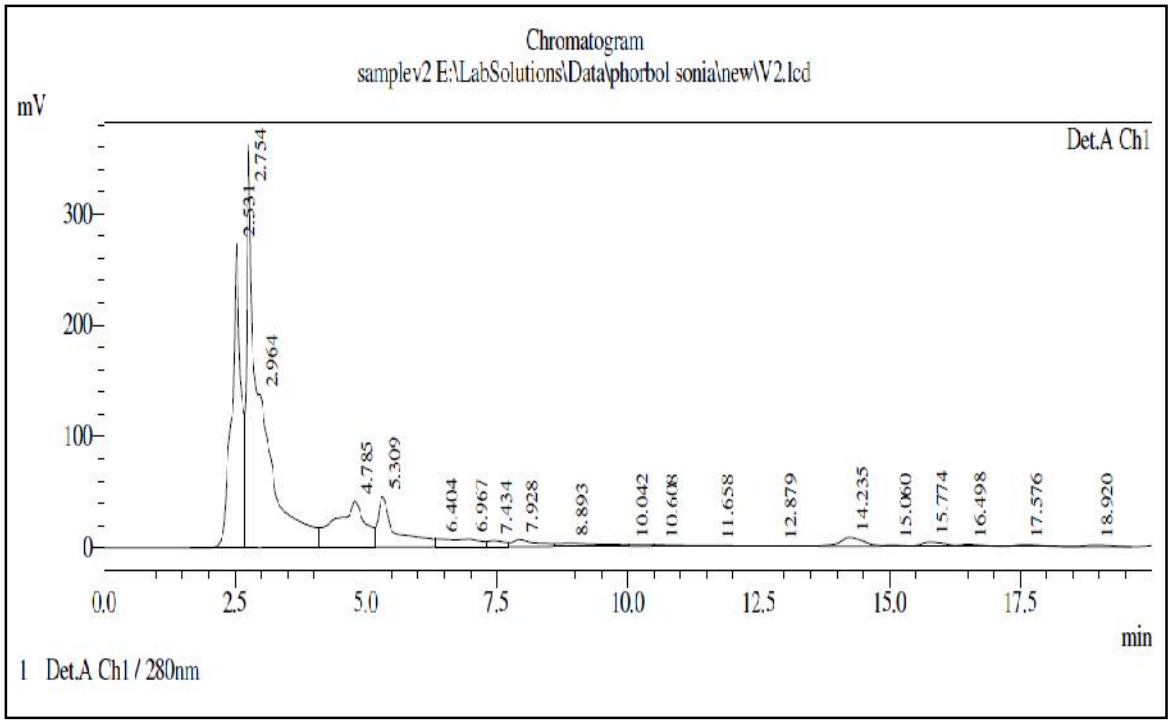

Fig. 4i : Chromatogram of phorbol esters extracted from V_2 accession

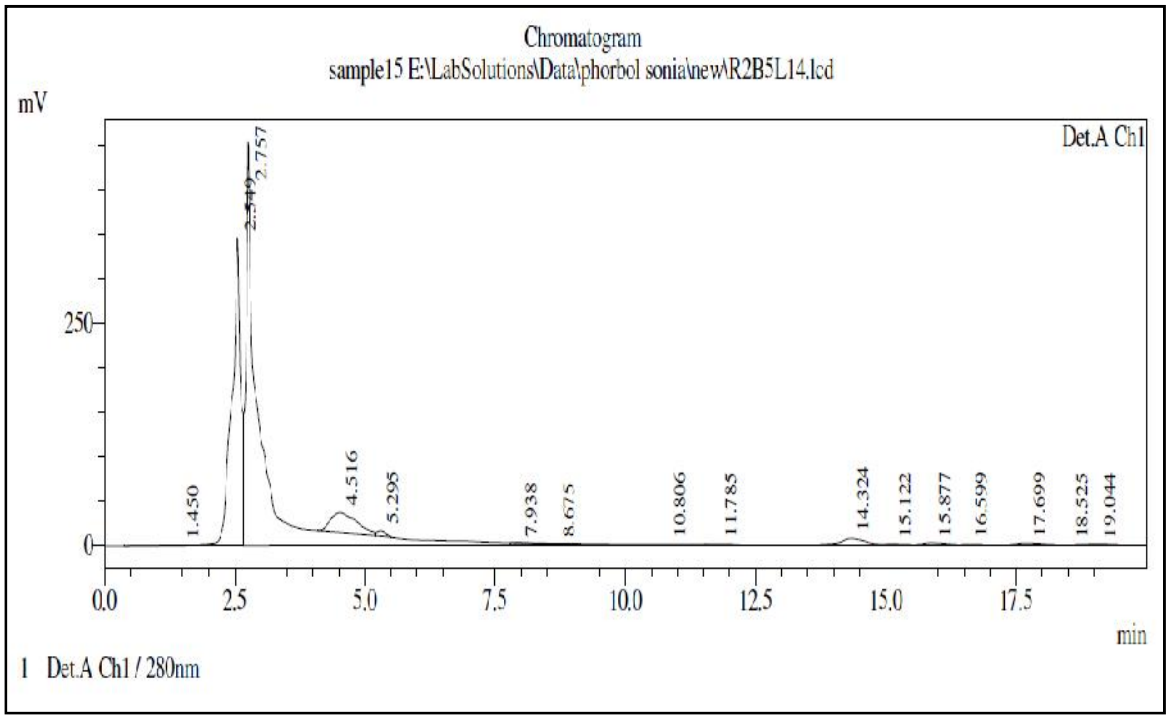

Fig. 4j : Chromatogram of phorbol esters extracted from $R_2B_5L_{14}$ accession

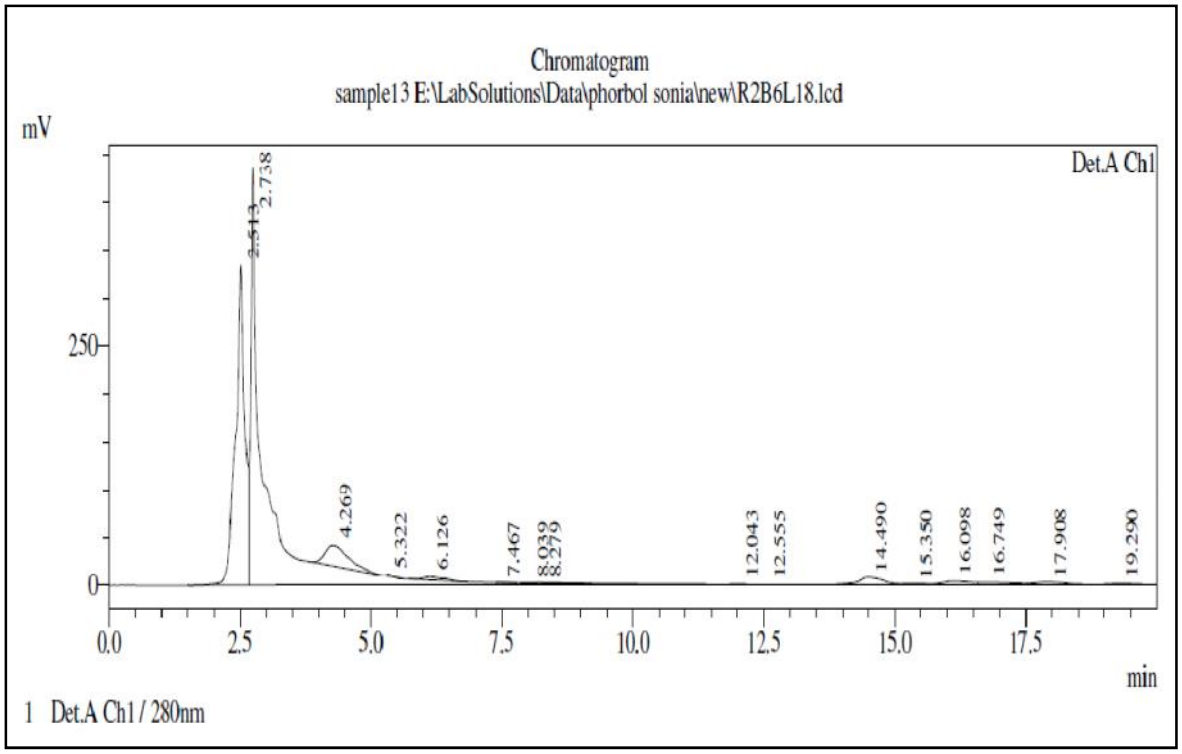

Fig. 4k : Chromatogram of phorbol esters extracted from $R_2B_6L_{18}$ accession

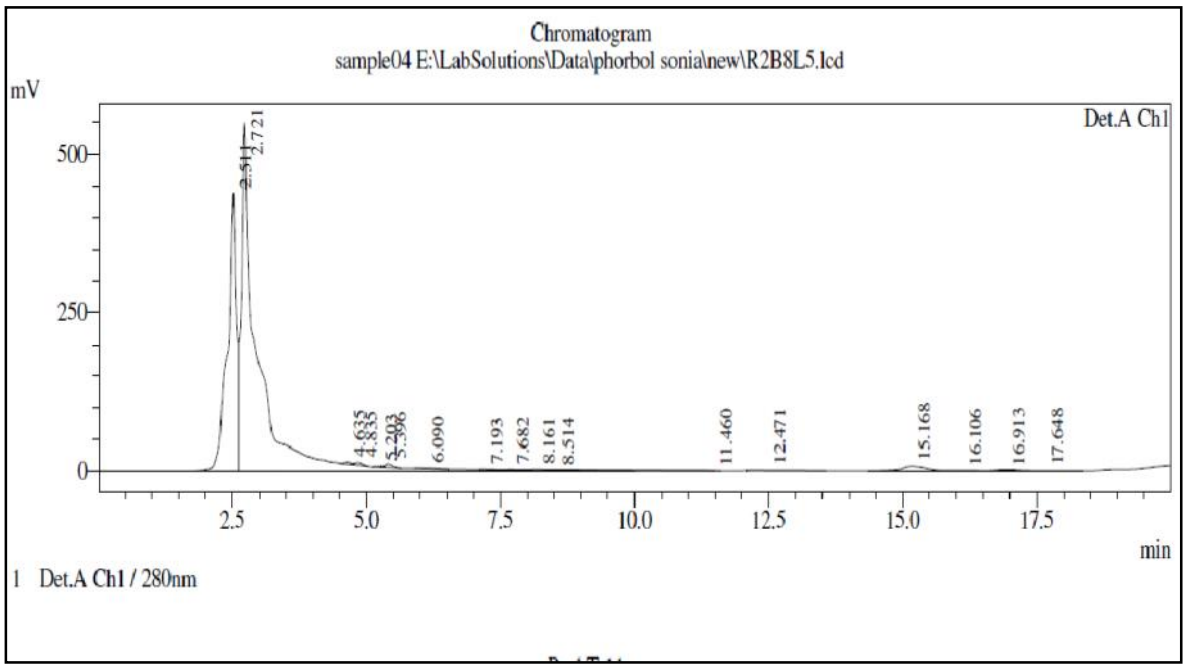

Fig. 4l : Chromatogram of phorbol esters extracted from $R_2B_8L_5$ accession

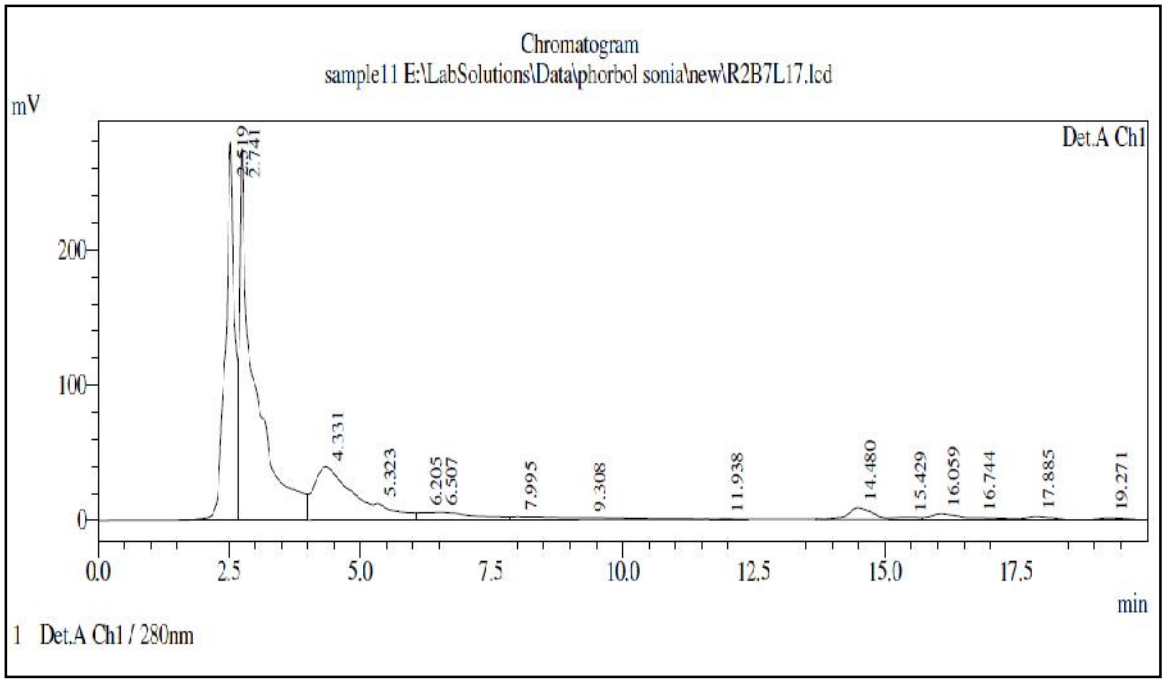

Fig. 4m : Chromatogram of phorbol esters extracted from $R_2B_7L_{17}$ accession

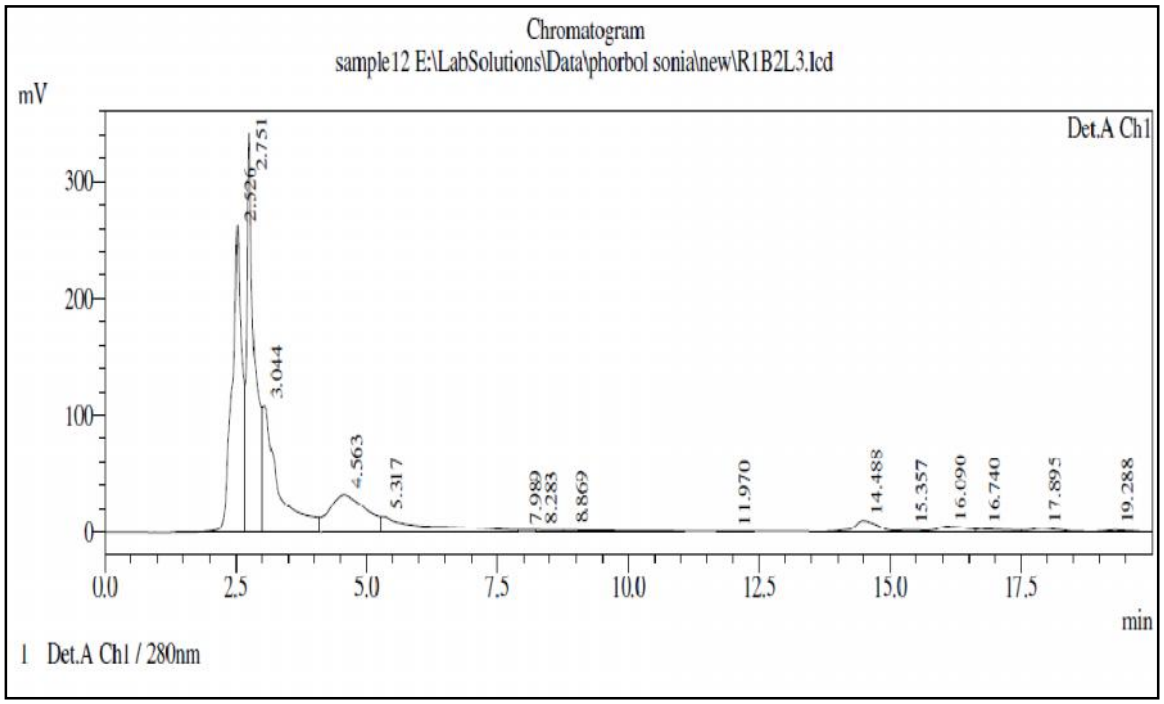

Fig. 4n : Chromatogram of phorbol esters extracted from $R_1B_2L_3$ accession

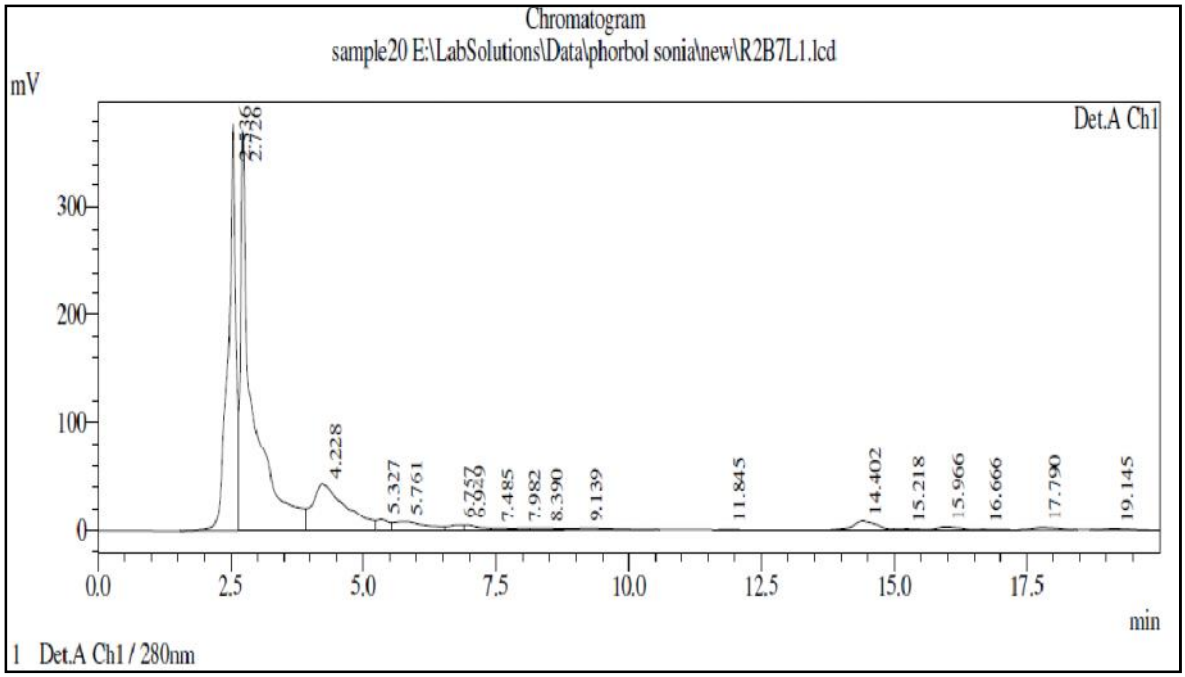

Fig. 4o : Chromatogram of phorbol esters extracted from $R_1B_7L_1$ accession

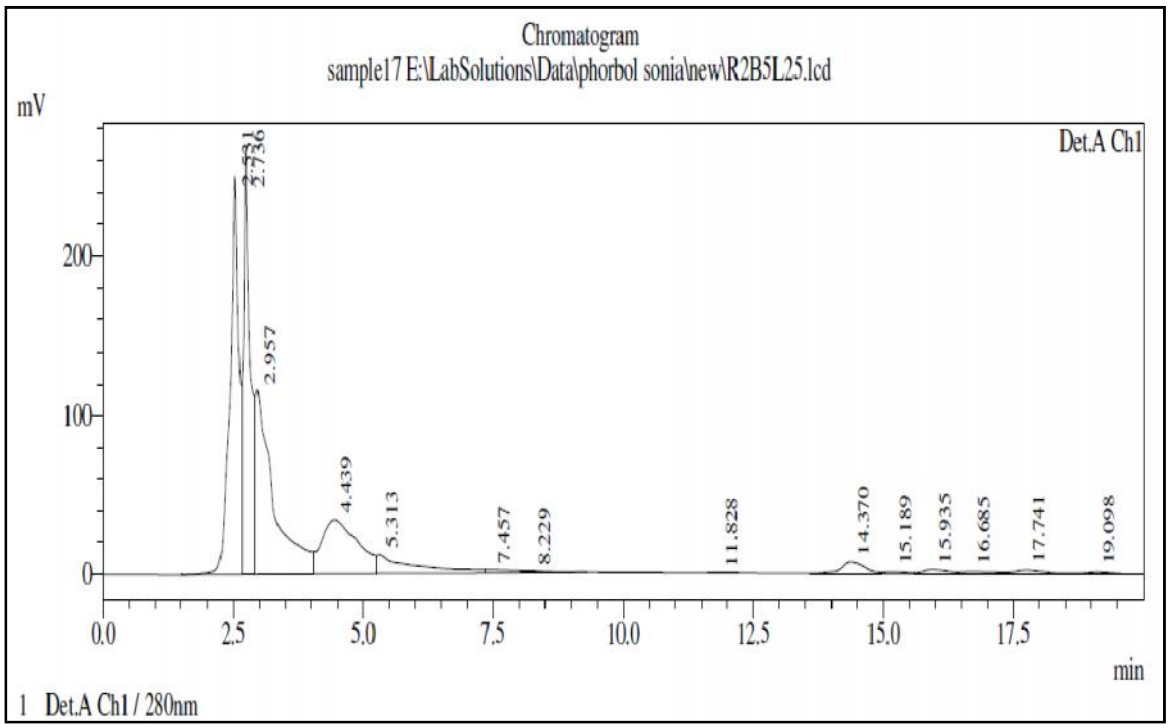

Fig. 4p : Chromatogram of phorbol esters extracted from $R_2B_5L_{25}$ accession

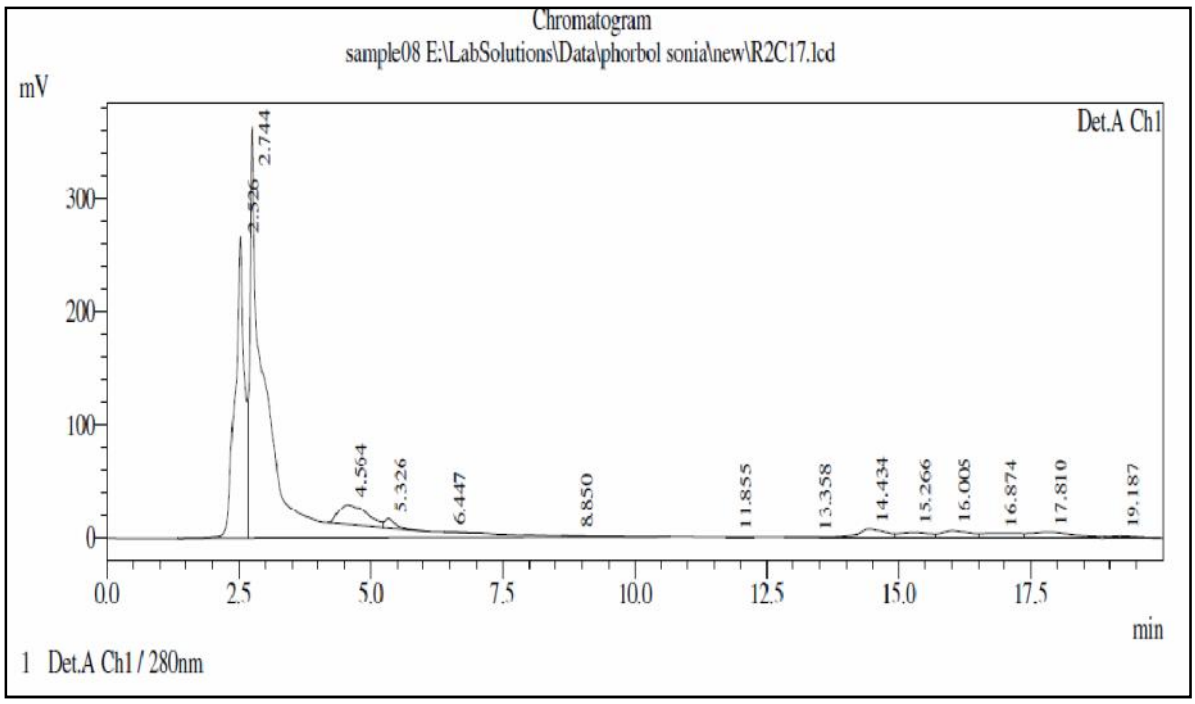

Fig. 4q : Chromatogram of phorbol esters extracted from R_2C_{17} accession

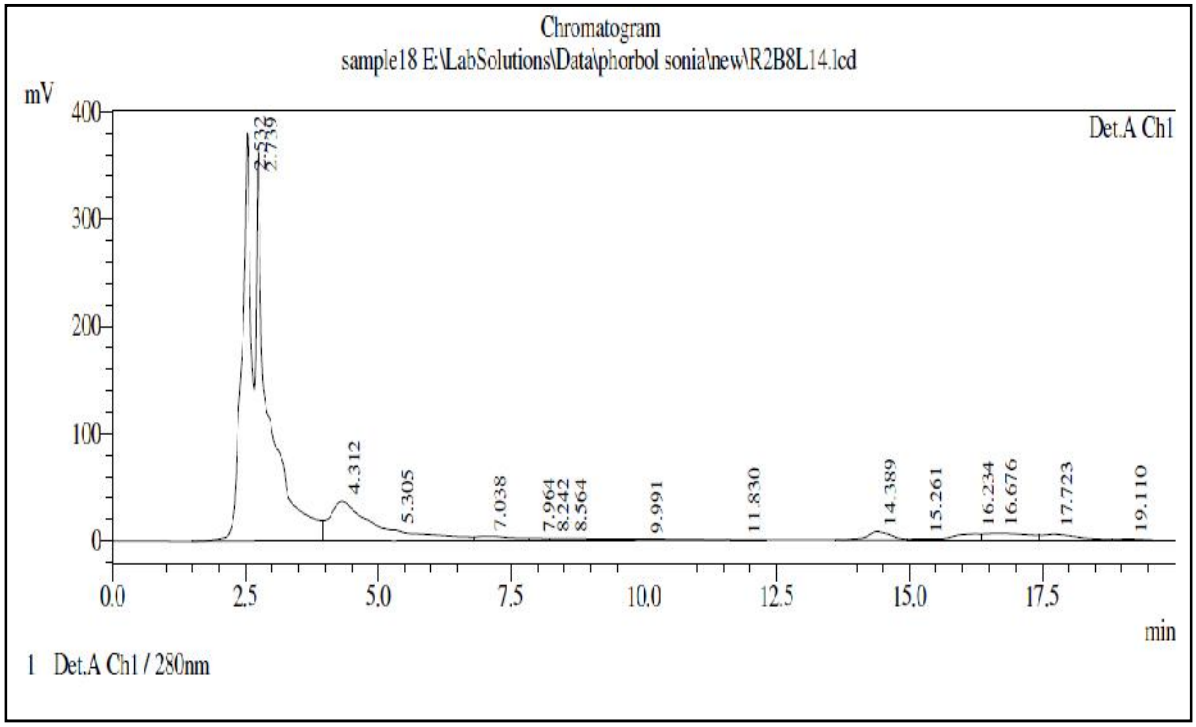

Fig. 4r : Chromatogram of phorbol esters extracted from $R_2B_8L_{14}$ accession

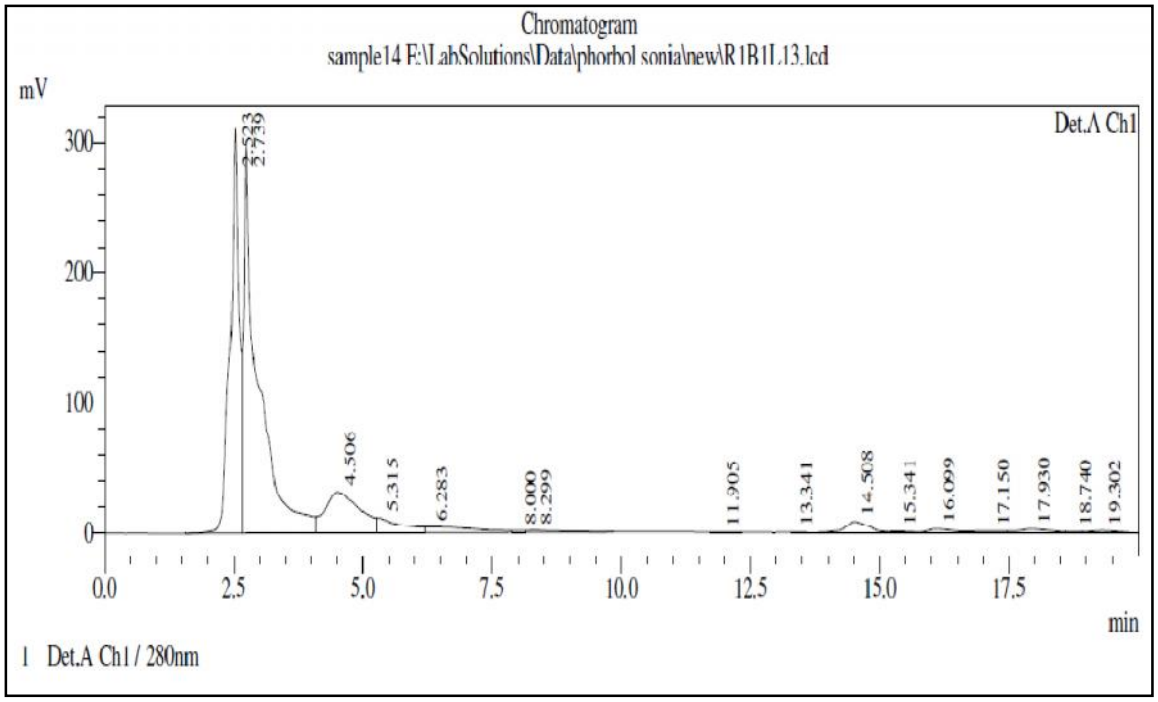

Fig. 4s : Chromatogram of phorbol esters extracted from $R_1B_1L_{13}$ accession

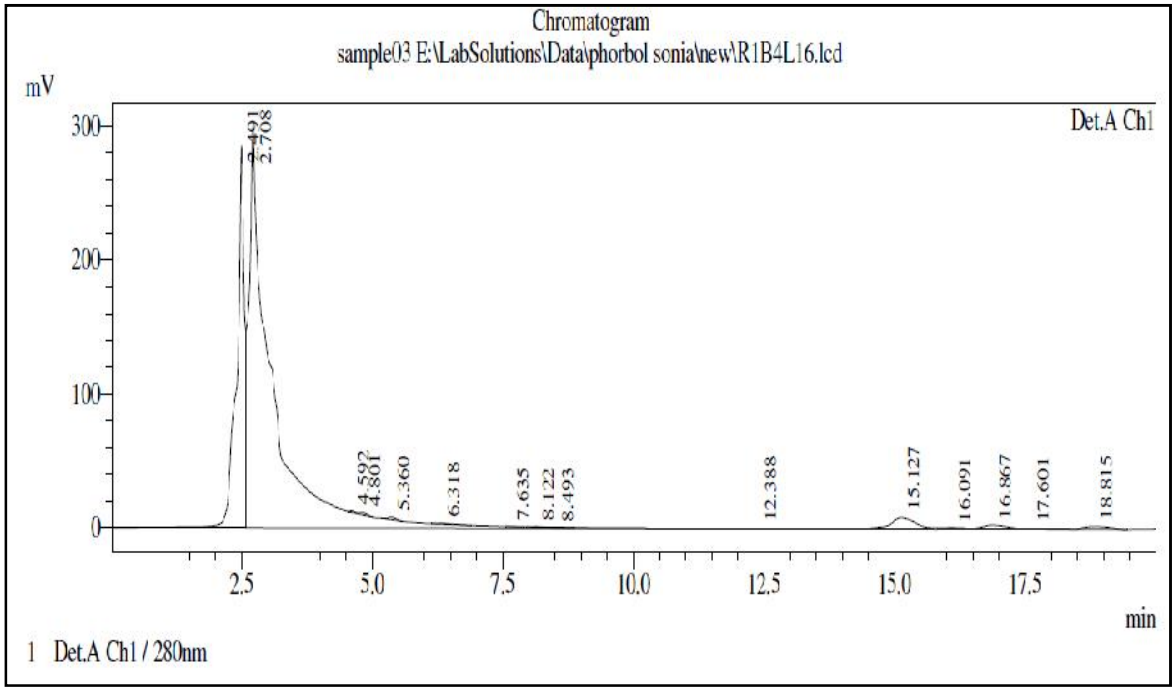

Fig. 4t : Chromatogram of phorbol esters extracted from $R_1B_4L_{16}$ accession

4.4. Correlation between oil content, seed yield per plant and phorbol esters in J*atropha curcas* L.

Correlation between oil content (%), seed yield (g) and phorbol esters (mg/g) is presented in the Table 5 and Fig. 5-7. Results obtained revealed that there was a positive correlation between the oil content and the yield in the selected 20 accessions. But they did not vary significantly.

But the correlation co-efficient between phorbol esters +seed yield and phorbol esters + oil content in selected accession showed a negative correlation and the data showed that they were non significant.

4.5. Treatment effects on phorbol esters

The high concentration of phorbol esters present in Jatropha seeds has been identified as the main toxic agent responsible for *J.curcas* toxicity. Hence, it is necessary to find feasible means for detoxification. The oil, meal and biodiesel was subjected to different treatments to detoxify the phorbol esters and the results are presented below.

4.6. Detoxification of *Jatropha curcas* L. oil

The effect of several types of refining process on the content of phorbol esters in the *Jatropha curcas* seed oil was quite varying and data presented in Table 6 and Fig. 8. The content of phorbol ester in the untreated oil was 3.08 mg/g. The influence of degumming was very low whereas the treatment with methanol leads to significant reduction of phorbol esters.

By the process of degumming this could be reduced to only 2.36 mg/g. Treated degummed oil was further neutralized with sodium hydroxide or potassium hydroxide which lead to soap formation and hence neutralizing could not be used as refining process. Methanol

Table 5 : Correlation of oil content, seed yield/plant and phorbol esters in *Jatropha curcas L.*

Parameters correlated	Correlation coefficient (r)
Oil content (%)-Seed yield per plant (g)	0.33^{NS}
Phorbol esters (mg/g)- Seed yield per plant (g)	-0.33^{NS}
Phorbol esters (mg/g) - Oil content (%)	-0.29^{NS}

NS-Not Significant

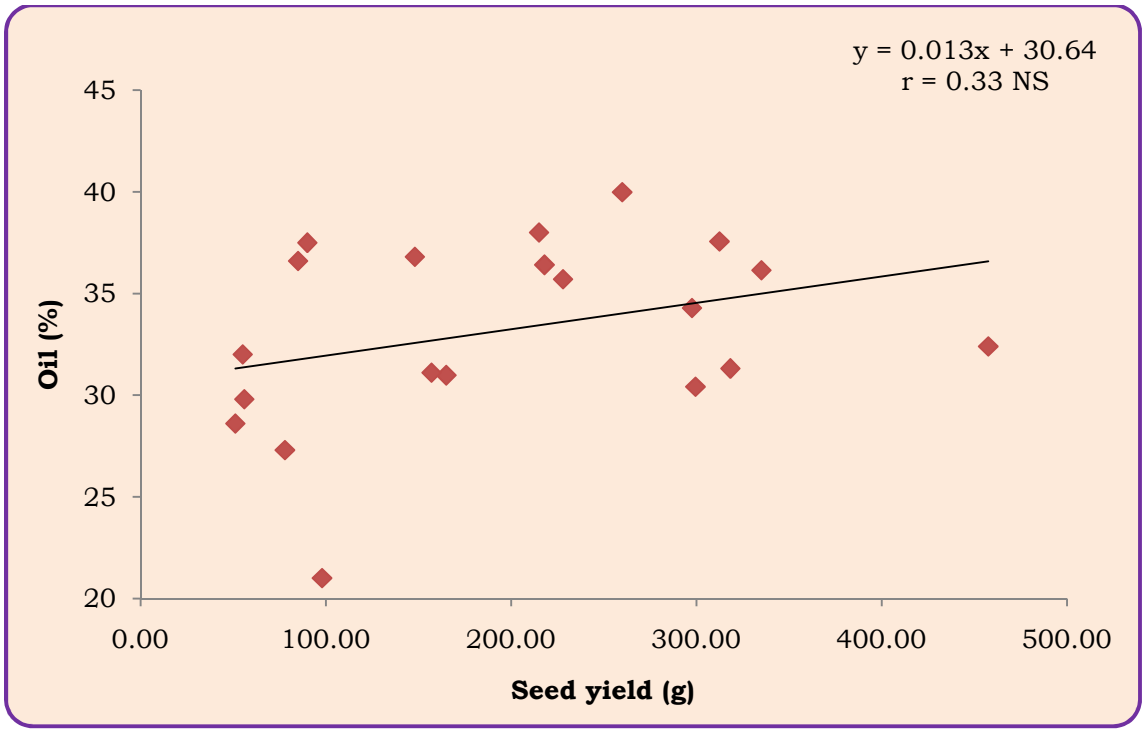

Fig. 5 : Correlation between oil and seed yield

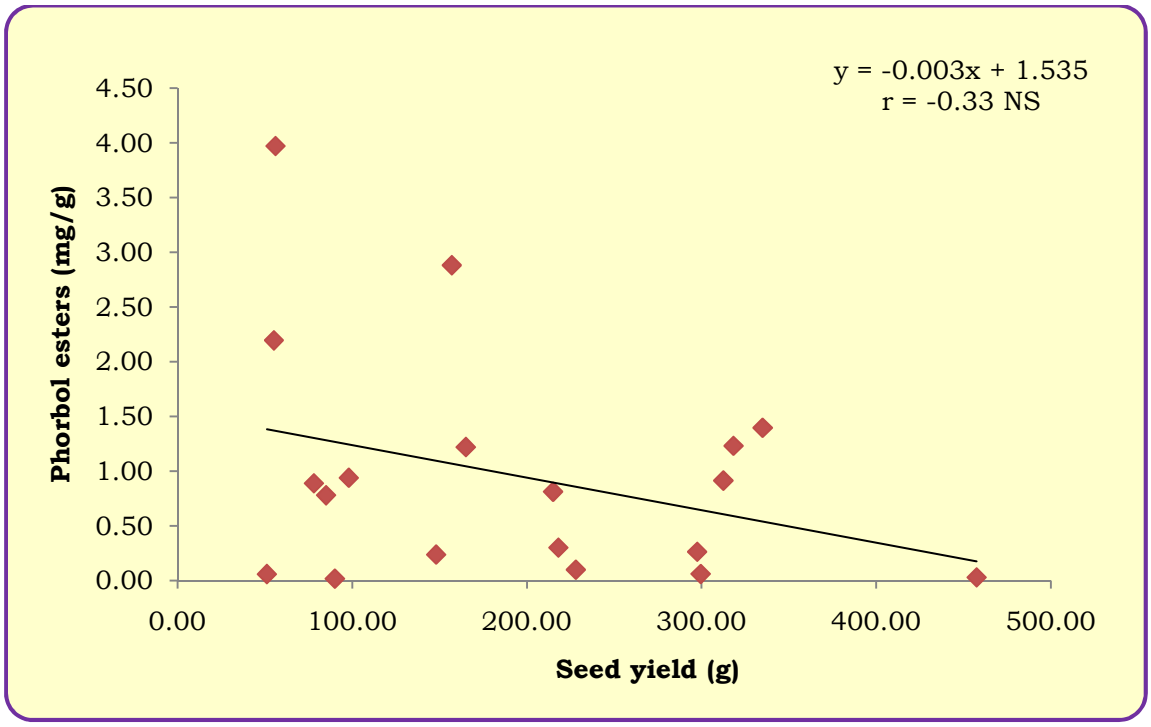

Fig. 6 : Correlation between phorbol esters and yield

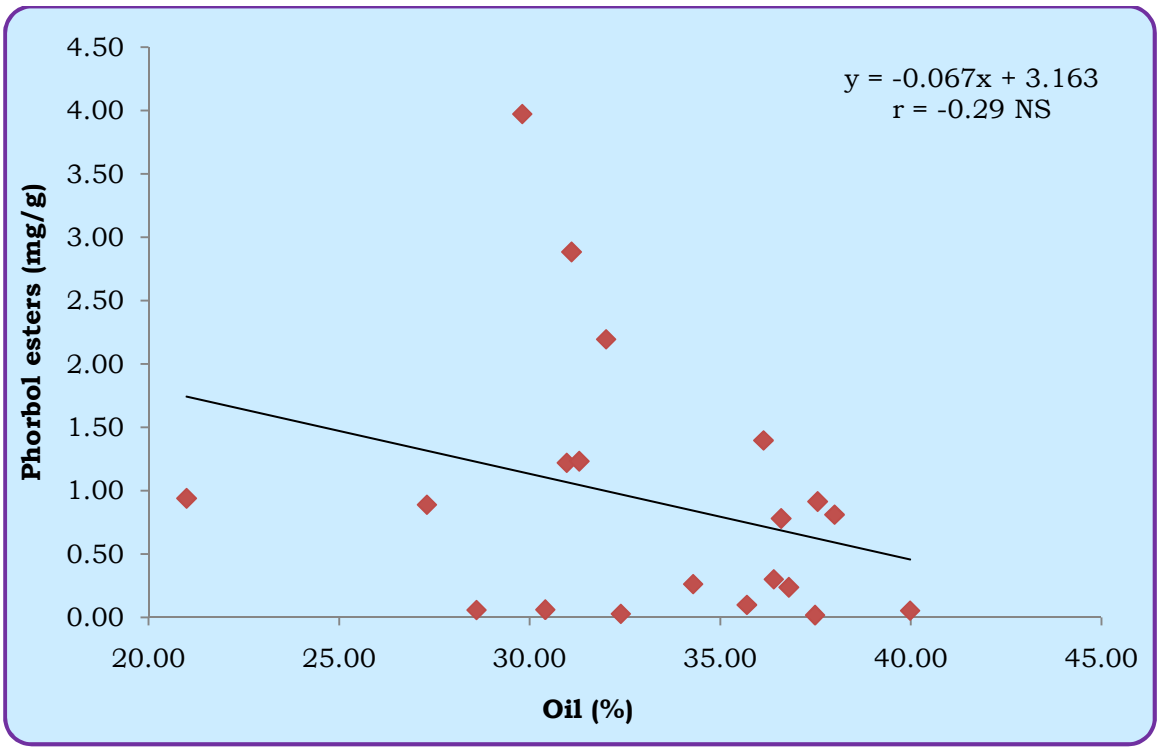

Fig. 7 : Correlation between phorbol esters and oil

Table 6 : Phorbol esters in treated oil

Treatment No.	**Treatments**	**Phorbol esters (mg/g)**	**% Reduction in phorbol esters over control**
Tc.	Control	3.08	-
T_1.	Methanol	1.44	53.25
T_2.	Degumming	2.36	23.70
T_3.	Bleaching	1.97	36.36
	F	*	
	CV(%)	O.52	
	S.Em $\pm$	0.01	
	CD at 5%	0.02	

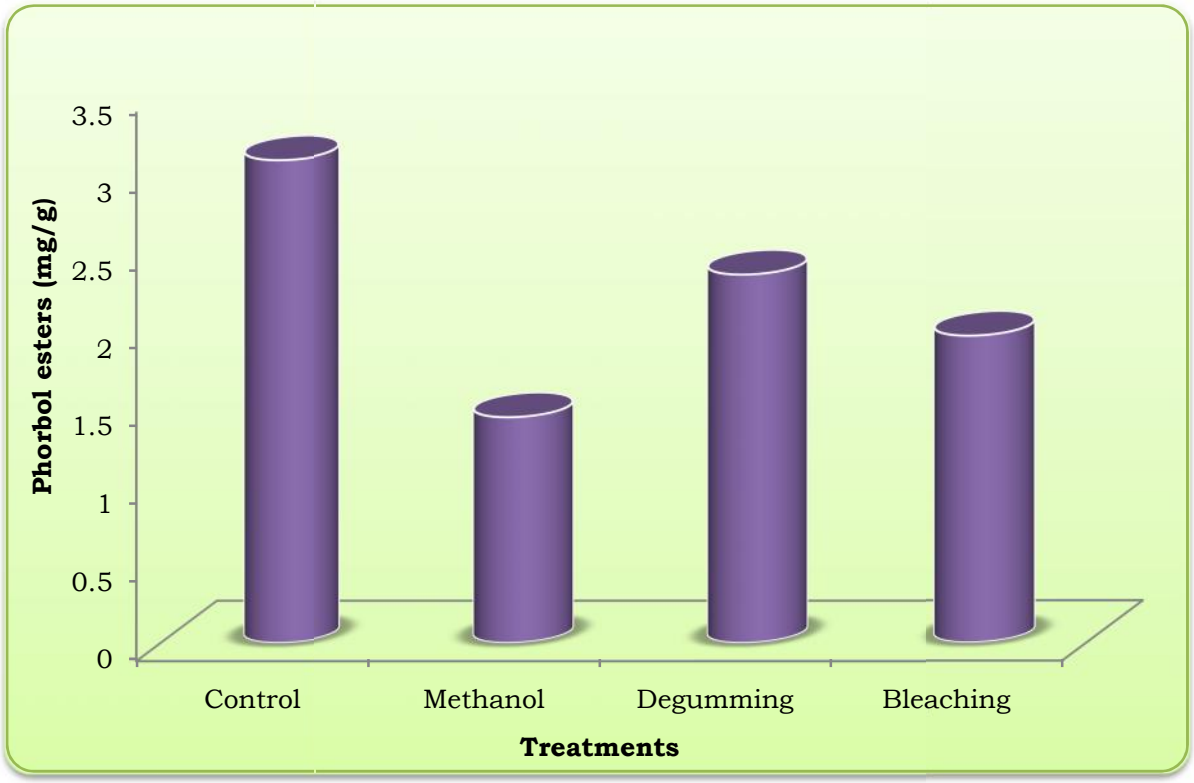

Fig. 8 : Phorbol esters in treated Oil

Fig. 9 : Chromatogram of phorbol esters extracted from treated oil samples of *Jatropha curcas L.*

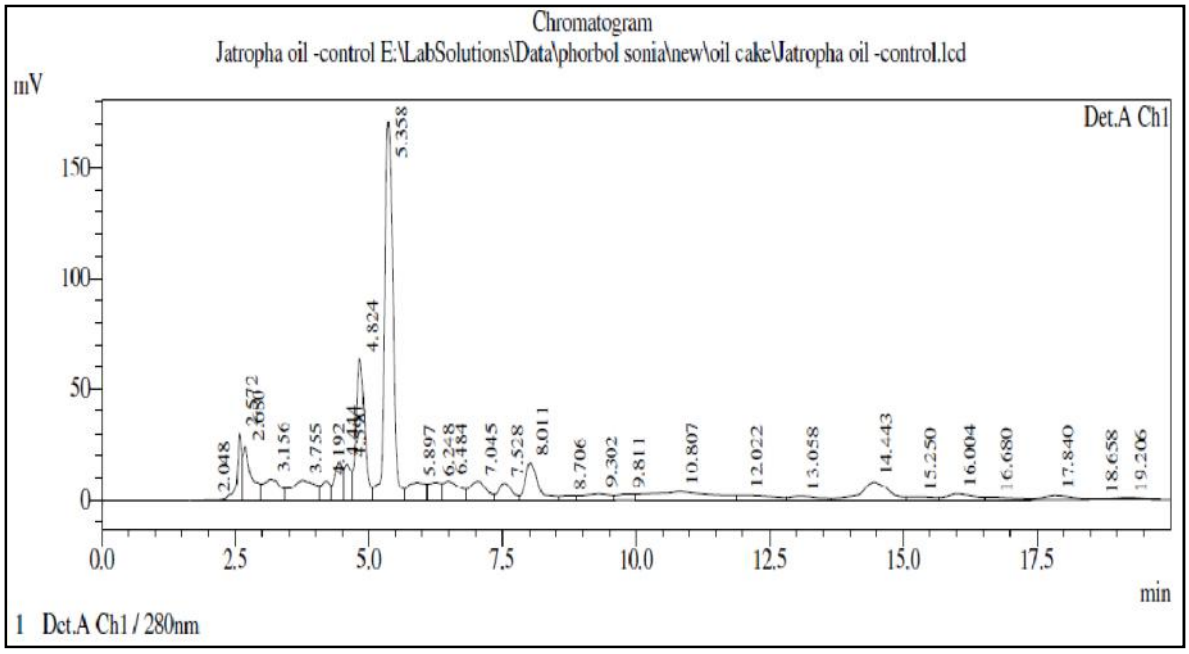

Fig. 9a : Chromatogram of phorbol esters extracted from untreated oil

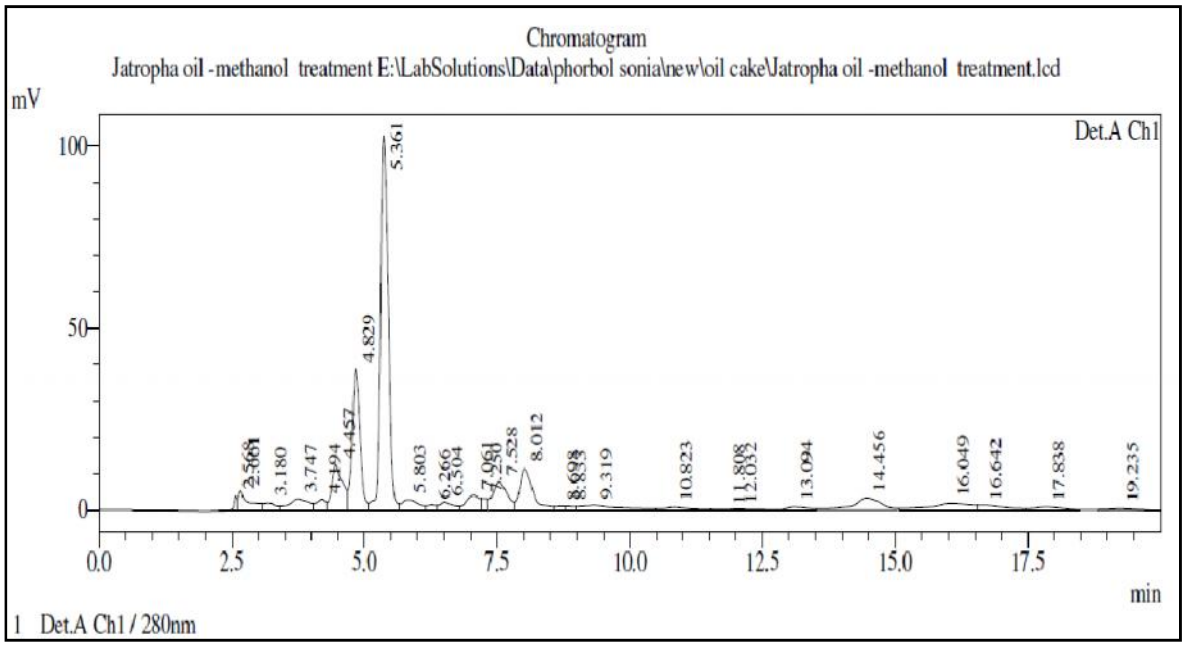

Fig. 9b : Chromatogram of phorbol esters extracted after methanol treatment

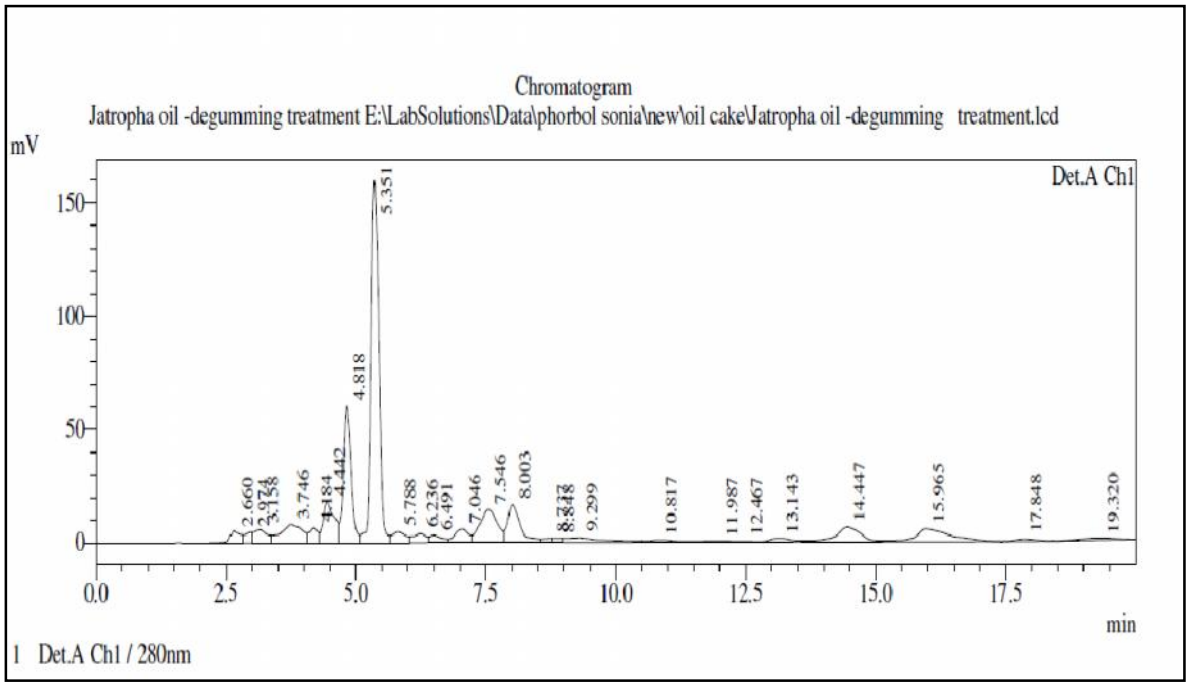

Fig. 9c : Chromatogram of phorbol esters extracted after degumming

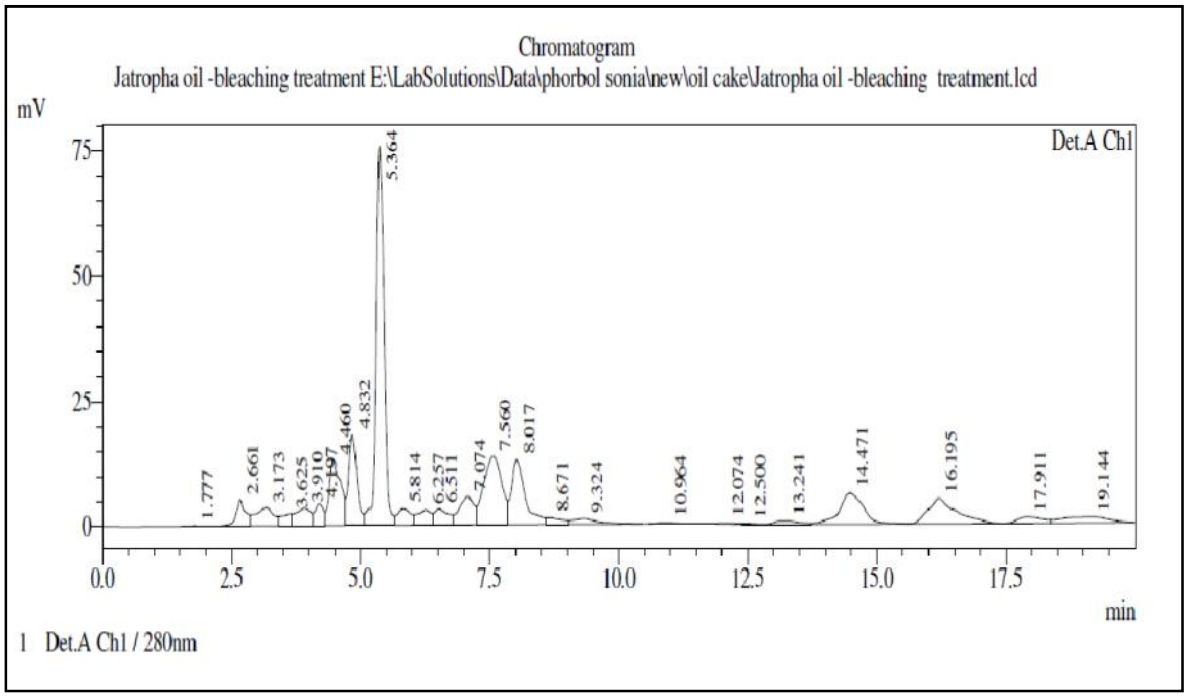

Fig. 9d : Chromatogram of phorbol esters extracted after bleaching

treatment was most effective and reduced the phorbol ester to1.44 mg/g. This was followed by bleaching treatment which showed1.97 mg/g. There was marginal reduction of phorbol esters in oil subjected to degumming treatment. Chromatograms are presented in Fig.9. Statistical analysis showed that there was a significant variation at 5 per cent.

4.7. Detoxification of oilcake

Table 7 and Fig. 10 presents the final concentration of phorbol esters obtained for each treatment. Results showed that untreated oilcake contains 3.07 mg/g of phorbol esters. Detoxification experiments which included heat treatment along with different chemical treatments were done to reduce the concentration of phorbol esters and hence can be used as animal feed.

The concentration of phorbol esters in defatted and treated meal was significantly lower at 5 per cent than in untreated meal. The most effective treatment was hydrochloric acid where the phorbol ester content was reduced to 0.09 mg/g followed by sodium hydroxide along with 3 times wash with 92 per cent methanol treatment where the phorbol ester concentration was reduced to 0.18 mg/g from 3.07 mg/g.

Other treatments such as roasting (2.27 mg/g), water soaking (2.16 mg/g), calcium oxide (0.88 mg/g), sodium hydroxide with sodium hypochlorite (0.84 mg/g), autoclaving (0.81 mg/g), sodium hydroxide followed by 3 times water wash (0.73 mg/g) and sodium bicarbonate (0.26 mg/g) also had increasing effectiveness in removing phorbol esters. Chromatograms are presented in Fig. 11.

Phorbol esters were reduced to a tolerable level when *Jatropha curcas* was treated with hydrochloric acid and sodium hydroxide along with 3 times wash with 92 per cent methanol. These treatments were the better treatments for detoxifying the meal. This treatment is promising

Table 7 : Phorbol esters in treated oilcake

Treatment No.	Treatments	Phorbol esters (mg/g)	% Reduction in phorbol esters over control
Tc.	Control	3.07	-
T_1.	Autoclave	0.81	73.53
T_2.	Sodium hydroxide followed by washing with 92% methanol	0.18	94.12
T_3.	Sodium hydroxide and sodium hypochlorite	0.84	72.55
T_4.	Calcium oxide	0.88	71.57
T_5.	Sodium bicarbonate	0.26	91.5
T_6.	Hydrochloric acid	0.09	97.06
T_7.	Roasting	2.27	25.82
T_8.	Sodium hydroxide followed by water wash	0.73	76.14
T_9.	Water soaking	2.16	29.14
	F	*	
	CV(%)	0.82	
	S.Em $\pm$	0.01	
	CD at 5%	0.02	

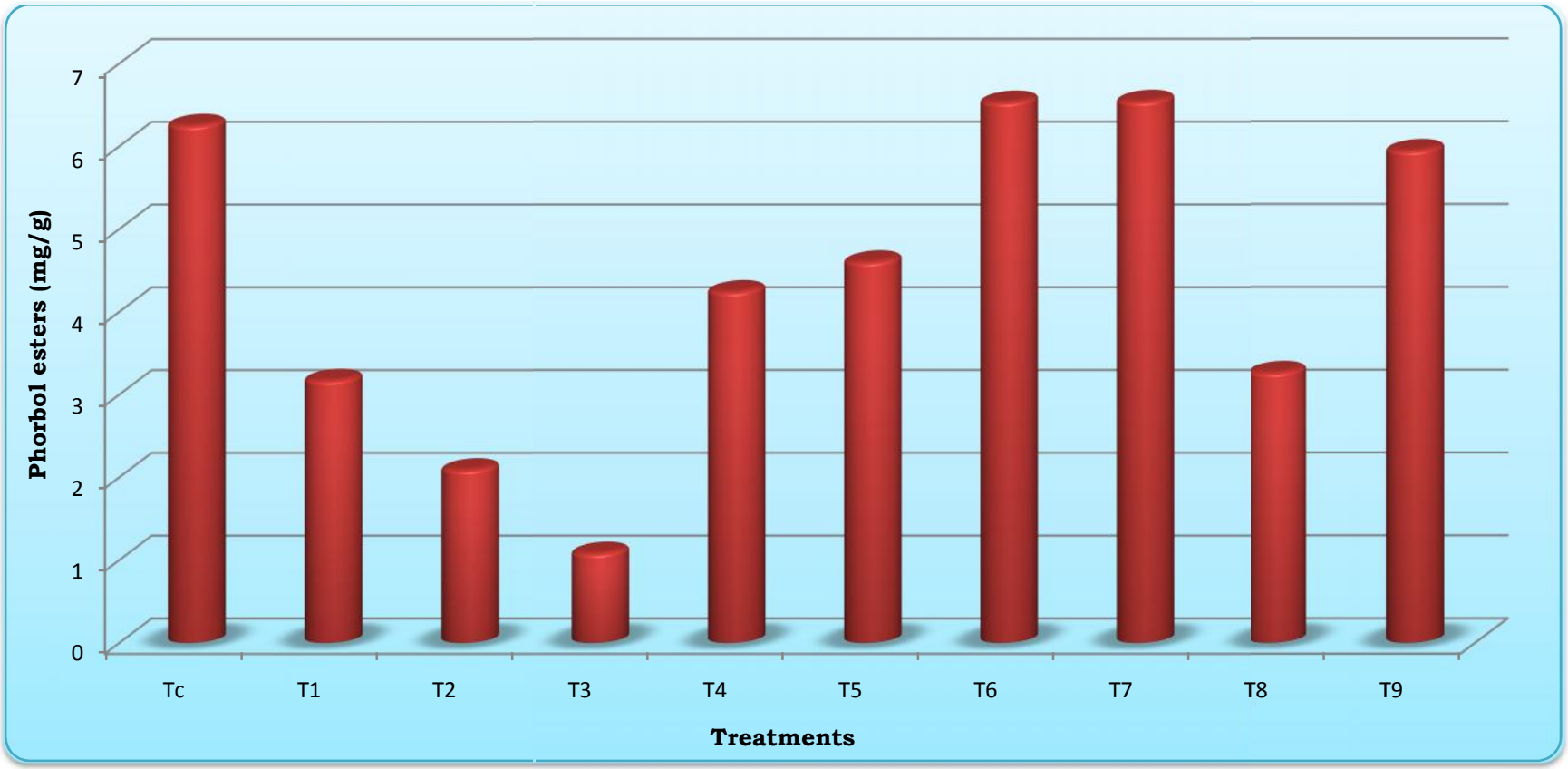

Fig. 10 : Phorbol esters in treated oilcake

Note:

Tc-Control
T_1-Autoclave
T_2-Sodium hydroxide followed by 92% methanol wash
T_3-Sodium hydroxide and sodium hypochlorite
T_4-Calcium oxide
T_5-Sodium bicarbonate
T_6-Hydrochloric acid
T_7-Roasting
T_8-Sodium hydroxide followed by water wash
T_9-Water soaking

Fig. 11 : Chromatogram of phorbol esters extracted from treated oilcake samples of *Jatropha curcas L.*

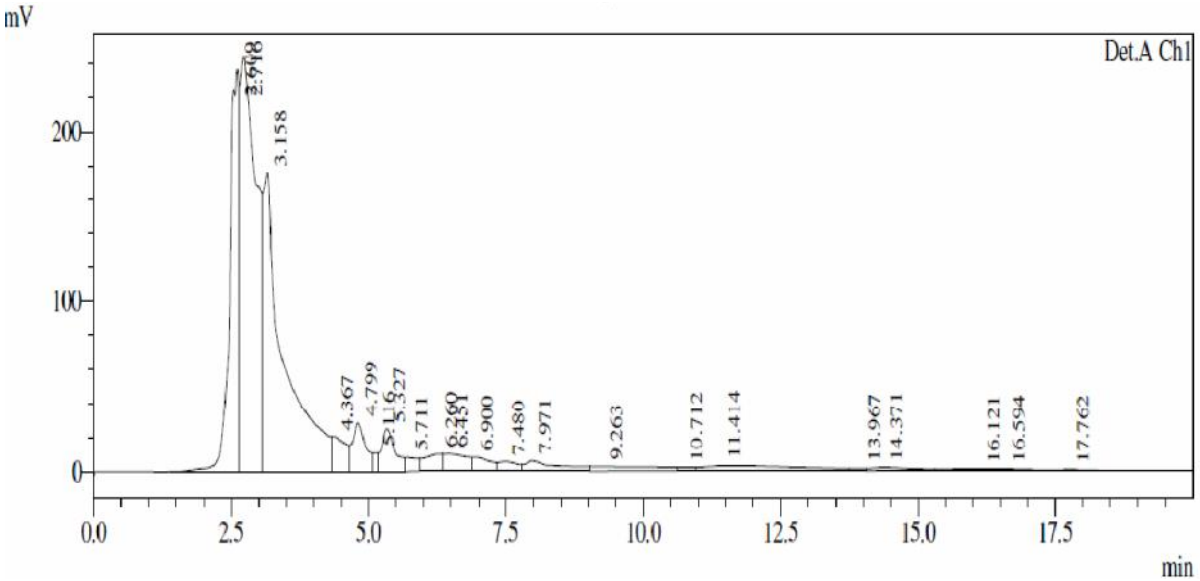

Fig. 11a : Chromatogram of phorbol esters extracted from untreated oilcake

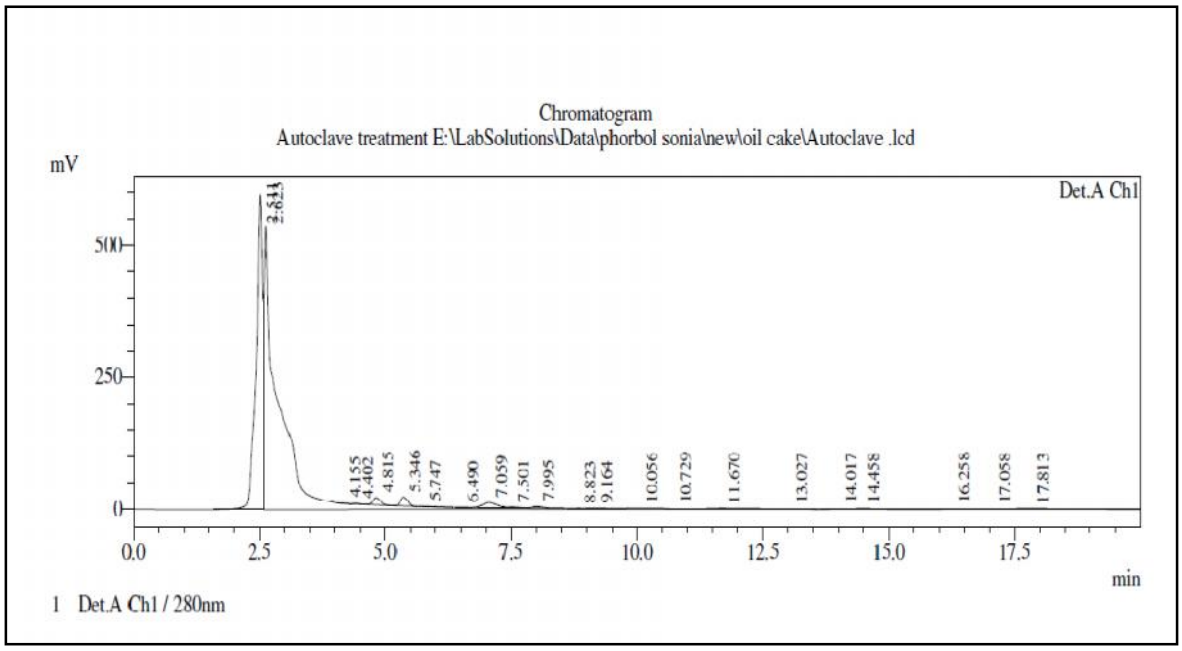

Fig. 11b : Chromatogram of phorbol esters extracted after autoclaving

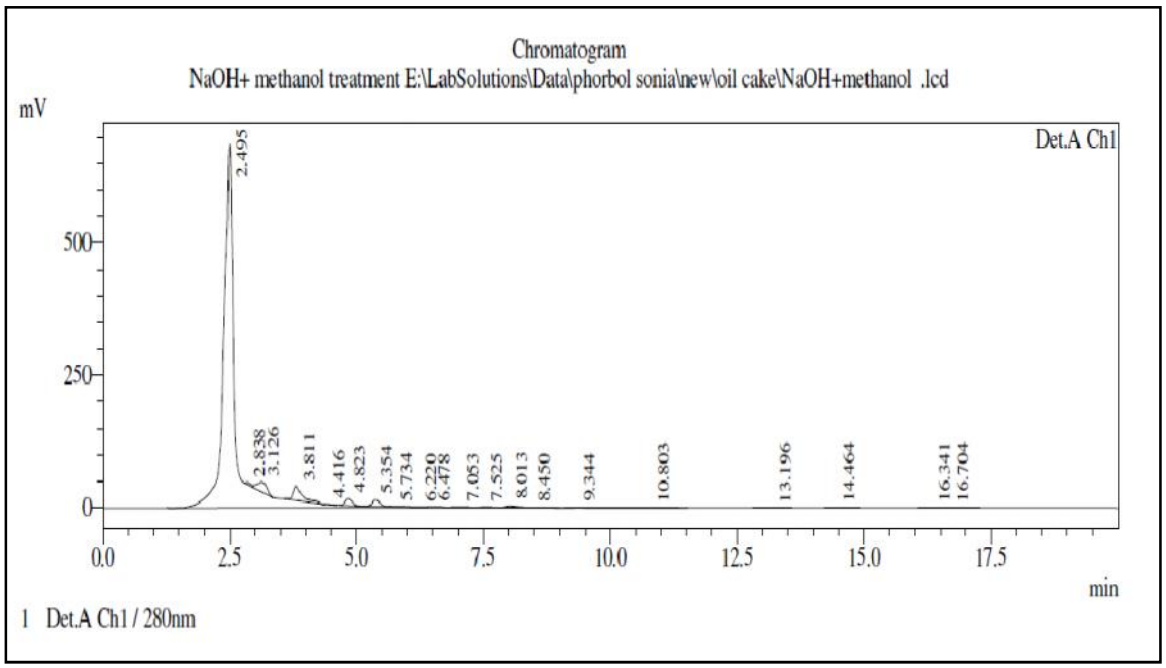

Fig. 11c : Chromatogram of phorbol esters extracted after sodium hydroxide followed by methanol wash

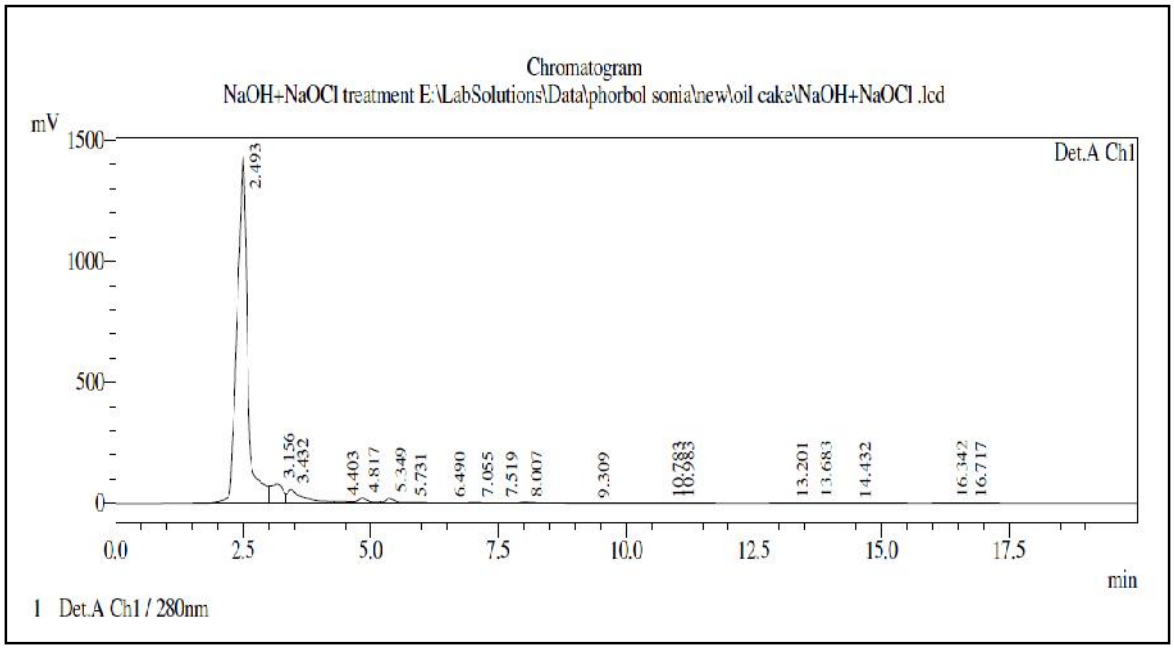

Fig. 11d : Chromatogram of phorbol esters extracted after sodium hydroxide and sodium hypochlorite

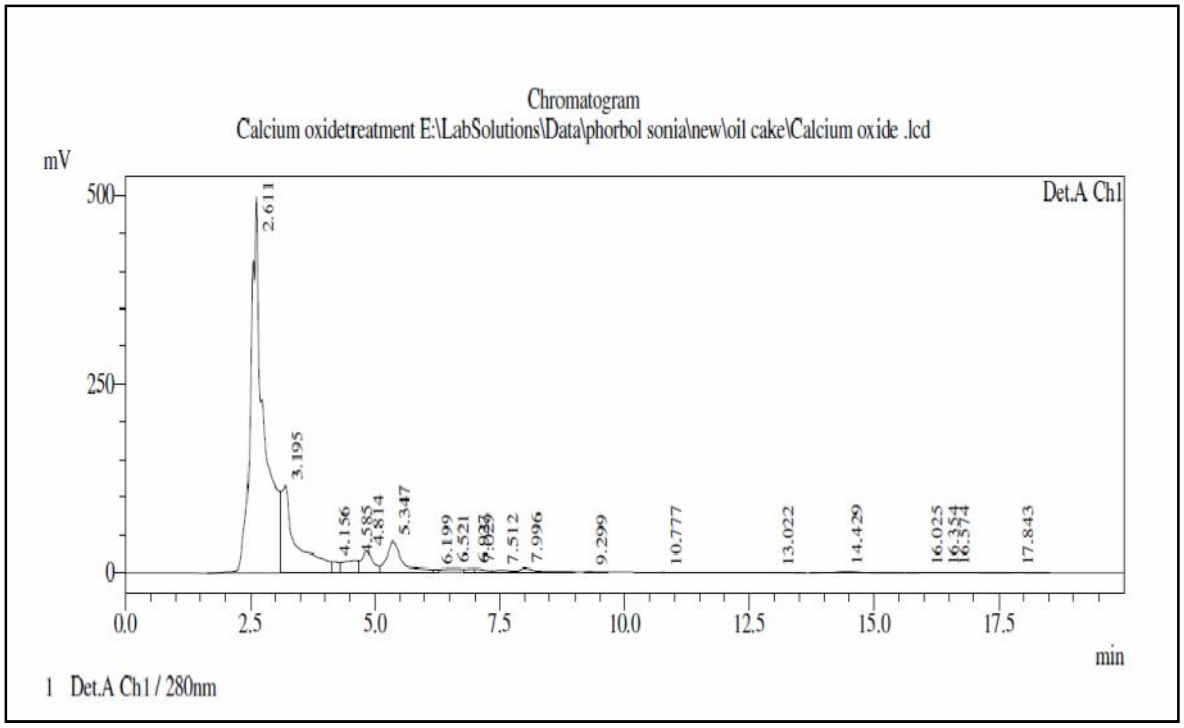

Fig. 11e : Chromatogram of phorbol esters extracted after calcium oxide treatment

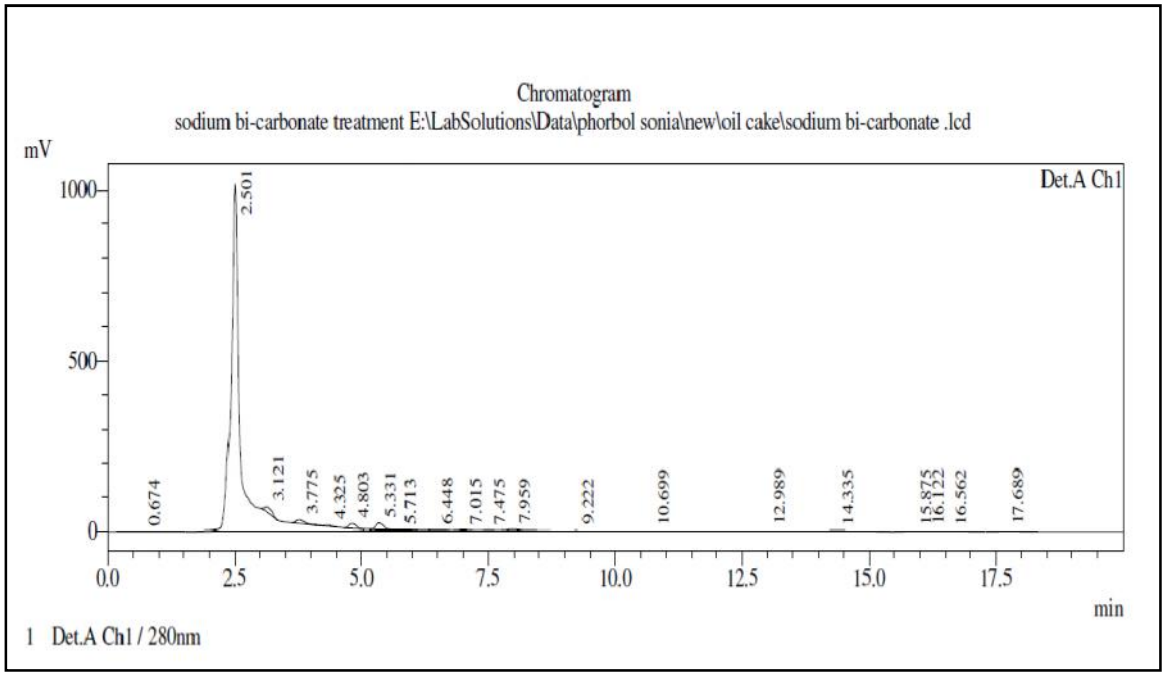

Fig. 11f : Chromatogram of phorbol esters extracted after sodium bi-carbonate treatment

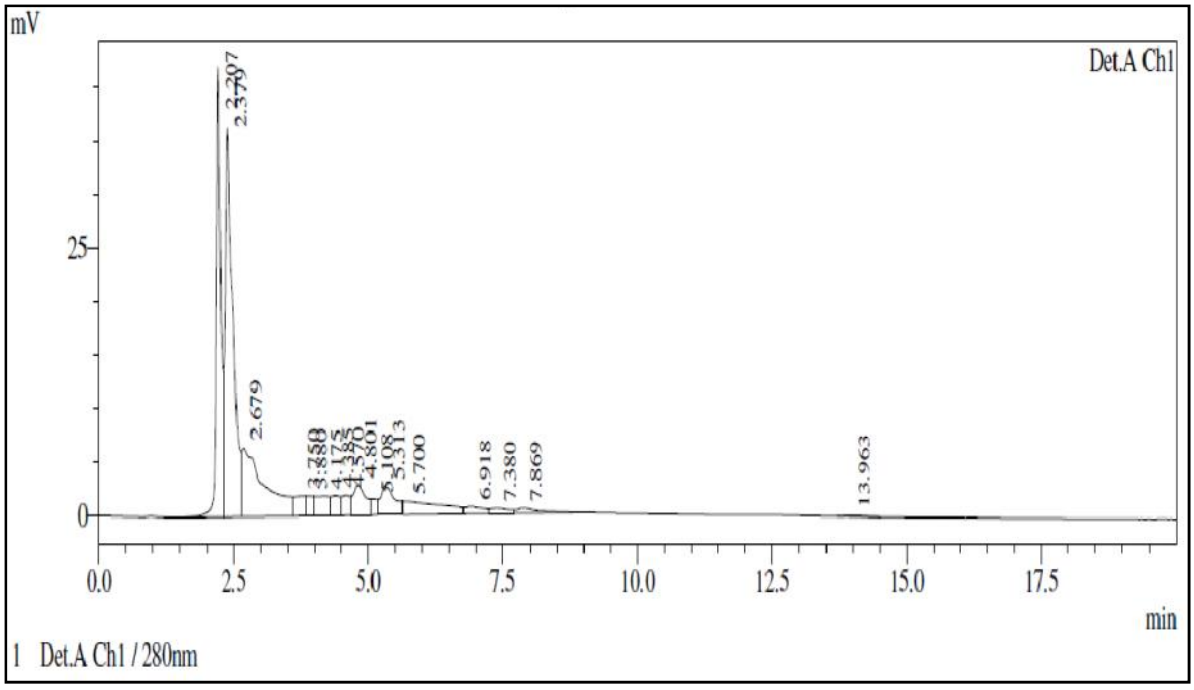

Fig. 11g : Chromatogram of phorbol esters extracted after hydrochloric acid treatment

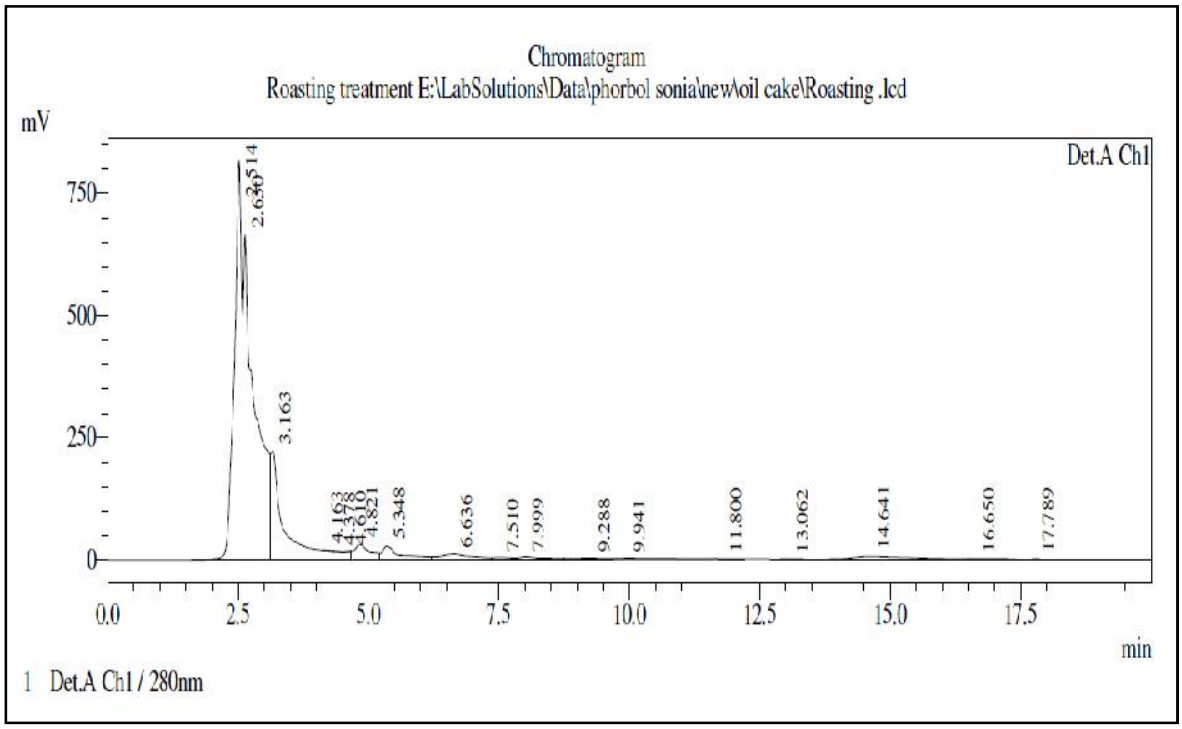

Fig. 11h : Chromatogram of phorbol esters extracted after roasting treatment

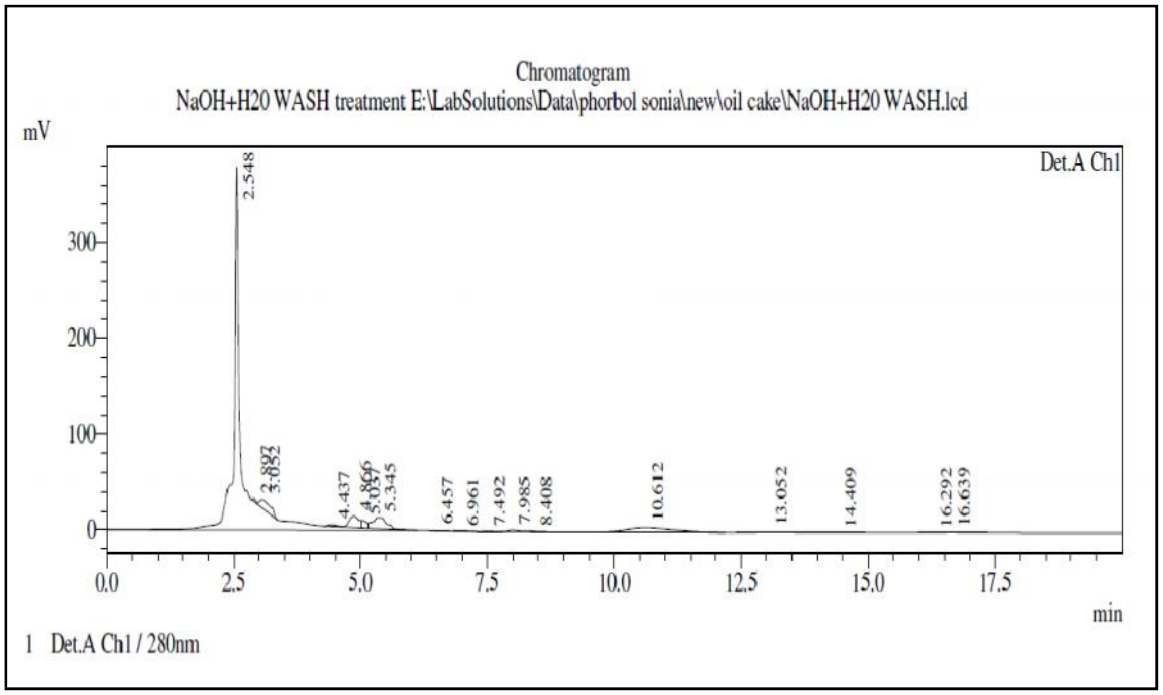

Fig. 11i : Chromatogram of phorbol esters extracted after sodium hydroxide followed by water wash

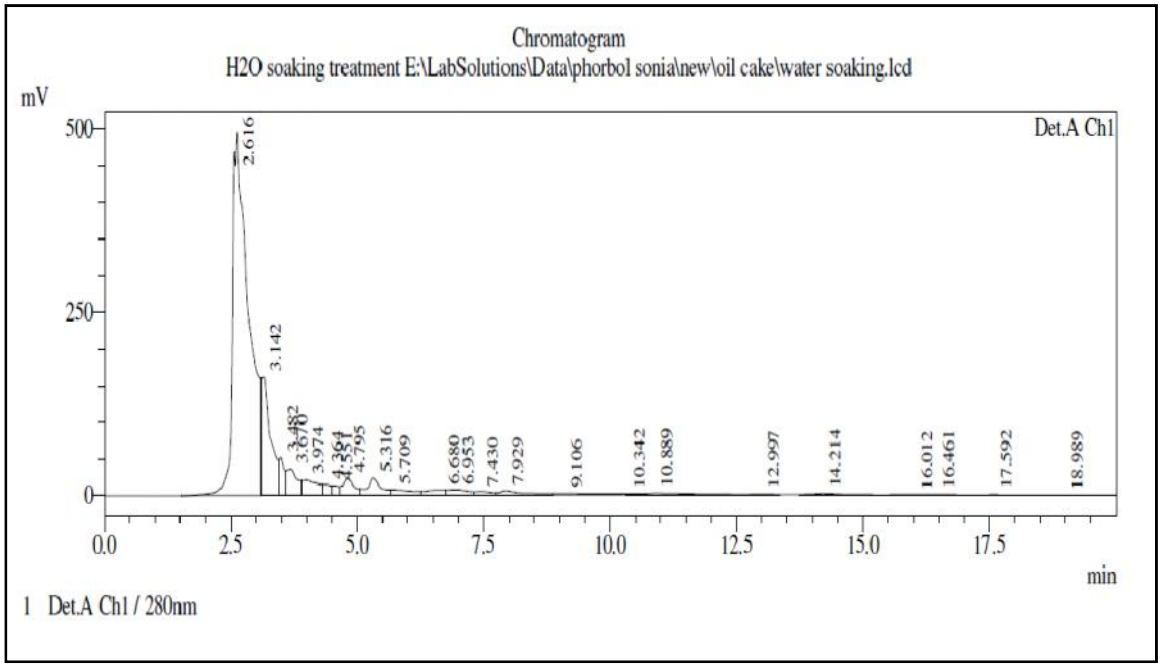

Fig. 11j : Chromatogram of phorbol esters extracted after water soaking

but in economic terms it is expensive to produce *Jatropha curcas* meal from it. But it can be exploited through small scale industry. The price can be reduced if the hydrochloric acid and methanol can be recovered.

4.8. Chemical composition of *Jatrohpa curcas* L.

The results of chemical compositions for different treatments of *Jatrohpa curcas* L. oilcake is presented in Table 8.

4.8.1. Oil content in oilcake

The oil content of *Jatropha curcas* oilcake was determined by using the soxtherm instrument. There was a significant variation in the oil content in oilcake that was subjected to different treatments (Fig. 13).

The oil content ranged from high of 6.25 per cent in untreated control to a low of 1.08 per cent in cakes treated and with sodium hydroxide along with sodium hypochlorite showing maximum effects. Marginal increase in oil content was observed in hydrochloric acid (6.53%) and roasting (6.54%) treatments over the control showing the ineffectiveness. Sodium hydroxide followed by washing with 92 per cent methanol, autoclaving, sodium hydroxide followed by washing with water, calcium oxide, sodium bicarbonate and water soaking treatments showed resulted oil contents of 2.09, 3.17, 3.26, 4.24, 4.60 and 5.94 per cent indicating decreasing effectiveness.

4.8.2. Carbohydrate content in oilcake

Carbohydrate was estimated by adding contents of components such as ash, oil per cent, moisture, crude fibre and protein and subtracting values from 100. Usually carbohydrate is the major nutritive component that is present in the sample.

Carbohydrates content ranged from 30.04-50.96 per cent (Fig. 12). The least content of carbohydrates was present in the sample that was treated with sodium hydroxide and sodium hypochlorite (30.04 per cent). Hydrochloric acid treated sample had the highest carbohydrates content which was about 50.96 per cent. The untreated Jatropha oilcake had 45.05 per cent of carbohydrates. The difference in the carbohydrates value among different treatments was statistically significant.

Treatments with sodium bicarbonate (41.38%), sodium hydroxide followed by washing with water (41.94%), sodium hydroxide followed by washing with 92 per cent methanol (42.65%), calcium oxide (43.67%), water soaking (47.19%) and roasting (48.48%) showed increasing effectiveness in retaining more carbohydrates.

4.8.3. Protein content in oilcake

Protein was estimated by using nitrogen value and multiplying by 6.25.

Protein content ranged from 24.67 to 32.5 per cent (Fig. 12). The control sample had protein content of 27.08 per cent .The treatment with sodium hydroxide and washing with 92 per cent methanol had the highest content of protein which is 32.54 per cent followed by autoclave treatment (29.56 per cent). Least protein content was present in the sample that was treated with hydrochloric acid (24.67 per cent) followed by sodium hydroxide along with sodium hypochlorite treatment (25.21 per cent). However, data obtained showed statistically significant difference at 5 per cent level among the different treatments.

Water soaking, sodium hydroxide followed by washing with water, sodium bicarbonate, calcium oxide, and roasting treatments showed 25.38, 28.44, 29.04, 29.67 and 30.77 per cent indicating decreasing effectiveness in reducing protein.

4.8.4. Moisture content in oilcake

The moisture content of the oilcake was estimated by using high constant temperature hot air oven method. There was a significant difference in the moisture contents of the oilcake with different treatments (Fig. 13).

The moisture content of the oilcake ranged from 3.2 to 23.03 per cent. The sample treated with sodium hydroxide along with Sodium hypochlorite treatment had the highest moisture content (23.03%) and roasting treatment had reduced the moisture content to 3.2 per cent. The oilcake without any treatment which was considered as control had moisture content of 9.06 per cent. Treatments with sodium hydroxide along with 3 times washing with water, sodium hydroxide along with 3 times washing with 92 per cent methanol, sodium bicarbonate, calcium oxide, autoclaving and hydrochloric acid showed the moisture contents of 11.95, 11.37, 9.43, 8.43, 6.41 and 5.88 per cent indicating increased effectiveness in reducing moisture content.

4.8.5. Crude fibre in oilcake

Crude fibre was estimated by using defatted sample with H_2SO_4 and sodium hydroxide and finally washing with distill water, diethyl ether and alcohol (AOAC, 1980).The data on crude fibre presented in the Table 8 exhibited statistical significant difference among treatments.

The crude fibre content ranged from a low of 5.09 per cent to a high of 11.85 per cent among different samples (Fig. 13). It was found to be higher in water soaking treatment (11.85%) followed by autoclave treatment (9.77%). Roasting treatment had the least crude fibre content (5.09%). Untreated *Jatropha curcas* L. oilcake had 6.36 per cent of crude fibre. Treatments with hydrochloric acid, calcium oxide, sodium hydroxide along with 3 times washing with 92 per cent methanol, sodium

Table 8 : Chemical composition in oilcake of *Jatropha curcas* L.

Treatments	Oil	Carbohydrates	Protein	Ash	Crude fibre	Moisture
Control	6.25	45.05	27.08	6.20	6.36	9.06
Autoclave	3.17	45.12	29.56	5.96	9.77	6.41
Sodium hydroxide followed by washing with 92% methanol	2.09	42.65	32.54	2.84	8.50	11.37
Sodium hydroxide and sodium hypochlorite	1.08	30.04	25.21	12.74	7.90	23.03
Calcium oxide	4.24	43.67	29.67	5.58	8.55	8.43
Sodium bicarbonate	4.60	41.38	29.04	7.52	8.02	9.43
Hydrochloric acid	6.53	50.96	24.67	2.20	9.75	5.88
Roasting	6.54	48.48	30.77	5.91	5.09	3.20
Sodium hydroxide followed by water wash	3.26	41.94	28.44	6.83	7.57	11.95
Water soaking	5.94	47.19	25.38	4.15	11.85	5.46
F	*	*	*	*	*	*
CV (%)	2.3	S0.91	0.67	1.86	1.34	3.44
S.Em $\pm$	0.06	0.23	0.11	0.06	0.07	0.19
CD at 5 %	0.18	0.67	0.33	0.19	0.19	0.55

Note: All the values are expressed in percentage

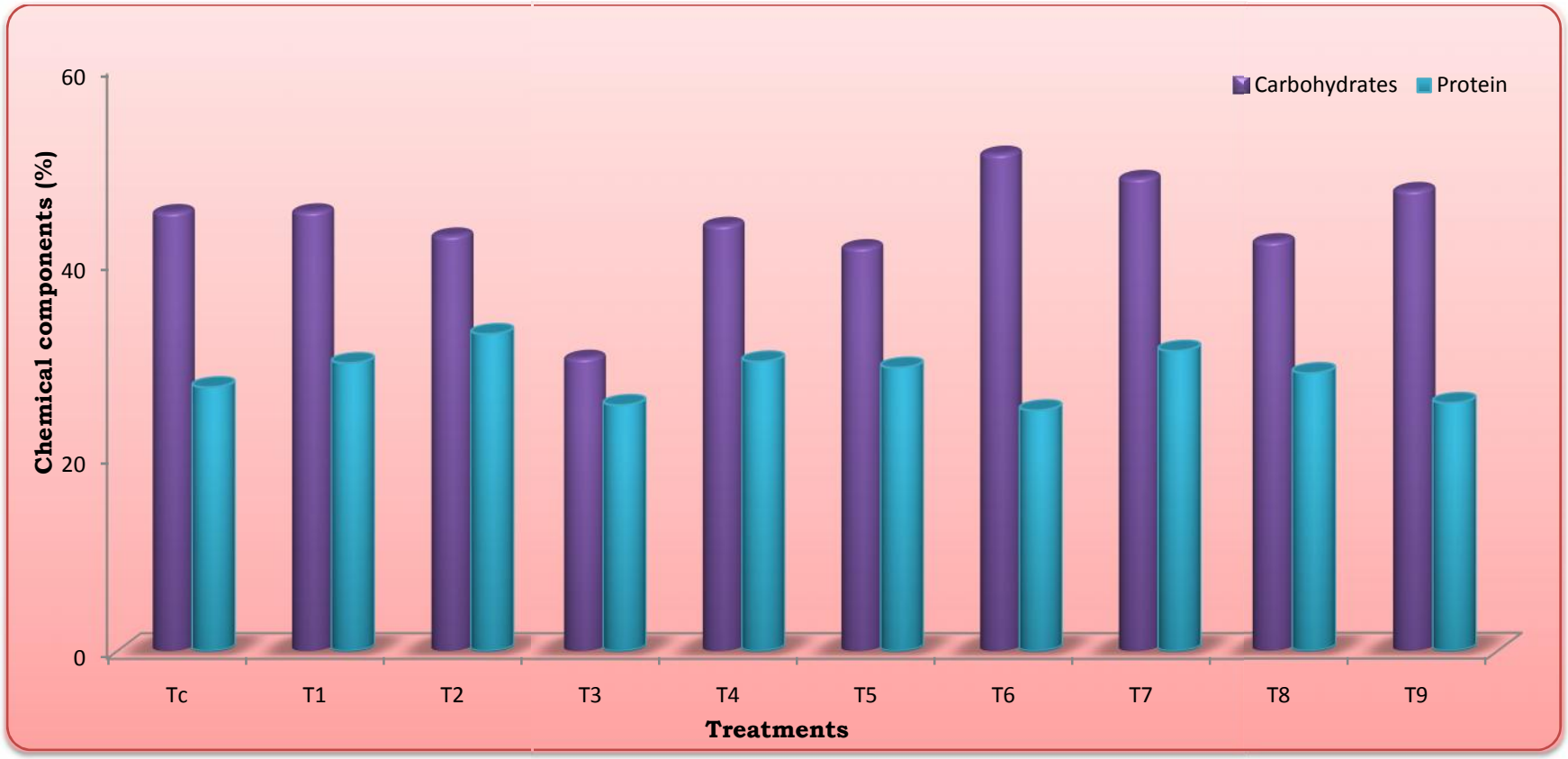

Fig. 12 : Effect of treatments on protein and carbohydrate content in oilcake of *Jatropha curcas* L.

Note:

Tc-Control
T_1-Autoclave
T_2-Sodium hydroxide followed by 92% methanol wash
T_3-Sodium hydroxide and sodium hypochlorite
T_4-Calcium oxide
T_5-Sodium bicarbonate
T_6-Hydrochloric acid
T_7-Roasting
T_8-Sodium hydroxide followed by water wash
T_9-Water soaking

bicarbonate, sodium hydroxide along with sodium hypochlorite, sodium hydroxide along with 3 times washing with water showed the crude fibre of 9.75, 8.55, 8.5, 8.02, 7.9 and 7.57 per cent indicating differential effectiveness of treatments.

4.8.6. Ash content in oilcake

Ash is one of the important nutrition components and it was estimated by taking the sample in the crucible and placed in the muffle furnace at 600^0 C and calculated by taking the difference between the initial and final weights (AOAC, 1980).

The ash content of the oilcake ranged from 2.20 to 12.74 per cent (Fig. 13). The oilcake without any treatment had an ash content of 6.2 per cent. The highest ash content was recorded in the sample treated with sodium hydroxide along with sodium hypochlorite with 12.74 per cent. Least ash content was observed in the sample treated with hydrochloric acid with 2.20 per cent. Statistical analysis showed significant difference among the treatments.

Treatments with sodium hydroxide followed by washing with 92 per cent methanol (2.84%), water soaking (4.15%), calcium oxide (5.58%), Autoclaving (5.96%), roasting (5.91%), sodium bicarbonate (7.52%) and sodium hydroxide along with 3 times washing with water (6.83%) showed decreasing effectiveness in reducing the ash contents.

4.9. Nitrogen, phosphorus and potassium content in oilcake

The results of nitrogen, phosphorus and potassium contents in oilcake for different treatments of *Jatrohpa curcas* L. is presented in Table 8 and Fig. 14.

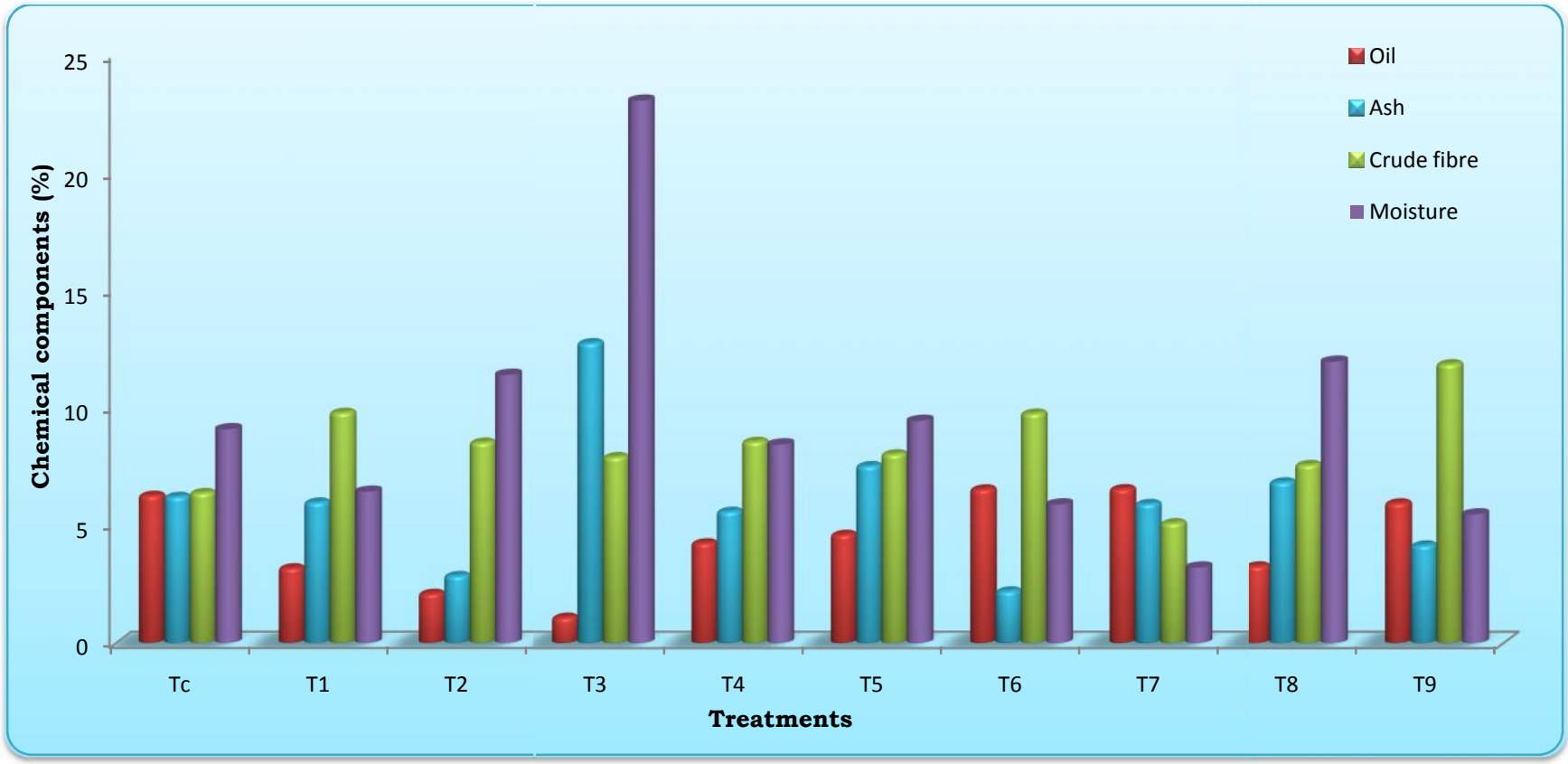

Fig. 13 : Effect of treatments on chemical components in oilcake of *Jatropha curcas* L.

Note:

Tc-Control
T_1-Autoclave
T_2-Sodium hydroxide followed by 92% methanol wash
T_3-Sodium hydroxide and sodium hypochlorite
T_4-Calcium oxide
T_5-Sodium bicarbonate
T_6-Hydrochloric acid
T_7-Roasting
T_8-Sodium hydroxide followed by water wash
T_9-Water soaking

4.9.1. Nitrogen in oilcake

The total nitrogen of Jatropha curcas oilcake sample was determined by Kjeldhal method where sample was digested using concentrated sulphuric acid along with digestion mixture and distilled in alkaline media and trapped in boric acid and finally titrated against hydrochloric acid.

The untreated *J. curcas* sample contained 4.33 per cent nitrogen. Nitrogen content in different treated samples ranged from 3.91 to 5.2 per cent. The highest percentage of nitrogen is 5.2 per cent which was present in the sample that was treated with sodium hydroxide along with 92 per cent methanol. Least percentage of nitrogen was present in hydrochloric acid treated sample which is about 3.91 per cent. Statistical analysis showed a significant variation in the nitrogen content among the various treatments at 5 per cent level.

Treatments with sodium hydroxide followed by washing with 92 per cent methanol (4.03%), water soaking (4.09%), sodium hydroxide with sodium hypochlorite (4.57%), sodium bicarbonate (4.67%), calcium oxide (4.17%), autoclaving (4.73%) and roasting (4.91%), showed decreasing effectiveness with respect to nitrogen contents, which has helped to retain higher nitrogen, indirectly protein.

4.9.2. Phosphorus in oilcake

Phosphorus was estimated by using vandomolybdphosphoric yellow colour method in nitric acid system (Jackson 1973).

Difference in phosphorus contents was in narrow range of 1.31-1.68 per cent. The control sample had phosphorus content of 1.43 per cent. The highest percentage of phosphorus is 1.68 per cent which was present in the sample which was treated with methanol along with 92

per cent methanol followed by autoclaving (1.61%). Least per cent of phosphorus was present in sample treated with sodium hydroxide along with sodium hypochlorite treatment (1.31%) followed by water soaking (1.37%). However statistical analysis showed a significant variation in the phosphorus content among the various treatments at 5 per cent level.

Other treatments like sodium hydroxide followed by washing with water, hydrochloric acid, sodium bicarbonate, roasting and calcium oxide showed 1.4, 1.46, 1.57, 1.59 and 1.6 per cent phosphorus which indicates retention of higher levels of phosphorus, the major nutrient.

4.9.3. Potassium in oilcake

Potassium was estimated by using the principle followed with flame photometry as described by Jackson, 1973.

Potassium content ranged from 0.91 to 2.13 per cent. The control sample had phosphorus content of 1.12 per cent. The highest per centage of phosphorus is 2.13 per cent which was present in the sample which was treated with roasting followed by calcium oxide (2.08%). Least per cent of phosphorus was present in sample treated with hydrochloric acid treatment (0.91%) followed by water soaking (1.07%). The difference in the potassium value among different treatments was statistically significant at 5 per cent level.

Treatments with water soaking (1.15%), sodium hydroxide with sodium hypochlorite (1.37%), sodium hydroxide followed by washing with 92 per cent methanol (1.77%), sodium bicarbonate (2.01%) and autoclaving (2.05%) showed decreasing effectiveness in respect to potassium content which has helped to retain more of potassium, one of the major nutrients in the treated oilcake samples.

Table 9 : Nitrogen, phosphorus and potassium content in oilcake of *Jatropha curcas* L.

Treatment No.	Treatments	N (%)	P (%)	K (%)
Tc.	Control	4.33	1.43	1.12
T_1.	Autoclave	4.73	1.61	2.05
T_2.	Sodium hydroxide followed by washing with 92% methanol	4.03	1.68	1.77
T_3.	Sodium hydroxide and sodium hypochlorite	4.57	1.31	1.37
T_4.	Calcium oxide	4.71	1.6	2.08
T_5.	Sodium bicarbonate	4.67	1.57	2.01
T_6.	Hydrochloric acid	3.91	1.46	0.91
T_7.	Roasting	4.91	1.59	2.13
T_8.	Sodium hydroxide followed by water wash	5.2	1.4	1.07
T_9.	Water soaking	4.09	1.37	1.15
	F	*	*	*
	CV(%)	2.50	1.52	1.36
	S.Em $\pm$	0.07	0.01	0.12
	CD at 5%	0.20	0.04	0.04

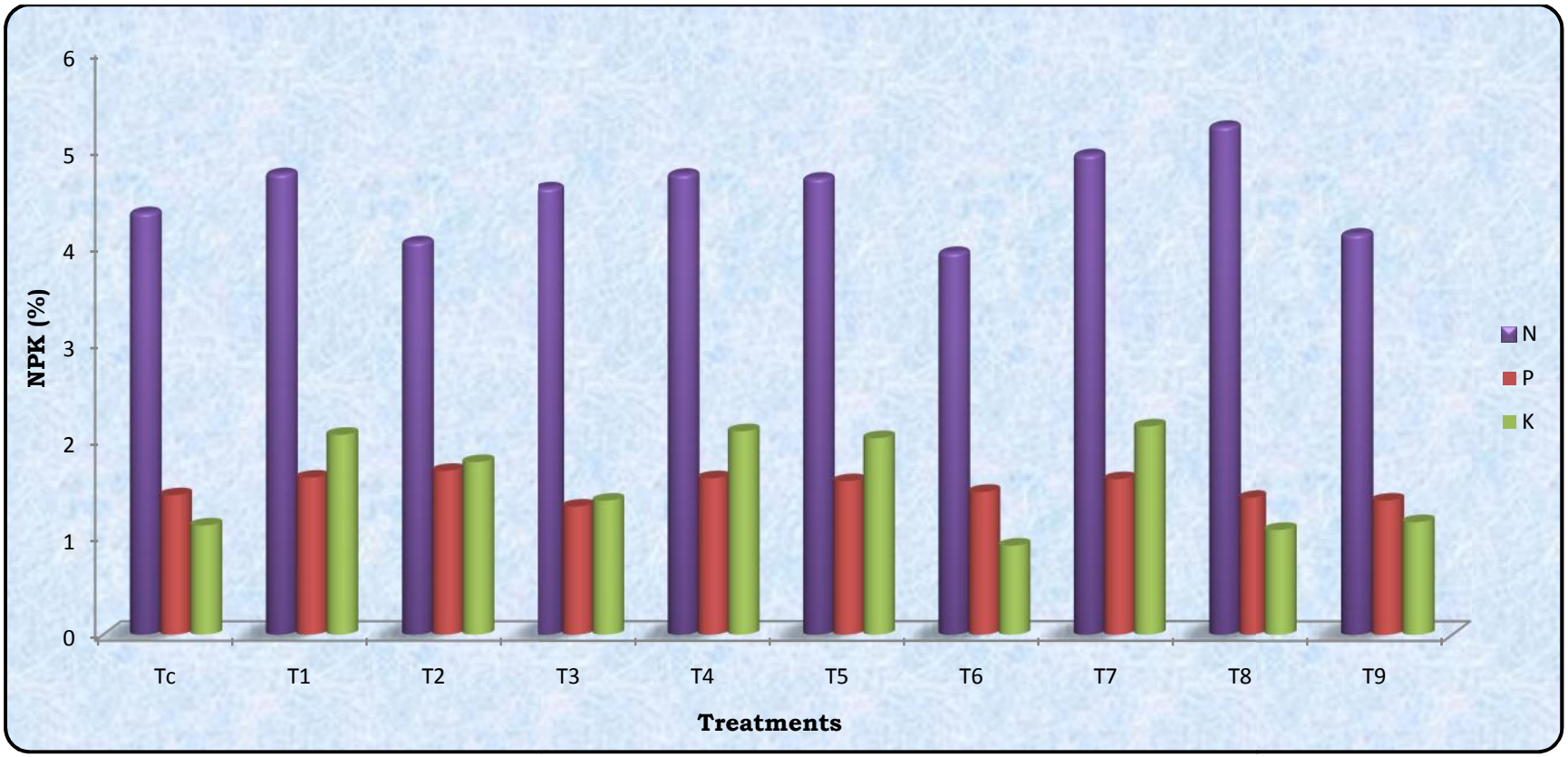

Fig. 14 : NPK content in oilcake of *Jatropha curcas* L.

Note:

Tc-Control
T_1-Autoclave
T_2-Sodium hydroxide followed by 92% methanol wash
T_3-Sodium hydroxide and sodium hypochlorite
T_4-Calcium oxide
T_5-Sodium bicarbonate
T_6-Hydrochloric acid
T_7-Roasting
T_8-Sodium hydroxide followed by water wash
T_9-Water soaking

4.10. Detoxification of biodiesel

Results showed that biodiesel prepared contain higher amount of phorbol esters (5.38mg/g).Detoxification experiments were carried out for removal of phorbol esters from biodiesel samples and presented in Table 10 and Fig. 15. Only bleaching could be done where there was significant reduction of phorbol esters (2.39 mg/g). Neutralizing with sodium hydroxide lead to soap formation in biodiesel. During methanol treatment separation of layers between biodiesel and methanol was not possible. Due to this only bleaching could be done where the phorbol esters was reduced to 55.58 per cent. Chromatograms are presented in Fig. 16.

Table 10 : Phorbol esters in biodiesel

Sl. No.	Treatments	Phorbol esters (mg/g)	% Reduction in phorbol esters over control
1.	Control	5.38	-
2.	Bleaching	2.39	55.58

Note : Other treatments such as neutralizing, methanol and hot water were tried but the attempts was a failure.

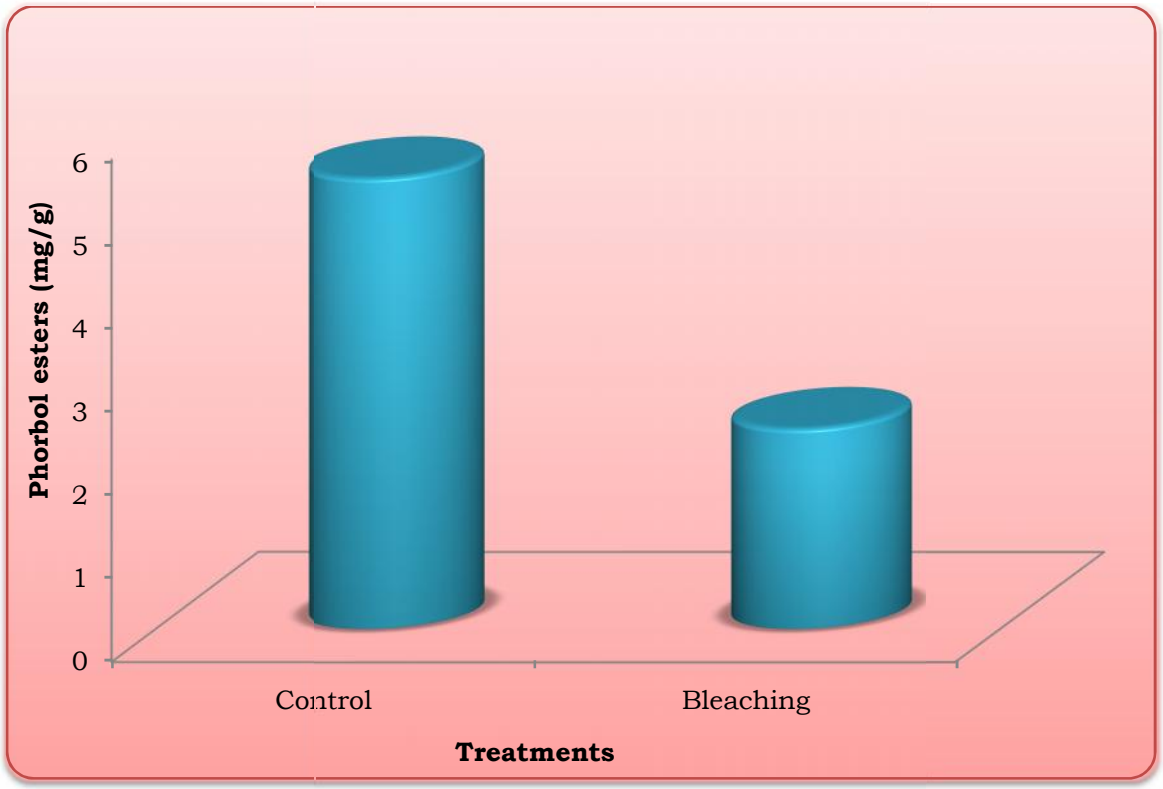

Fig. 15 : Phorbol esters in biodiesel

Fig. 16 : Chromatogram of phorbol esters extracted from treated biodiesel sample of *Jatropha curcas L.*

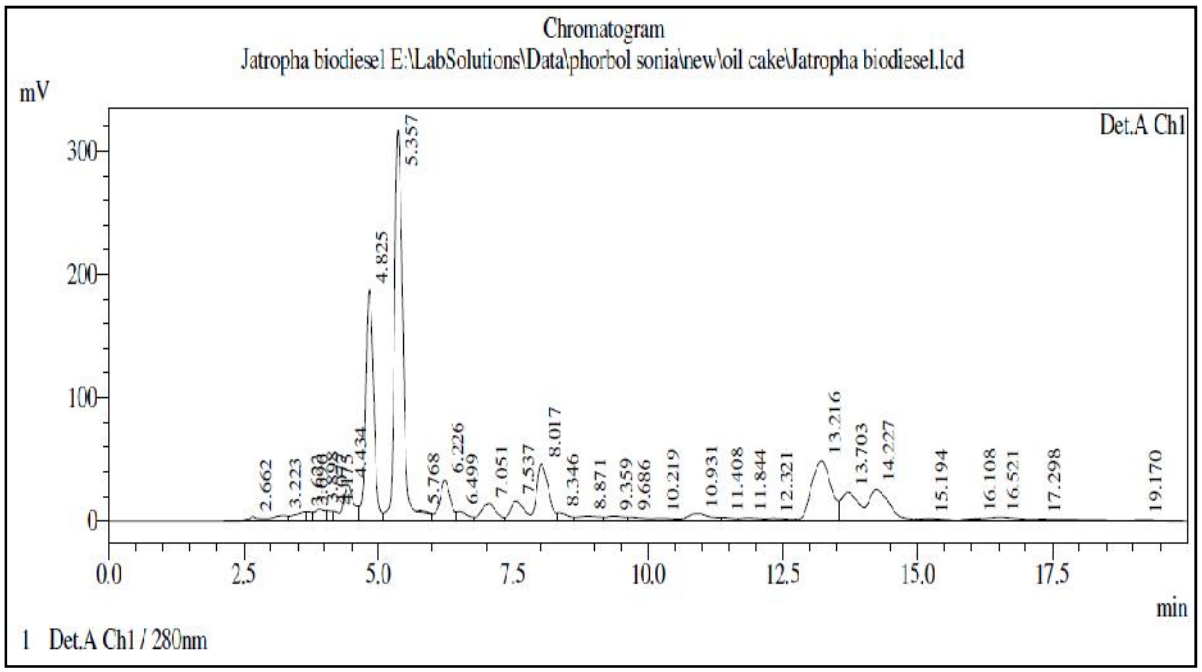

Fig. 16a : Chromatogram of phorbol esters extracted from untreated biodiesel

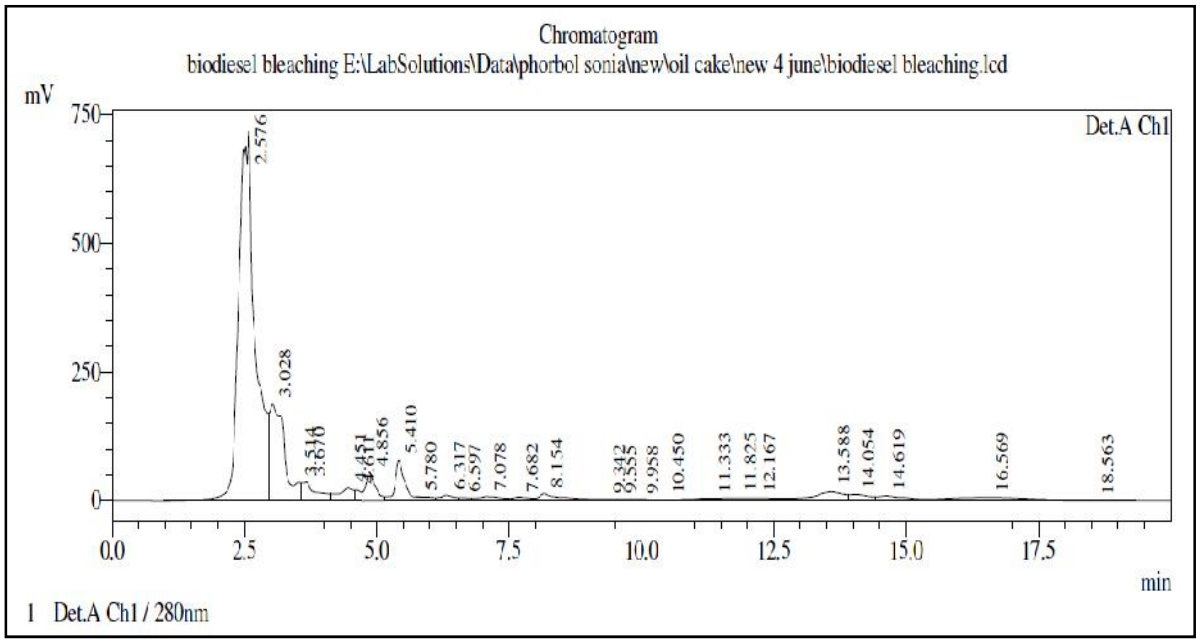

Fig. 16b : Chromatogram of phorbol esters extracted after bleaching treatment

Discussion

V. DISCUSSION

Ever since the automobiles were discovered, there was need for fuel to run them as faster means of movement from place to place. Earlier they were run on wood based fuels. Discovery of fossil fuels and processing lead to revolution in automobiles and other areas of industry in general. Rapid industrialization, economic improvement across world and India in particular has lead to enormous increase in demand for fossil fuel, more by transport sector.

Phorbol esters are natural compounds and highly toxic, carthatic skin irritant and tumor promoting agents. The presence of phorbol esters in the plant, keeps the animals away from browsing and birds from eating the seeds. Detoxification has to be done to remove the phorbol esters for use as feed or fodder because of the rich source of nitrogen, phosphorus and potassium in the plant and the byproducts.

Therefore, attempts have been made to quantify the phorbol esters present in *Jatropha curcas* and different methods employed to detoxify by removal of compounds that are present in oil, oilcake and biodiesel. The results obtained from the study are discussed in brief, here under.

5.1. Seed yield of *Jatropha curcas* L.

The yield projections were for single plant for one season. Yield of *Jatropha curcas* seeds among different accessions also differed significantly, which may be due to ecotypic differences or climatic variations either of location or period. This is due to availability of water, nutrients and growth and lipid metabolism are dependent upon environmental conditions. The yield ranged from 51 to 458 grams.

5.2. Per cent oil yield

The oil percentage results showed that, the oil per cent ranged between 21-40 per cent in the seed. The per cent of oil varied in the accessions from different locations. The environmental factors might affect the oil yield from one location to other.

Similar results were reported by Pant *et al.*, (2006) who reported that, the average oil content in Jatropha was 30.16- 43.19 per cent of their seed weight. They also mentioned that Jatropha oil content varied depending on the genotype and climatic conditions, but mainly on altitude where it is grown.

Manian and Gopalakrishan, (1995) also reported similar findings, where there was a higher utilization of photo assimilation for the plant growth compared to oil production at different altitude. Devappa *et al.,* (2009) reported that *Jatropha curcas* seeds are rich in oil (28-32%), which can be converted to high quality biodiesel.

Similar findings were reported by Ginwal *et al.*, (2004) where the maximum oil yield was 39.12 per cent from whole seed and 58.12 per cent from kernel. The oil content ranged from 33.02 to 39.12 per cent in whole seeds and 47.08 to 58.12 per cent in kernel, across the seed sources. Results indicate that genetic differences exist between the seed sources of *J. curcas.*

Kaushik *et al.*, (2007) studied the variability in seed oil content of 24 accessions of *Jatropha curcas* collected from different agro climatic zones and oil variability ranged from 28.00 per cent to 38.80 per cent. Seed weight had positive correlation with seed length, breadth, thickness and oil content. Krishnamurthy, (2005) studied two species of *Jatropha* namely *J. curcas* and *J. glandulifera.* Although only *J. curcas* is being promoted for bio-diesel, *J. glandulifera* is known for its beautiful flowers.

The seeds of *J. curcas* contain 48% oil, while that of *J glandulifera* contain 27 per cent oil. This implies that oil content also varies with species.

Makkar *et al.,* (1997) found similar oil content from *Jatropha curcas* L. Eighteen different provenances of *Jatropha curcas* from countries in West and East Africa, North and Central America, and Asia were characterized for nutrient and antinutritional factors. The study revealed that oil content in *Jatropha curcas L.* was 33–39 per cent.

These reports and present findings indicate that the agroclimatic and/or edaphic factors have influenced lipid metabolism and oil contents. It may also be due to minor ecotypic or genetic differences, leading to differences in oil contents in seeds of accessions pooled from different locations varying in soil, climatic and rainfall patterns.

5.3. Phorbol esters

Phorbol esters are the major toxic and this was quantified by using HPLC where an isocratic mixture of 80 per cent acetonitrile and 20 per cent water was used and the retention time of phorbol esters was, 6-11 minutes.

Haas and Mittelbach, (2007) reported similar findings where the isocratic mixture of 80:20 acetonitrile and water which reduced the retention time of phorbol esters by about 30 minutes to 6-11 minutes, similar to the results obtained. This was also compared with Wink.*et al.* (1997) where 60:40 per cent acetonitrile and water was used and found the retention time of the phorbol esters was 40-48 minutes.

Gaudani *et al.*, (2009) concluded that the peaks of phorbol esters were detected between the retention time 20-25 minutes, and they suggested that efficient protocol has to be developed for isolation and

characterization of phorbol esters. The difference in the retention time was due to the adoption of gradient solvent system.

5.4. Phorbol esters content in seeds

The phorbol esters were extracted from the seeds of *Jatropha curcas.* The extraction was done by using methanol because it has greater affinity towards phorbol esters. The concentration of phorbol esters ranged from 0.02 to 3.97 mg/g in the seeds (Table 4).

The contraction of high concentration of phorbol esters may be due to genetic and environmental factors. Similar results were reported by Herrera *et al.,* (2006) where the phorbol esters ranged from 0.08-3.85mg/g in the seeds. Gaudani *et al.,* (2009) also reported similar findings where the phorbol esters ranged from 0.44-2.15 mg/g in case of oilcake and 3.99- 5.48 mg/g in case of oil. This variation in the phorbol esters was due to partial solubility of oil in methanol during the extraction of phorbol esters. Aregheore *et al.*, (2003) reported that the content of phorbol esters was 1.78mg/g. Winks *et al.*, (1997) and Makkar *et al.,* (1997) found similar results in the level of phorbol ester which ranged from 0.87-3.32 mg/g.

The use of seed oil as a cooking medium or meal cake as an animal feed is not been possible due to the presence of toxic compounds (Gubitz *et al.,* 1999). There are scanty reports in the literature with respect to detoxification procedures.

The present study has evaluated accessions from across Southern Karnataka and could recognize the occurrence of clones with least toxicity were TERI-1 (R_3 V_1), Kollegal, (R_1 B_2 L_{19}), Arasikere, (R_2 B_5 L_{14}). GKVK-IV, (R_2 C_{17}) and Magadikaimara, (R_1 B_4 L_{16}).

5.5. Phorbol esters in oil

Phorbol ester present in *Jatropha curcas* oil has been identified as a major toxic agent that is responsible for Jatropha curcas toxicity. Due to this, different detoxifying methods were adopted to reduce the Phorbol ester content (Table 6) where it signifies that methanol treatment (1.44 mg/g) was best followed by bleaching (1.97 mg/g).

Similar work was conducted by Haas and Mittelbatch, (2000) where they followed different refining process and found that the content of phorbol esters in untreated seed oil was 0.31 per cent which reduced to 0.17 per cent, thus about 45 per cent of phorbol were removed.

Ahmed and Salimon, (2009) also conducted the work on phorbol ester content of three different provenances of tropical *Jatropha curcas seed* from Malaysia, Indonesia and India and they found that there was a significant variation in the oil content and phorbol ester content of the seed from three provenances. Phorbol esters level was low in Malaysian seed oil (0.23%), whereas the level of phorbol esters on Indonesian and Indian seed oil were 1.58 per cent and 0.58 per cent respectively. Neutralization and bleaching led to the significant reduction of phorbol esters content.

5.6. Phorbol esters in oilcake

Phorbol esters are one of the major toxic compounds that are present in *Jatropha curcas.* Different detoxifying methods were employed to reduce the phorbol ester content (Table 7). Results showed that hydrochloric acid treatment followed at present was best method where the phorbol ester content was reduced from 3.07 mg/g to 0.09 mg/g.

Rakshit *et al.,* (2008) reported that, the cake after oil extraction is a rich source of protein but due to its toxic nature it can't be used as

animal feed and hence the meal was subjected to alkaline and heat treatments to deactivate the phorbol esters. The phorbol esters content under various alkaline treated meal ranged between 2.4 and 4.8 mg/g and hence the diet consumption and growth rate was poor. The phorbol esters content depends on the meal after processing.

Heat treatment alone could not decrease the concentration of phorbol esters. Aregheore *et al.,* (2003) have reported that, chemical treatments in addition to heat treatment are necessary to bring down concentration of phorbol esters content. Therefore in the present study heat treatments such as autoclaving and roasting were employed and estimated phorbol ester contents were 0.81 and 2.27 mg/g respectively.

Areghreore *et al.,* (2003) reported that the diet consumption in alkali treated meal group was higher compared to dehulled and ghani pressed meal. The results indicated that calcium hydroxide and sodium hydroxide at 2 per cent level were better in removing the phorbol esters. They have further reported that the concentration of 0.13 mg/g of phorbol esters present in the meal which was obtained after treatment was used to prepare the meal where the concentration was reduced from 2.08 mg/g. This was similar to the result obtained where the concentration of phorbol esters was reduced from 3.06 mg/g to 0.18 mg/g in case of alkaline treatments.

Herrera *et al.*, (2006) reported that autoclaving reduced the phorbol ester content to the extent of 95.8 per cent. They also reported that the high level of phorbol esters was due to genetic or environment factors. Behura *et al.*, (2008) also found similar results where there was decrease of toxic compounds in simarouba.

The treatment with hydrochloric acid, followed by sodium hydroxide along with washing with 92 per cent methanol treatments

seems to be ideal at present to detoxify the cake. However, other ways and means may have to be found by experimentation to remove not only phorbol esters but also other toxins that are identified/unidentified. However, the cake treated with hydrochloric acid may be tested for its fitness as animal feed.

5.7. Chemical composition of oilcake

Nutritional components are the most important factors that determine the quality of the *Jatropha curcas* seed/oilcake. Nutritional studies were carried out before and after treatment and the results are tabulated in the Table 8.

5.7.1. Residual oil in oilcake

The oil estimated by using soxtherm apparatus showed oil content to be in the range of 1.08-6.54 per cent. Maximum oil yield was obtained during roasting process i.e. 6.54 per cent. Treatment with sodium hydroxide and sodium hypochlorite to reduce residual oil showed maximum reduction (1.08%). Similar effect was observed in sodium hydroxide followed by methanol wash treatment (2.09%). Significant reduction in other treatments was also noticed.

Sirisomboon and Kitchiya, (2009) reported that at high drying temperatures the oil yield also increases which is similar to results obtained where the oil was increased from 6.25 to 6.54 per cent. The increase in the oil yield results in the increase in the acid value. This indicates the high free fatty acid which caused high abrasion of metals. But the total oil content remained the same with decrease in moisture content in the roasted sample which indicates increase in oil content.

5.7.2. Carbohydrate in oilcake

Carbohydrates are main source of ready energy. Carbohydrate values calculated showed that it ranged from 30.04 to 50.96 per cent. The variation in the carbohydrate content was due to high presence of other nutrients such as oil, protein, crude fibre and moisture content. Possible hydrolysis breakdown is contributing to the increase of carbohydrate content. Treatments such as alkaline hydrolysis by sodium hydroxide followed by 92 per cent methanol, sodium hydroxide with sodium hypochlorite, sodium bi-carbonate and sodium hydroxide along with water wash may be the cause for reduced carbohydrate levels, whereas acid (hydrochloric acid) treatments might have been responsible for the breakdown other substances like protein, ash etc to higher values of carbohydrates.

According to Raghuram *et al.*, (1997) 60-70 per cent of total calories should be contributed through carbohydrates. But in the present study it ranged from 30 to 50.96 per cent.

5.7.3. Protein content in oilcake

Calculated protein values with different treatments ranged from 24.67 to 32.54 per cent. Maximum increase was found in sodium hydroxide treated with methanol. During the treatment other chemical components was lost at higher proportion and hence lead to increase in the protein content.

High protein per cent (32.54%) in cakes treated with sodium hydroxide with 92 per cent methanol was followed by roasting (30.77%), calcium oxide (29.67%), autoclave (29.56%), sodium bi-carbonate (29.04%) and sodium hydroxide with water wash (28.44%) over the control sample (27.08%) which may be due to higher loss of other

chemical component. Decrease in calculated protein values in hydrochloric acid treatment may be due to protein degradation.

This can be compared with the results obtained by Aregheore *et al.*, (2003) where they also found increase in the protein content from 25.6% to 55% which was due to higher loss of non-protein component. Defatted meal has been found to have high amount of protein which was 50% (Makkar *et al.,* 1997, Herrera *et al.*, 2006).

5.7.4. Moisture content in oilcake

Determination of moisture is one of the crucial aspects of seed quality assessment. Moisture was determined by different methods (Table 8). Among them hot air oven method was found to be reproducible. The results on moisture content ranged from 3.20 to 23.03 per cent with significant variation within the treatments. Roasting was very effective in reducing moisture content to maximum extent. The other treatments where alkali either alone or in combination, enhanced the moisture per cent in the cake, showing increase in water hydration of cake indirectly by alkali.

Similar results were recorded by Garnayak *et al.,* (2008) where the moisture content ranged from 4.75 – 19.51 per cent. This may be due to the physical properties of the seed which includes the length, width, thickness etc. this was similar to the results obtained when the seed was treated.

The drying characteristic and physical properties of *Jatropha curcas* after heat treatments were investigated by Sirisomboon and Kitchiya, (2009). They also found that there was reduction in moisture content as the temperature increases.

5.7.5. Ash content in oilcake

Ash is one of the nutritional parameter. Ash content ranged from 2.2 g – 12.74 per cent. During the treatment of sample with sodium hydroxide there was a increase in the ash content. Similar results were obtained in the study conducted by Behura *et al.,* (2008).

Greater ash contents in samples treated with sodium hydroxide + Sodium hypochlorite and sodium bi-carbonate may be due to residual effects of chemicals used. Whereas water soaking might have complete solubility of some water soluble material and hence there was decrease in ash content. The most effective treatments i.e hydrochloric acid and sodium hydroxide followed by 92 per cent methanol wash may be due to acid hydrolysis, alkaline effect and dehydration.

5.7.6. Crude fibre content in oilcake

Crude fibre content ranged from 5.09-11.85 per cent. Crude fibre content usually raised from the untreated sample. These results were similar to the work conducted by Behura *et al.,* (2008).

Reduction in crude fibre (5.09%) over the control (6.36%) due to roasting may be attributed to oxidation and removal of some simple fibre components. Whereas increase in fibre contents in other treatments, may be due to additive effects of some cake components becoming insoluble.

5.8. N, P, K content in *Jatropha curcas L.* oilcake

The N, P and K content of the oilcake of *Jatropha curcas* L. oilcake was estimated using the standard procedures. The results indicated that the oilcake obtained from the *Jatropha curcas* seeds consisted of good amounts N, P and K (3.95% of N, 0.9-2.14% of P and 1.31-1.68% of K). This showed that the oilcake could be used as manure in agriculture where it can act as a source of plant nutrients.

The results are in confirmation with the results of Srinivasappa, (2004) where he analysed the N, P and K content in Jatropha and found it contained a good amounts of nitrogen (3.2-4.4%), phosphorus (1.4-2.09 %) and potassium (1.2-1.68%). Ramanathan, (2006) also treated the byproduct of Pongamia seed extraction as a source of organic plant nutrients as it contains 3.9 per cent N, 1.0 per cent of P and 1.3 per cent of K. The available N, P and K content of *Jatropha curcas* seed cake is almost similar to that of Pongamia seed oilcake and it could be used as a soil amendment and also a source of plant nutrition in organic farming which helps to increase the yield or productivity of crops or plants.

5.9. Detoxification of biodiesel

Biodiesel is an environmental friendly renewable fuel. Jatropha seeds can be a feedstock to produce a valuable amount of oil to be converted to biodiesel using transesterification process. As an alternative to diesel fuel, biodiesel must be technically feasible, economically competitive, environmentally acceptable and readily available.

Biodiesel is produced from oil through a process of transesterification, toxic compound that is phorbol esters is present in higher quantity in biodiesel also. Hence efficient methods were also employed for the detoxification of biodiesel. Only bleaching could be adopted for the reduction of phorbol esters (Table 10).

During the process of sodium hydroxide treatment, the conversion of biodiesel into soap formation was observed. Due to this the yield and quality of biodiesel was decreased. When biodiesel was treated with methanol for detoxification, separation of methanol from biodiesel was not observed on settling. Hence methanol leads to be evaporated during which phorbol esters could not be removed. During the process of biodiesel production, biodiesel was already treated with hot water to

remove the contaminants like catalysts, glycerol and other water soluble which could not remove the phorbol esters from biodiesel.

When biodiesel was extracted with methanol, the separation was not possible by centrifugation because biodiesel was completely solubalized by methanol and therefore resulted in higher concentration of phorbol esters i.e. 5.39mg/g than Jatropha oil and oilcake.

Similar observations were recorded by Gaudani *et al.,* (2009) in Jatropha oil and oilcake where oil had more phorbol esters than cake and this is due to partial solubility of oil in methanol during the extraction of phorbol esters from the oil sample. These results can be comparable with the results obtained in case of detoxification of biodiesel.

In toto the treatments like hydrochloric acid, sodium hydroxide followed by washing with 92 per cent methanol wash, sodium bicarbonate, which exhibit removal of phorbol esters from the oilcake to make it edible sum to be very promising. The oilcake to make it edible was promising. The oil contents in the cake in these treatments were 6.53, 2.09 and 4.60 per cent. The corresponding carbohydrates of the treated cake were 50.96, 42.65 and 41.38 per cent. Crude fibre in cake was 9.75, 8.50 and 8.02 and protein contents were 24.67, 32.54 and 29.04 per cent.

Keeping in view the objectives stated earlier to remove the toxic substance i.e., the phorbol ester and make the oilcake fit for edible purposes, the above experiment was carried out employing different treatments and the results showed that the treatments cited above had significantly reduced the toxic components from the cake. At the same time the nutrients like oil, carbohydrates, crude fibre and protein seem to be retained at higher levels and these nutrient factors along with NPK

showed that the oilcake detoxification is effective in making it fit for consumption as feed or food. However, further refinement of the technologies may be necessary to make the process of complete detoxification of oilcake and retention of the nutritional composites intact.

Summary

VI. SUMMARY

Present study was conducted at the Department of Forestry and Environmental Science, University of Agricultural Sciences, GKVK, Bengaluru during 2010-2011. Quantitative and qualitative factors of seed, oil, seed cake and estimated the phorbol ester content in the oil and in the cake. Since these factors are related to toxicity and are the deterrents in the use of oil and oilcake either as animal feed or for human consumption. Different detoxification methods were adopted to know the treatment effects in removing the toxic component, the phorbol esters. The objectives of the study involved (a) to estimation of the toxicity levels in selected *Jatropha* accessions (b) to find out the relationship of toxin with oil and yield parameters (c) to develop methods to reduce the toxic level in oil, oilcake and biodiesel.

The seed samples were collected from plants assembled from different location in southern Karnataka and maintained as germplasm collection in UAS, GKVK, Bengaluru. Fruits from different accessions were collected and seed yield per plant was calculated. Seed samples of 20 accessions were estimated to know the oil percentage. It was found that significant differences occurred in the oil contents of samples within the range of 21-40 per cent.

The seeds were extracted with methanol and by using HPLC, the content of phorbol esters were was found out which ranged from 0.02-3.97mg/g. Out of 20 accessions, 6 accessions contained phorbol esters less than 0.1mg/g which can be considered as non-toxic accessions. The accessions collected from GKVK (II and III) contained the highest amount of esters and hence this can be considered as most toxic accessions. Finally, correlation was done using above three parameters such as oil, yield and phorbol esters but the values were not significantly different.

By the above analysis we can conclude that, both toxic and non-toxic accession of *Jatropha curcas* L. were found in southern Karnataka. Due to this the process of detoxification was carried out using the samples (oil, oilcake and biodiesel) collected from GKVK.

Four different treatments were given to detoxify the oil which also contained the highest amount of phorbol ester (3.08 mg/g). Methanol treated sample gave the best results where the phorbol esters content was reduced to 1.44 mg/g followed by the bleaching (1.97 mg/g) and degumming (2.36 mg/g). During detoxification neutralizing of oil could not be done because sodium hydroxide treatment leads to soap formation in oil.

Different treatments like autoclave, sodium hydroxide followed by three times washing with 92 per cent methanol, sodium hydroxide and sodium hypochlorite, calcium oxide, sodium bicarbonate, hydrochloric acid, roasting, sodium hydroxide followed by three times washing with water and water soaking were carried out for detoxifying the oilcake samples. The results showed that, the untreated samples contained highest amount of esters (3.07 mg/g). This was reduced by different treatments and among them oilcake treated with hydrochloric acid was found to be the best (0.09 mg/g) treatment along with sodium hydroxide followed by three times washing with 92 per cent methanol in reducing phorbol ester levels.

Biodiesel also contained highest amount of phorbol esters which was higher than oil and oilcake (5.38 mg/g). This was due to complete dissolution of biodiesel in methanol. Detoxification of biodiesel was also performed by using different treatments. Neutralizing of oil could not be done due the formation of soap during the process. Methanol treatment was tried but separation of the layers was not possible due to complete

dissolution. Hence only bleaching was done which reduced the phorbol esters content 2.39 mg/g.

Detoxification was mainly done to remove toxic substances and make it fit for use as cooking oil, animal feed etc. Hence, the chemical composition of the oilcake was determined by using different methods. There was a large variation in the chemical composition of the oilcake before and after treatment. Protein content and crude fibre were increased after detoxification for most of the treatments. Similar results were obtained for nitrogen, phosphorus and potassium content in oilcake.

Finally it is concluded that, hydrochloric acid treated samples can be done to reduce the toxic compounds and thus can be used as animal feed. Detoxification of oilcake was to see that it can be used as a supplementary feed stock for animals and also as protein supplement in human diet. Though there was success in detoxification of cake to a great extent, further refinement is needed.

Future line of work

The recognition of elite clones with fairly good yield with least toxicity is the major finding of the study. Trial studies are required in different agroclimatic zones to see the impact of environment on the formation of phorbol esters. Following these efforts, accession could be further improvised for taking them for large scale growing along bunds and borders in different agro climatic regions.

Effective vegetative propagation protocols need to be established for large scale production of the planting material.

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