Rational Use of *Jatropha curcas* L. in Food and Medicine

From Toxicity Problems to Safe Applications

Muhamad Insanu

The research described in this thesis was conducted at the Department of Pharmaceutical Biology (Groningen Research Institute of Pharmacy, University of Groningen, The Netherlands) according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences, University of Groningen, The Netherlands).

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CHAPTER 1

Introduction and scope of thesis

As time goes by, the number of vehicles in Indonesia is increasing rapidly. Based on statistical data, the total number of vehicles in 1991 was 9,582,138, whereas in 2011 the number had increased almost 10 times to 85,601,351 (Fig. 1). It was followed by an enormous increase in fuel usage, from 15,191 million BOE (Barrel Oil of Equivalent) in 1990 to 388,241 million BOE twenty years later. This is quite worrying, both from an environmental as from an economical viewpoint. Consequently, the crude oil prices have increased whereas in 2004 the price was only 36 $ per barrel, in 2012 the price rose to 112 $ per barrel (Fig. 2). These conditions were followed by a decline of Indonesian national oil reserves. According to statistics (Fig. 3) the number was 740,824 billion barrels in 2012. This means that the reserves will be exhausted within the next two decades if there were no alternative sources.

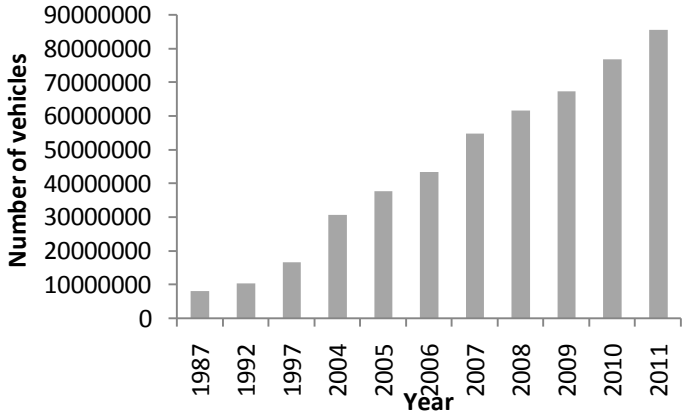

Figure 1. Total number of vehicles in Indonesia [1]

One hot issue that is connected with the increase of fuel usage is global warming. It affects weather and climate. The mainland and water temperature increase and they lead to the occurrence of storms with great power and cause forest fires. This produces thick smoke and has negative effects to the human respiratory system. Other effects of global warming are the thawing of glaciers at both poles, the rise of the sea level posing a potential flooding in some areas, the disruption of the ecological balance in the ocean and polar areas that might lead to the extinction of animals and plants species. The most dangerous effect is sociocultural when there is a war between human beings to fight for certain region.

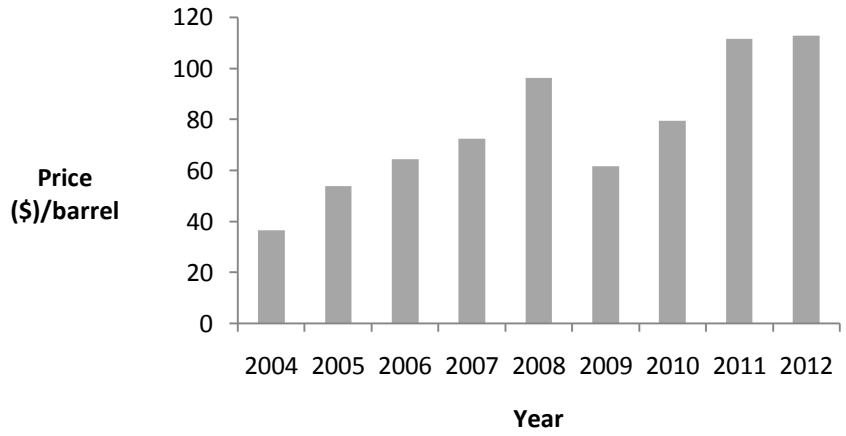

Figure 2. The price of crude oil in the world [2]

Considering so many side effects that may occur because of the increase of fuel usage, the government of the Republic of Indonesia issued Presidential Regulation No. 5 in 2006. It was about the national energy policy to develop alternative energy sources instead of fossil fuels. The regulation puts emphasis on natural resources, which can be renewed as an alternative energy. The policy was strengthened with the Presidential Instruction No. I in 2006, which regulated the supply and the use of biofuels as alternative energy sources.The support from government policies led to an increase in researchsearching for alternative energy sources from plant materials. Some sources may be used such as corn, palm oil, and *Jatropha curcas* oil (Tab. 1). Based on previous reports, in 2006 corn plantation area in Indonesia was 3.5 million hectares, with an average yield of 3.47 tonnesdry weight per hectares (ha), the national production was 12.145million tonnes. Palmoil was investigated as another source. Until now there is 7,641,753 ha area in Indonesia used as palm plantations. This industry has developed quite rapidly. During 1980-1990, the plantation areas increased by 11% per year, which increasedthe production rate by 9.4% per year. In 2001-2004 the growth in area was 3.97% while the growth in production rate was 7.25% [3]. Based on estimations, in 2020 Indonesian Crude Palm Oil production will be about 17 million tonnes. *Jatropha* oil was selected as alternative source. Based on the productivity calculation, per year, one tree produces 3.5-

4.5 kg seeds. In 1 ha it contained 2500-3300 trees. If we calculated with an average oil content of 35% in the fruit, *Jatropha* produces 2.5-5 tonnes oil per year per ha.

From the three sources mentioned, *Jatropha* oil is more attractive than others, because the oil is not edible so there will be no price competition with food as is the case with palm and corn oil. Besides that, the oil content in the fruit can reach 60% and the cost of production is quite low, so the estimated selling price of *Jatropha* oil would be cheaper compared to petroleum diesel and crude palm oil.

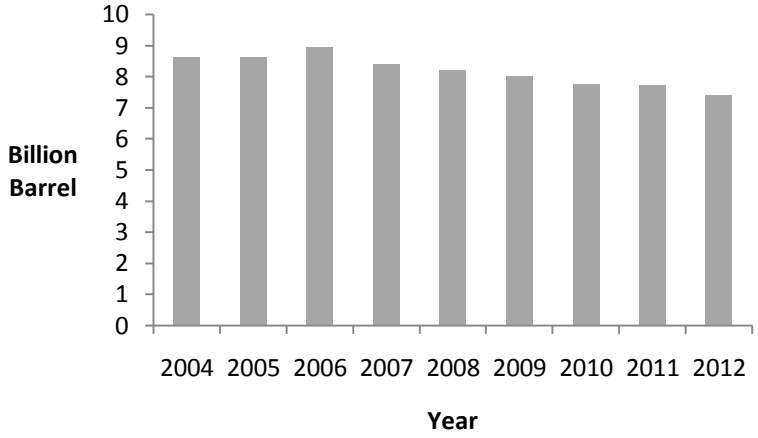

Figure 3. National oil reserves [3]

Jatropha curcas L. is classified as euphorbiaceae family. This plant has economically of potential value, because different parts of the plant body have their own values. In Thailand whole parts of the plant are traditionally used as live fence. It controls

soil erosion. *J. curcas* L.is also known as the source of biodiesel, because from the seed, oil can be isolated by direct compression. This oil is used as a biofuel, candle and soap production, lighting and lubricant. In Europe the deoiled seedcake is believed to be suitable as animal feedstock and biofertilizer. In some rural areas in Indonesia the latex was traditionally used for treating toothache.

Since *J. curcas* L. is considered as a future source of biodiesel, many people in Indonesia plant it in a huge plantation. they think if they can produce high amounts of oil from *J. curcas* L., it will replace the petroleum usage. Although, *J. curcas* L.is known to have many other usages, but the farmer did not realize they thought this plant only produced oil without any beneficial usage. So, they will be loss. This situation leads to a new concept that *J. curcas* L. should not only be used as biodiesel source, but it should give additional values to a farmer who plants this crop.

Table 1. Plants as alternative energy sources [4]

No	Species	Part	Oil (%)	Edible
1	*Ricinus communis*	seed	45-50	Non edible
2	*Jatrophacurcas*	kernel	40-60	Non edible
3	*Arachis hypogea*	kernel	35-55	Edible
4	*Ceiba pentandra*	kernel	24-40	Non edible
5	*Hevea brasilensis*	kernel	40-50	Non edible
6	*Cocos nucifera*	kernel	60-70	Edible
7	*Moringa oleifera*	seed	30-49	Edible
8	*Aleurites moluccana*	kernel	57-69	Non edible
9	*Sleichera trijuga*	kernel	55-70	Non edible
10	*Azadirachta indica*	kernel	40-50	Non edible
11	*Adenanthera pavonina*	Kernel	14-28	Edible
12	*Elais guineensis*	Pulp + Kernel	46-54	Edible
13	*Theobroma cacao*	Seed	54-58	Edible
14	*Sterculia foetida*	kernel	45-55	Non edible
15	*Callophyllum inophyllum*	kernel	40-73	Non edible
16	*Shorea stenoptera*	kernel	45-70	Edible
17	*Sesamum orientale*	seed	45-55	Edible
18	*Croton tiglium*	kernel	50-60	Non edible
19	*Annona muricata*	kernel	20-30	Non edible
20	*Annona squamosal*	seed	15-20	Non edible
21	*Cinnamomum burmanni*	seed	30	Edible
22	*Oryza sativa*	Bran	20	Edible
23	*Zea mays*	Germ	33	Edible

The aim of this thesis is to give an overview of the additional values of *Jatropha curcas* L. plant by characterization of its natural products that can be used as a safe pharmaceutical product. In addition the detoxicification of the plantcake allowing it to be used for animal stock has been researched. This thesis is a part of larger project for valorization *Jatropha curcas* L. plantation, especially in Indonesia.

Recent developments in the technology of detoxification process and application of this ethnomedicinal plant to new fields of experimental medicine are reviewed in **chapter 2**. In this chapter recent data on biological activities, concepts and strategies for turning a toxic plant into a valuable crop with high pharmaceutical value are also discussed.

A group of toxic compounds, which are relevant to study in *J. curcas,* are phorbol esters (PEs) since they are known as tumour promoter. In analysing those phorbol esters, phorbolmyristic acetate is used as a standard. This compound has two isomers which are α and β. In **chapter 3** the differences between both isomers are discussed using LC-UV and LC-MS. Selecting wrong standard can lead to quantification error of PEs.

From different parts of *J. curcas* some interesting compounds were isolated. Some of them were investigated for their biological activity, but others were only chemically analysed. Curcacycline A and B were isolated from *J. curcas* latex. In **chapter 4** full synthetic approach, structure elucidation and

biological activities of both curcacyclines were described. Some assays like antibiotic, cytotoxic, ecotoxic and mutagenic activities were investigated to shed light on the pharmacological activity of those compounds.

J. curcas seeds contain up to 60% of oil. The residues from oil processing are called *Jatropha* kernel meal. This meal can be used for animal feed. But the use is limited since it contained toxic substances. Some studies have been done to detoxify the meal and in this thesis the detoxified meal has been analysed. In **chapter 5**, the validation of detoxification processes are performed using cytotoxic and mutagenic assays. Colo 205 and OVCAR3 were used as cell lines in a cytotoxicity test while *Salmonella typhimurium*TA 98 and TA mix *(*TA 7001, TA 7002, TA 7003, TA 7004, TA 7005, TA 7006) were used in a mutagenicity assay.

References

1. Indonesian Statistic Centre, 2013, Perkembangan Jumlah Kendaraan Bermotor 1987-2012.

2. Indonesian Directorate of Energy, 2013, Statistik Minyak Bumi

3. Directorate General of cultivated plantation production, 2005, Pokok-Pokok Rencana Makro Pengembangan Agribisnis Komoditi Perkebunan 2005-2009

4. Soerawidjaja, T. H., 2006, Fondasi-Fondasi Ilmiah dan Keteknikan dari Teknologi Pembuatan Biodiesel, Yogyakarta

CHAPTER 2

Rational use of *Jatropha curcas* L. in food and medicine: from toxicity problems to safe applications

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Abstract

Jatropha curcas L. has become an important plant for biorefinery and production of biodiesel. From its ethnobotanical use, the plant is known for several activities which are associated with high toxicity. The latest development in engineering technology enables detoxification of native oil and other parts of the plant for new pharmaceutical purposes. Hence a revised look to the rich metabolic spectra of partly structurally rare secondary compound becomes an interesting field of research to be explored. In this review, we discuss recent developments in the technology of detoxification process and give insight about how this ethnomedicinal plant can be applied to new fields of experimental medicine. The review highlights recent data on biological activities and discussed concepts and strategies for turning a poison plant into a valuable crop with high pharmaceutical potential.

2.1 Introduction

Jatropha curcas L. widely known as physic nut or purging nut, is one of the oldest members of Euphorbiaceae. From the fossils founded in Belem, Peru, the age of this plant is approximately 70 million years. The name was given by Karl von Linné in 1743 which means doctor (iatros) and food (trophe) [1]. The plant is found in tropical regions of Africa, South America, South East Asia and India [2]. *Jatropha curcas* L. is classified as a large shrub or a small perennial tree because it can attain 5 m in height, while under several conditions the height can reach 8 or 10 m [3]. It has soft wood with subtle grey bark and when it is cut, it produces white and milky latex [4].

Jatropha curcas L.is a plant with multiple uses and considerable economic potential. In the tropical countries, it serves as a live fence in the fields and settlements and in arid areas it is cultivated to control soil erosion. The deoiled seedcake can be used for organic fertilizer without any detectable phorbol ester both in the crops and soil [5].

Jatropha curcas L. has a potential for controlling environmental pollution. Grounded seeds of *J. curcas* L. have been demonstrated as an effective natural coagulant for industrial effluent. Treatment of contaminated waters or soils is an approach that gains popularity. Although the conventional physical, chemical and thermal waste treatments are fast and controllable, it requires high energy that renders them very costly.

This plant is also known as source of biodiesel, the seed consist of 60-68% of kernel which contain up to 60% oil depends on geographical location (humidity, altitude, temperature, etc.). The oil can be used directly or in methylester form as biodiesel [6-9].The oil has been traditionally used for soap or candle production, lighting and lubricant. It was observed that this fatty acid composition of the oil was suitable for human nutrition. The kernel also contains a high amount of crude protein (up to 32%), which could be used as an animal feed [10].

Jatropha curcas L. is known as two genotypes, the toxic and non-toxic. The difference between both types, is the presence of phorbol esters in the seed. Non-toxic varieties from Mexico contain very low to undetectable amounts of phorbol ester while the other one contain up 3500 ppm. No differences were found in the level of amino acids, trypsin inhibitor, lectin, phytate,curcin and saponin between these two genotypes [7, 11-13].

The objectives of this review are to make a validation of the secondary natural products of *Jatropha curcas* L., to review its toxic principles and the possibility of detoxification process for safe use in animal feed and pharmaceutical purposes.

2.2 Chemical Composition

2.2.1 Compounds from the primary metabolism

Chemical analysis of *Jatropha curcas* L. revealed the presence of primary metabolism in the seeds of the plant. Chemical analysis of *Jatropha curcas* L. kernel and seed meal as well as the fatty acid composition of the oil was reported [14] (Tab. 1 and 2). The presence of *cis*-11-eicosaenoic acid (20:1) and *cis*-11,14-eicosadienoic acid (20:2) in *Jatropha curcas* L. seed oil from four Mexican provenances was investigated. The same study revealed that the content of starch and total soluble sugars was below 6%, while the levels of essential amino acids except for lysine were higher than those of FAO/WHO reference protein for a five year old child on a dry matter basis [15].

Table 1. Average chemical composition of *J. curcas* L. kernel and meal

Constituent	Kernel (%)	Defatted Meal (%)
Dry matter	94.2-96.9	100
Crude protein	22.2-27.2	56.4-63.8
Lipid	56.8-58.4	1.0-1.5
Ash	3.6-4.3	9.6-10.4
Neutral detergent fibre	3.5-3.8	8.1-9.1
Acid detergent fibre	2.4-3.0	5.7-7.0
Acid detergent lignin	0.0-0.02	0.1-0.4
Gross Energy (MJ/Kg)	30.5-31.1	18.0-18.3

Table 2. Average fatty acid composition of *J. curcas* L. oil [14]

Fatty Acid	Chain length	Composition (%)
Myristic acid	14:0	0-0.1
Palmitic acid	16:0	14.1-15.3
Stearic acid	18:0	3.7-9.8
Arachidic acid	20:0	0-0.3
Behenic acid	22:0	0-0.2
Palmitoleic acid	16:1	0-1.3
Oleic acid	18:1	34.3-45.8
Linoleic acid	18:2	29.0-44.2
Linolenic acid	18:3	0-0.3

2.2.2 Compounds from secondary metabolism

Phytochemical analysis of the *Jatropha curcas* L. roots showed the presence of many secondary metabolites including terpenoids, steroids, tannins, alkaloids and saponins (Tab. 3) [16]. Among the group of terpenoids the biological important phorbols were isolated from this plant which can be classified by different backbones structure like lathyrane, podocarpane, rhamnofolane, tigliane, daphnane (Fig.1). The toxicological relevant phorbol esters in *Jatropha curcas* L. have the tigliane and lathyrene skeleton in common. Phorbols with a rhamnofolane skeleton were isolated for the first time in 1986. These were curcusone A, B, C, and D [17] and curcusone E later in 2011 [18]. By the same author two lathyrane diterpenoids were isolated in 1986 and named curculathyrane A & B [17].

From aerial parts and stem of *Jatropha curcas* L., three lathyrane types ((4E)-15-*O*-Acetyl-15-epijatrogrossidentadione, (14E)-14-*O*-Acetyl-5,6-epoxygrossien-tadione, (4E)-15-epijatrogrossidenta-dione)), two podocarpane types (3β-acetoxy-12-methoxy-13-methylpodo-carpane-8,11,13-trien-7-one, 3β,12-dihydroxy-13-methylpodocarpane 8,10,13-triene), one dinorditerpenetype (heudelotinone) and three deoxy-preusomerins (Palmarumycin CP1, JC1, and JC2) were isolated [19, 20]. The presence of alkaloids in *Jatropha curcas* L. was marked by 5-OH-pyrrolidin-2-one and pyrimidin-2,4-dione [21], while the presence of coumarins was marked by marmesin, tomentin, propacin and jatrophin [19, 22]. Flavonoids like apigenin, vitexin and isovitexin were also found in leaves [21, 23]. Two cyclopeptides were isolated from latex. Curcacycline A possess eight amino acids in the order c[Gly-Leu-Leu-Gly-Thr-Val-Leu-Leu] and curcacycline B possess nine amino acids which were c[Gly-Ile-Leu-Gly-Ser-Pro-Ile-leu-Leu]. Curcacycline B can bind to human cyclophilin B and increase by 60% its peptidyl-prolil *cis trans* isomerase (PPI-ase) activity at 30μM [24, 25].

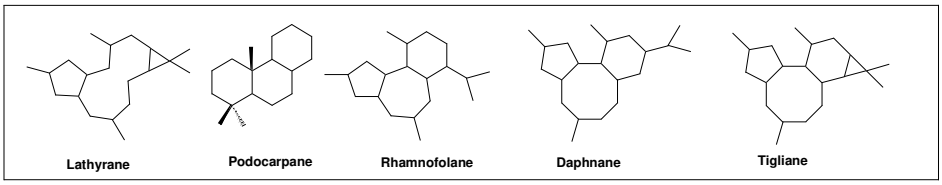

Figure 1. Basic structures of *Jatropha curcas* L. diterpenoid skeletons

2.3 Human toxicity and case reports

Acute poisoning with seeds of *Jatropha* curcas L. was reported. Abdu reported intoxication in two children aged three and five [26]. In 2005, twenty children were admitted to hospital in India because of *Jatropha curcas* L. seed ingesting. The age varied between 8-13 years. All cases showed complain of vomiting, diarrhea, abdominal pain, sensation in the throat. Vomiting was the predominant symptom (95%), diarrhea (50%), headache (40%), asymptomatic (5%). Intravenous fluid and antiemetic were given to the children, the recovery rate was six hours and after 24 hours they were discharged from medical services [27].

Table 3. Phytochemical compounds present in *J. curcas* L.

No	Chemical compounds	Type	Sources	Ref.
1.	5-OH-pyrrolidin-2-one	Alkaloid	Leaf	[21]
2.	Pyrimidine-2,4-dione	Alkaloid	Leaf	[21]
3.	2-methylanthraquinone	Antraquinone	Aerial part	[19]
4.	Marmesin	Coumarin	Root	[22]
5.	Tomentin	Coumarin	Root	[20]
6.	Propacin	Coumarino-Lignan	Root	[22]
7.	Jatrophin	Coumarino-lignane	Root	[22]
8.	Curcacyline-A	Cyclic peptide	Latex	[24]
9.	Curcacycline-B	Cyclic peptide	Latex	[25]
10.	(4*E*)-15-O-Acetyl-15-epi-jatrogrossidentadione	Diterpenes	Aerial part	[19]
11.	(14*E*)-14-O-Acetyl-5,6-	Diterpenes	Aerial part	[19]

epoxygrossidentadione			
12. (4*E*)-15-epijatrogros-sidentadione	Diterpenes	Aerial part	[19]
13. 3β-acetoxy-12-methoxy-13-methylpodocarpa-8,11,13-trien-7-one	Diterpenes	Aerial part	[19]
14. 3β, 12-Dihydroxy-13-methylpodocarpane 8,10,13-triene	Diterpenes	Aerial part	[19]
15. Heudelotinone	Diterpenes	Aerial part	[19]
16. *Epi*-isojatrogrossidione	Diterpenes	Aerial part	[19]
17. 2α-hydroxy-*epi*-isojatrogrossidione	Diterpenes	Aerial part	[19]
18. Spirocurcasone	Diterpenes	Root	[18]
19. Curculathyrane-A	Diterpenes	Root	[28]
20. Curculathyrane-B	Diterpenes	Root	[28]
21. Curcusone-A	Diterpenes	Root	[17]
22. Curcusone-B	Diterpenes	Root	[17]
23. Curcusone-C	Diterpenes	Root	[17]
24. Curcusone-D	Diterpenes	Root	[17]
25. Curcusone-E	Diterpenes	Root	[18]
26. Jatrophone	Diterpenes	Root	[29]
27. Jatrophalactam	Diterpenes	Root	[30]
28. Palmarumycin CP1	Diterpenes	Stem	[20]
29. Palmarumycin JC1	Diterpenes	Stem	[20]
30. Palmarumycin JC2	Diterpenes	Stem	[20]
31. Apigenin	Flavonoid	Leaf	[21]

32. Vitexin	Flavonoid	Leaf	[23]
33. Isovitexin	Flavonoid	Leaf	[23]
34. Curcin	Lectin	Seed	[31]
35. Tetradecyl-*E*-ferulate	Lignane	Aerial part	[19]
36. 12-deoxy-16-hydroxy phorbol-C13-C16 diesters	Phorbol ester	Seed	[9, 32]
37. Factor 1	Phorbol ester	Seed	[9]
38. Factor 2	Phorbol ester	Seed	[9]
39. Factor 3	Phorbol ester	Seed	[9]
40. Factor 4	Phorbol ester	Seed	[9]
41. Factor 5	Phorbol ester	Seed	[9]
42. Factor 6	Phorbol ester	Seed	[9]
43. β-sitosterol	Phytosterol	Phytosterol	[33]
44. Curcain	Protease	Protease	[34]
45. Stigmasterol	Triterpenes	Leaf	[33]
46. 3-O-(*Z*)-coumaroyl oleanolic acid	Triterpenes	Aerial part	[19]
47. Acetoxyjatropholone	Diterpenes	Root	[18]
48. Multidione	Diterpenes	Root	[35]

2.4 Toxic principles of *Jatropha curcas* L.

Jatropha curcas L. toxicity is mainly characterized by the presence of phorbol ester and ribosome inactivating proteins (RIP). From the last group of compound one of the representing is curcin. It can be classified as type I ribosome inactivating protein. The mechanism of action is due to depurination of the α-

sarcin loop of large rRNA. Curcin can demolish *N*-glycosidic linkage between polyphosphate backbone of the 28S rRNA and adenine at A_{4324} (alpha sarcin loop) of the rat liver ribosome. This will block protein translation. Curcin (28.2 kD) consist of 251 amino acids with the composition:Asx(31), Val(26), Leu(24), Glx(22), Ala(22), Lys(18), Ser(16), Thr(15), Gly(15), Ile(14), Tyr(14), Phe(12), Pro(9), Met(2), Arg(7), His(2), Cys(1) and Trp(1)[36]. Its biosynthesis is induced in leaves under stress conditions like drought, temperature and fungal infection, serving thus plant defense purposes [37]. It was stored in endosperm and tegmen of Madagascar and Mexican varieties [13]. Previous result reported that the curcin gene coding region has similar amino acid sequences with gelonin, bryodin, trichosantin, momorcharin, ricin A-chain and abrin A-chain [38]. The gene of the protein was inserted into the PQE30 vector. After introduction into *E. coli* this protein was successfully expressed in strain M_{15}. Although the yield in this observation was low, the result showed that 0.5 mM IPTG was suggested as an optimum inducer [39].

It was reported that curcin strongly inhibit protein synthesis in reticulosyte lysate, gastric cancer cell line (SGC-7901), mouse myeloma cell line (sp 2/0), and human hepatoma with IC_{50} (95% confidence limit) of 0.19 (0.11-0.27) nmol/L, 0.23 (0.15-0.32) mg/L, 0.66 (0.35-0.97) mg/L, 3.16 (2.74-3.58) mg/L, respectively [40]. Curcin showed no activity on Hela and MRC cells (human embryo lung diploid cell line).

In vivo application of curcin was done in mice. After 12 hours of curcin administration, some symptoms appeared in the animal, such as hypersensitivity, declining pineal and corneal reflexes, locomotorious activity, losing of grip strength and righting reflex, defecations and palpebral closure. Autopsy on dead animal showed hyperemia of the intestine and wounds in the spleen, pancreas and liver. Death occurred after 48 hours of curcin administration. LD_{50} of curcin was 9.11 IU intraperitoneal [31].

In nature we find four types of diterpenoid esters. The basic structure of the compounds is a tigliane, daphnane, ingenane, and lathyrane skeleton. The classification was based on their basic pattern containing tri and tetracyclic ring systems [41]. Tigliane is the basic skeleton of phorbol ester (PE). Hydroxylation is found in the position C12 and C13 of tigliane backbone. Esterification with various fatty acids gives a broad spectrum of phorbol ester in this plant [42].The target of PE are phospolipid membrane receptors. They activate protein kinase C (PKC), that is important for signal transduction leading to cell differentiation and cell growth regulation [43, 44]. Under normal physiological conditions, diacylglycerol (DAG) activates PKC, and enhances PKC's affinity to bind phosphatidyl serine(PS)-containing membranes. Whereas DAG is easily metabolized, PE is not and, therefore, PE acts as an agonist of DAG and uncontrollably activates the PKC with the consequence of

increasing cell proliferation. The toxic principle is that PKC activity can hardly be turned down and the system is out of regulatory control [45]. Carcinogenesis experiments on mouse skin revealed that phorbol esters stimulate tumour growth but do not induce tumours. Phorbol esters thus acts as cocarcinogens.

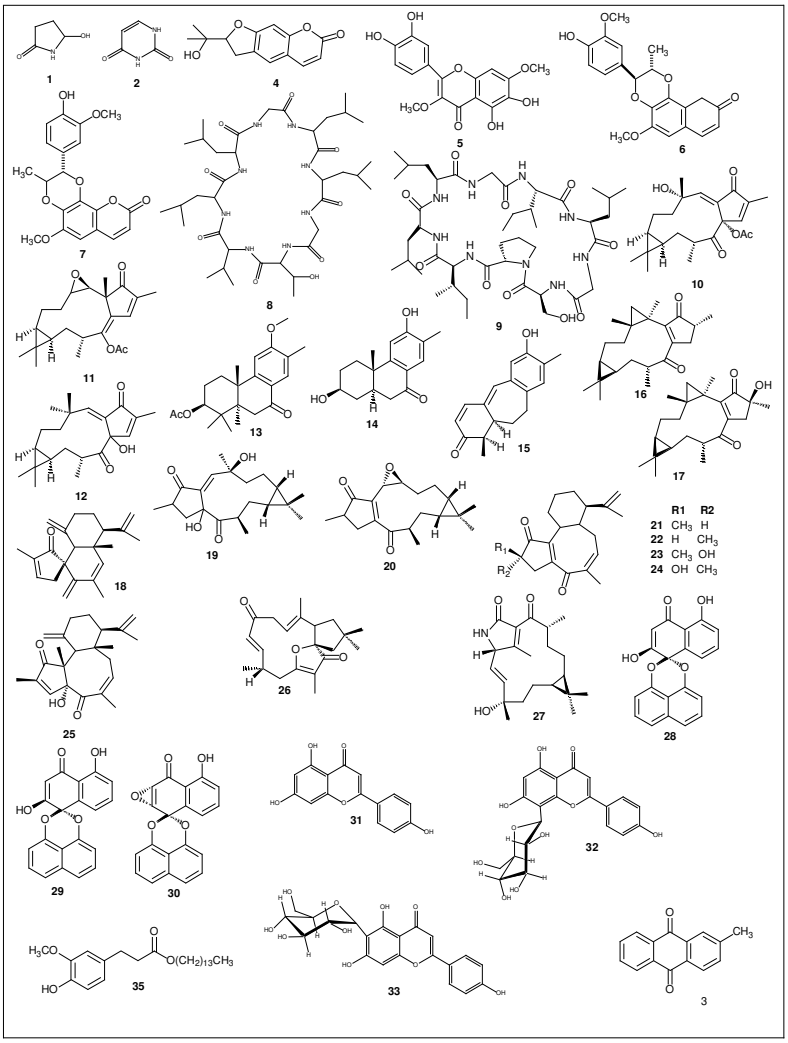

Figure 2. Various compounds identified in *J. curcas* L.

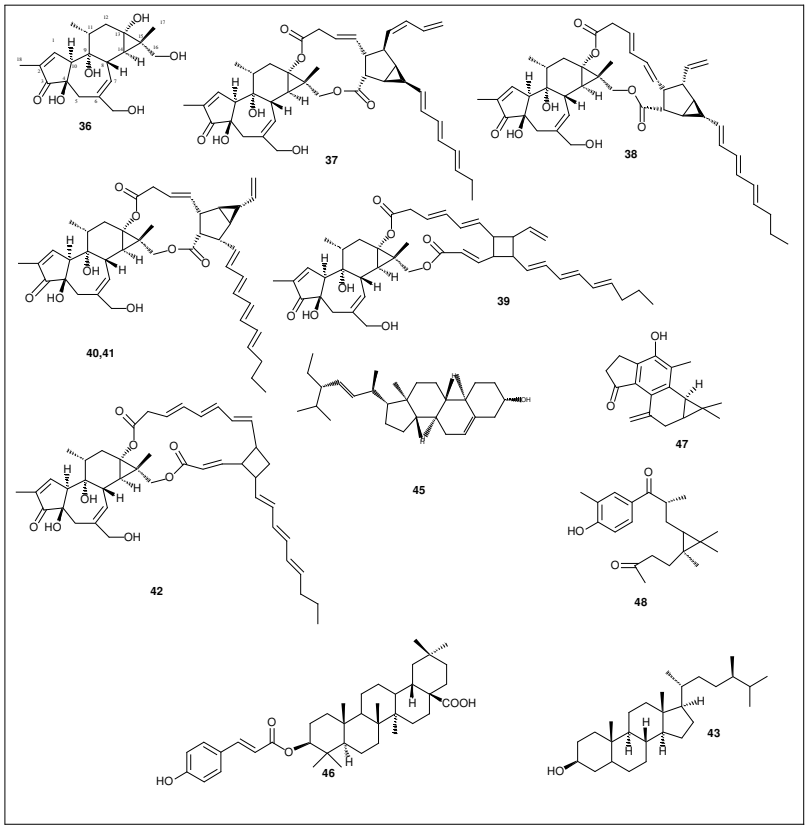

Figure 2. Continued

2.5 Detoxification Process

2.5.1 Detoxification of biomass

Many attempts have been performed to eliminate antinutritional components and toxic principles (trypsin inhibitor, lectins, phytate, phorbol esters) from the meal. Mexican people roasted the meal before it was eaten. Roasting the meal could only effect trypsin inhibitor and lectin activity [11], but the other components were

not affected by heat treatment [10]. The moist heat was more effective in decreasing lectin activity than dry heat[46]. It was observed that trypsin inhibitors and lectin were fully inactivated by double solvent extraction using hexane and ethanol coupled with moist heat treatment (20% moisture, 126^{o}C, 2 bar, 10 min) [47]. Double solvent extraction (petroleum ether and ethanol) mixed with chemical treatment using 0.07% $NaHCO_3$ eliminate 95.8% phorbol ester content. This treatment is accepted as the best method to reduce lectin activity [15]. The article described a complex detoxification strategy with protein extraction at basic pH followed by isoelectric precipitation and finally steam injection with different steps. By this procedure the level of trypsin inhibitors, phytate, tannins and saponin has reduced by more than 90% while toxic phorbol esters were not detected anymore in the meal [48].

Beside elimination of phorbol ester using physical and chemical methods, the influence of manganese chloride ($MnCl_2$) and *N*-ethylmaleimid in reducing phorbol ester biosynthesis in callus cultures were observed. Two concentrations of $MnCl_2$ were used (2 mM and 3 mM) and the content of phorbol ester in callus cultures was reduced to 30.5% and 30.6% respectively after 7 days. When *N*-ethylmaleimid was given to the callus in three different concentrations (0.6, 0.9, and 1.2 mM) the content of phorbol ester reduced to 26.6%, 6.2% and 32.2% respectively after 21 days [49].

2.5.2 Detoxification process for animal feed

Different animals show different physiological reactions to detoxified *Jatropha curcas* L. meal. For example, increasing time of heat treatment of *Jatropha* meal impacted the growth rate of fishes. Heat treatment provokes the loss of amino acid and structural changes in *Jatropha curcas* L. proteins. These changes made them difficult to be digested by fish trypsin, leading to low protein efficiency ratio and protein productive value [50]. It was reported that pigs which consumed treated *Jatropha curcas* L. meal, showed adverse effects with low level of percent packed cell volume, serum glucose, cholesterol concentration, serum alpha amylase activity ($p<0.001$) and serum triglyceride.

A method that comprises three major steps was patented. The first step was adding methanol and sodium hydroxide to form a mixture. Second step was heating the mixture, and final stap was separating it to obtain detoxified constituents. Analytical profiling of these materials using HPLC showed that PE was below the detection limit. Total crude protein content, available lysine value and protein digestibility were the same for both treated and untreated meal. Biological evaluation showed that these products were not harmful to mollusk and carps (*Cyprinus caprio*) [51].

Single step extraction with anazeotrop mixture of ethyl acetate and methanol at 62^{o}C and 1.2 bar with mixing rate 10

rpm, 6 cycles of 1 hour each was developed and patented. Desolventising was at 100°C for 80 min in a vertical steam desolventiser. For removing antinutrient factors, the meal was autoclaved with 120°C moist heat for 60 min. The recovery of oil was more than 50% of kernel weight while the content of the oil in the meal was less than 0.5% by weight. No differences in protein content and no residual PE was detected in the meal. Bioassays were carried out to determine remaining toxicity processed meal using Brine shrimps and *Drosophila* larvae assays. Based on these experiments, the processed meal showed no significant toxic effect on the larvae while 100% mortality was shown in control group with unprocessed meal [52].

The newest patent for removing phorbol ester from organic material was applied using microbial approach. *Bacillus subtilis* var natto (0.004-0.2 part by mass) with stirring and fermentation processes (37-50°C for two to four weeks) were selected for decomposing phorbol ester. Fermented *Jatropha curcas* seedcake showed better weight gain, feed intake and health condition than unfermented seedcake in mice. All of values were almost the same in fermented and soya bean groups [53].

Detoxification of *Jatropha curcas* L. meal was done using hydrolysis by enzymes (cellulase and pectinase) continued with ethanol washing was investigated. These treatments decreased the level of phorbol ester, trypsin inhibitor, lectin activity, tannin

and saponin to tolerable values. There were enhancement in crude protein and *in vitro* protein digestibility values from 60% to 75% and 82%to 92% respectively [54].

2.6 Biological Activities of *Jatropha curcas* L.

2.6.1 Antinutrient compounds in Jatropha curcas L.

The *Jatropha curcas* L. plant synthesizes some compounds that act as anti-nutrients or toxins in living organisms. Antinutrients have been characterized as substances that interfere with food utilization of animals. These phytochemicals can be generally divided into four groups: i. factors affecting protein digestion such as trypsin inhibitors, tannins, lectins; ii. factors affecting mineral utilization, which include phytates, gossypol pigments, oxalates, glucosinolates; iii. Antivitamins; iv. miscellaneous substances like mycotoxins, cyanogens, nitrate, alkaloids, phyto-estrogens and saponins [55]. High level of trypsin inhibitors content caused low *in vitro* rumen degradable proteins [10].The effect of trypsin inhibitor was decreasing protein digestibility, due to the interference with pancreatic proteolytic enzymes [56]. Phytate decreased bioavailability of protein and minerals (Ca^{2+},Mg^{2+}, Cu^{3+}, Fe^{3+})by forming complexes with them [57, 58].

2.6.2 Antimicrobial and antiparasitic

The latex of *Jatropha curcas* L. has strong antimicrobial activity when it was applied to both gram positive and negative bacteria [59].

Not only toxic to bacteria, *Jatropha curcas* L. latex was also toxic to parasites. No embryonation was found in ova of *Ascaris lumbricoides* and *Necator americanus* incubated in pure and 1:1 diluted latex. Mosquito eggs could not hatch in the latex of *Jatropha curcas* L. [59]. The methanol extract of the oil was also tested at two larvae stages (cercariae and miracidia) of *Schistosoma mansoni*. The extract showed toxicity to both of the larvae, but cercariae was more sensitive to the extract than miracidia. Decreasing the growth of *Schistosoma sp.* became important, since these species cause schistosomiasis which is one of the most serious parasitic diseases after malaria [60].

2.6.3 Antiviral activity

Water extract of stem bark of *J. curcas* L. showed good suppressive effects on HIV growth. The effect was determined by HIV-1 induced cytopathic effect on MT-4 cells, HIV-1 reversed transcriptase (RT) assay and HIV protease assay (PR) to identify the mode of action on inhibiting HIV growth while no inhibitory effects were found in both HIV-RT and HIV-PR assays. It was concluded that the results were significant and the activity had a

high selective index, although the mode of action is still unknown [61].

2.6.4 Insecticidal activity

Insecticidal activities of oil containing phorbol ester or concentrated phorbol ester fractions have been recorded for a large number of different insects like *Mandura sexta, Helicoverpa armigera, Aphis gossypii, Pectinophora gossypiella, Empoasca biguttula, Callosobruchus chinensis, Sitophillus zeamays, Phtorimaea opercullewla, Sesamia calamitis, Busseola fusca, Periplaneta americana, Blatella germanica, Oncopeltus fasciatus, Phaedon cochliariae, Platella xylostella, spodoptera frugiperda, Mycus persicae, Callosobruchus maculatus* and *Dinarmus basalis* [62, 63]. Jatropherol I, a phorbol ester diterpene, influenced some midgut enzymes of *Bombyx mori*.This influenced general esterase, carboxylesterase and acetylcholinesterase. Jatropherol It also caused pathological changes in endoplasmatic reticulum, chromatin, lysosome, mitochondria, and microvilli. LD_{50} values of these ester were 0.579 μg/mL, 0.217 μg/mL, 0.158 μg/mL after 48, 72 and 120 h administration, respectively [64].

2.6.5 Antisnail activity

Controlling snails is important because they act as the vector host of parasites that caused schistosomiasis (see above).

Seeds of *Jatropha curcas* L. were tested against *Biomphalaria glabrata, Bulinus truncatus, Bulinus natalensis, Oncomelania quadrasi* and *Oncomelania hupensis*. Methanolic extract of crude oil was reported to have high toxicity against all snails [60, 65].

2.7 Biosafety Studies

2.7.1 Acute toxicity in mice

Toxicological studies of *Jatropha curcas* L. seed have been conducted in mice. Adam reported that 1% seed material in diet did not cause mortality, but 5-50% of seed material was lethal in mice. During the experiment, mice suffered from diarrhea, they could not keep normal posture, lost their appetite (related to the taste of the food), decreased motor activity, showed depression and had increased respiration rate. The highest dose caused 100% mortality four days after feeding. Organs of death animals were examined and pathological changes were found such as catarrhal enteritis, erosion and widespread infarct of intestinal mucosa. Hemorrhage and congestion were detected in small intestine, heart and lungs. Other side effects were the increased hepatic and renal fat accumulation. There were blood clots in fecal material, but they were not found in peritoneal cavity. Hemorrhage and dilation of ascending colon were also detected in almost all groups [26, 66]. Crude oil and its fraction when

applied as topical application were toxic to the dorsal skin of the mice. Oil fraction caused eye hemorrhage, face swelling, and skin irritation while only small effect of damage caused by crude oil [6]. Acute toxicity of methanol extracts of *Jatropha curcas* L. seed was determined in mice intraperitoneally. The LD_{50} was 25.19 mg/kg body mass [67]. Another experiment of acute toxicity to male mice was carried out [68]. Phorbol esters were isolated from the oil and administered intragastricly to the animals. LD_{50} was determined at 27.34 mg/kg body weight and the highest dose (36 mg/kg body weight) caused abnormality of cortical neurons and cardiac muscle fibers. Glomerular sclerosis, atrophy in kidney and hemorrhage in the lung was shown in mice given a dose higher than 32.4 mg/kg body mass, while no changes were found in the animal organ at the lowest dose (21.26 mg/kg body mass) [68].

2.7.2 Acute Toxicity in rat

Acute toxicity of *Jatropha* oil was also observed in rats. Based on the result, the LD_{50} was 6 mL/kg body weight.At higher doses (9 and 15 mL/kg body mass) all of the rats died during the experiment. In topical application, oil fraction caused skin irritation and edema [6].Various extracts from the fruit of the *Jatropha curcas* L. also had an abortive effect to rats. They influenced the early stage of pregnancy [69]. Methanol extract of the seeds caused low hemoglobin concentration, low packed cell

volumes and also low red blood cell counts, but mean corpuscular volume & mean corpuscular hemoglobin values were high. These results indicated macrocytic anemia [67].

2.7.3 Acute toxicity in goat

Jatropha curcas L. seeds were also administered to goats. The doses ranged between 0.25 to 10 g/kg/day. During dosing period, some symptoms occurred in the goat like diarrhea and loosing ability to keep normal posture. Autopsy showed hemorrhage in the kidneys, lungs, reticulum, spleen and rumen. Necrosis and degeneration of the liver were marked by the elevation of glutamate oxalacetate transaminase and arginase activity, and depletion of glucose level in the goat serum [70]. The same symptoms were also found when the extract was given to a goatling, Abdel Gadir reported that the mortality of them occurred between day 7 and 21 after extract administration [71].

2.8 Conclusion and Perspective

Jatropha curcas L. has a great potential and value for cultivation as economic crop for biodiesel production. Not only because of the source of non-edible renewable biodiesel, but also containing secondary metabolites with interesting biological activities. As discussed, these secondary natural compounds were isolated and elucidated from different parts of *Jatropha curcas* L. Some experiments have been conducted in the past for validating

pharmacological uses. Curcin, a ribosome inactivating protein is a potential lead compound as anticancer and immune-suppressive drug. Curcusone B isolated from the root also has a good prospect for antiproliferative effect since it inhibits the growth of cancer cell lines. Jatropherol I has insecticidal activity because it changes pathological condition in endoplasmic reticulum, chromatin, lysosome, mitchondria and microvilli of insects. Curcacycline A shows moderate dose dependent inhibition on human T-cell proliferation while curcacycline B can bind to human cyclophilin B and increase its Peptidyl-prolil *cis trans* isomerase.

Phorbol ester has become a major issue regarding *Jatropha curcas* L. oil and toxicity. Since it is dissolved well in the oil, intoxication risk for workers in the oil producing industry is high, here more research is necessary to avoid the chronic harm of the risk. It was reported that multiple steps in oil refining process like degumming, deodorization, neutralization processes (with alkali hydroxides), bleaching and stripping process reduce the level of phorbol ester significantly. Detoxified oil can be used as a candidate of new basis for ointment and dermal application preparations. Further investigation is still needed for oil safety, because direct contact of remaining phorbol ester with skin have to be avoided in all cases. Studies about indoor pollution by *Jatropha curcas* L. oil should be conducted, in order to give clear

explanation between combustion products from the oil and related toxicity.

Jatropha curcas L. pressed cake contains a high protein yield. It can be used as animal feed, but the utilization is limited due to its toxic principles. Development strategy in detoxification process of the pressed cake has become high of interest. Physical and chemical treatments have been investigated in toxic removal. For safety reason, biosafety assays have been observed for this detoxified product to various *in vitro* and *in vivo* studies. These results show that detoxification of pressed cake is possible and safe for further use.

Recent progress on *Jatropha curcas* L. studies indicates that all parts of this plant are valuable. Utilization of the plant could improve valorization for huge plantation.

References

1. Becker K, Makkar HPS. *Jatropha curcas :* A potential source for tomorrow's oil and biodiesel. Lipid Tech. 2008;20(5):104-7.

2. Schmook B, Seralta-Peraza L. *J. curcas* : distribution and uses in the Yucatan Peninsula of Mexico. Gübitz G, Trabi M, Mittelbach M, editors1997.

3. Divakara BN, Upadhyaya HD, Wani SP, Gowda CLL. Biology and genetic improvement of *Jatropha curcas* L.: A review. Appl Energ. 2010;87(3):732-42.

4. Horiuchi T, Fujiki H, Hirota M, Suttajit M, Suganuma M, Yoshioka A, et al. Presence of tumor promoters in the seed oil of *Jatropha curcas* L. from Thailand. Jpn J Cancer Res. 1987;78(3):223-6. Epub 1987/03/01.

5. Srinophakun P, Titapiwatanakun B, Sooksathan I, Punsuvon V. Prospect of deoiled *Jatropha curcas* seedcake as fertilizer for vegetables crops- a case study. J Agr Sci. 2012;4(3):211-26.

6. Gandhi VM, Cherian KM, Mulky MJ. Toxicological studies on ratanjyot oil. Food Chem Toxicol. 1995;33(1):39-42.

7. Makkar HPS, Becker K, Sporer F, Wink M. Studies on Nutritive Potential and Toxic Constituents of Different Provenances of *Jatropha curcas*. J Agr Food Chem. 1997;45(8):3152-7.

8. Asseleih LMC, Plumbley RA, Hylands PJ. Purification and partial characterization of a hemagglutinin from seeds of *Jatropha Curcas*. J Food Biochem. 1989;13(1):1-20.

9. Haas W, Sterk H, Mittelbach M. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J Nat Prod. 2002;65(10):1434-40. Epub 2002/10/26.

10. Aderibigbe AO, Johnson COLE, Makkar HPS, Becker K, Foidl N. Chemical composition and effect of heat on organic matter- and nitrogen-degradability and some antinutritional components of *Jatropha* meal. Anim Feed Sci Tech. 1997;67(2-3):223-43.

11. Makkar HPS, Becker K, Schmook B. Edible provenances of *Jatropha curcas* from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. Plant Food Hum Nutr. 1998;52(1):31-6.

12. Makkar HPS, Aderibigbe AO, Becker K. Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem. 1998;62(2):207-15.

13. He W, King AJ, Khan MA, Cuevas JsA, Ramiaramanana DI, Graham IA. Analysis of seed phorbol-ester and curcin content together with genetic diversity in multiple provenances of *Jatropha curcas* L. from Madagascar and Mexico. Plant Physiol Bioch. 2011;49(10):1183-90.

14. Gubitz GM, Mittelbach M, Trabi M. Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresource Technol. 1999;67(1):73-82.

15. Martínez-Herrera J, Siddhuraju P, Francis G, Dávila-Ortíz G, Becker K. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chem. 2006;96(1):80-9.

16. Aiyelaagbe OO, Adeniyi BA, Fatunsin OF, Arimah BD. In vitro antimicrobial activity and phytochemical analysis of *Jatropha curcas* roots. Int J Pharm. 2007;3(1):106-10.

17. Naengchomnong W, Thebtaranonth Y, Wiriyachitra P, Okamoto KT, Clardy J. Isolation and structure determination of four novel diterpenes from *Jatropha curcas*. Tetrahedron Lett. 1986;27(22):2439-42.

18. Chianese G, Fattorusso E, Aiyelaagbe OO, Luciano P, SchroÌder HC, MuÌller WEG, et al. Spirocurcasone, a diterpenoid with a novel carbon skeleton from *Jatropha curcas*. Org Lett. 2011;13(2):316-9.

19. Ravindranath N, Ravinder Reddy M, Ramesh C, Ramu R, Prabhakar A, Jagadeesh B, et al. New lathyrane and podocarpane diterpenoids from *Jatropha curcas*. Chem Pharm Bull 2004;52(5):608-11. Epub 2004/05/11.

20. Ravindranath N, Reddy MR, Mahender G, Ramu R, Kumar KR, Das B. Deoxypreussomerins from *Jatropha curcas*: are they also plant metabolites? Phytochemistry. 2004;65(16):2387-90. Epub 2004/09/24.

21. Staubmann R, Schubert-Zsilavecz M, Hiermann A, Kartnig T. A complex of 5-hydroxypyrrolidin-2-one and pyrimidine-2,4-dione isolated from *Jatropha curcas*. Phytochemistry. 1998;50(2):337-8.

22. Naengchomnong W, Tarnchompoo B, Thebtaranonth Y. (+)-jatrophol, (+)-marmesin, propacin and jatrophin from the roots of *Jatropha curcas* (Euphorbiaceae). J Sci Soc. 1994;20(2):73-83.

23. Subramanian SS, Nagarajan S, Sulochana N. Flavonoids of some Euphorbiaceous plants. Phytochemistry 1971;10(10):2548-9.

24. Van den Berg AJ, Horsten SF, Kettenes-van den Bosch JJ, Kroes BH, Beukelman CJ, Leeflang BR, et al. Curcacycline A-a novel cyclic octapeptide isolated from the latex of *Jatropha curcas* L. FEBS Lett. 1995;358(3):215-8. Epub 1995/01/30.

25. Auvin C, Baraguey C, Blond A, Lezenven F, Pousset J-L, Bodo B. Curcacycline B, a cyclic nonapeptide from *Jatropha curcas* enhancing rotamase activity of cyclophilin. Tetrahedron Lett. 1997;38(16):2845-8.

26. Abdu-Aguye I, Sannusi A, Alafiya-Tayo RA, Bhusnurmath SR. Acute toxicity studies with *Jatropha curcas* L. Hum Exp Toxicol. 1986;5(4):269-74.

27. Kulkarni ML, H. Sreekar, K.S. Keshavamurthy, Shenoy N. *Jatropha curcas* - Poisoning. Indian J Pediatr. 2005;72(1):75-6.

28. Naengchomnong W, Thebtaranonth Y, Wiriyachitra P, Okamoto KT, Clardy J. Isolation and structure determination of two novel lathyrenes from *Jatropha curcas*. Tetrahedron Lett. 1986;27(47):5675-8.

29. Pletsch M, Charlwood BV. Accumulation of diterpenoids in cell and root-organ cultures of *Jatropha* species. J Plant Physiol 1997;150(1-2):37-45.

30. Wang X-C, Zheng Z-P, Gan X-W, Hu L-H. Jatrophalactam, A Novel Diterpenoid Lactam Isolated from Jatropha curcas. Organic Lett. 2009;11(23):5522-4.

31. Stirpe F, Pession-Brizzi A, Lorenzoni E, Strocchi P, Montanaro L, Sperti S. Studies on the proteins from the seeds of *Croton tiglium* and of *Jatropha curcas*. Toxic properties and inhibition of protein synthesis in vitro. Biochem J. 1976;156(1):1-6. Epub 1976/04/15.

32. Adolf W, Opferkuch H, Hecker E. Irritant phorbol derivatives from four *Jatropha* species. Phytochemistry. 1984;23(1):129-32.

33. Morton JF. Atlas of medicinal plants of middle America. Thomas CC, editor. IL: Springfield; 1981.

34. Nath LK, Dutta SK. Extraction and purification of curcain, a protease from the latex of *Jatropha curcas* Linn. J Pharm Pharmacol. 1991;43(2):111-4. Epub 1991/02/01.

35. Das B, Laxminarayana K, Krishnaiah M, Srinivas Y, Raju TV. Multidione, a novel diterpenoid from *Jatropha multifida*. Tetrahedron Lett. 2009;50(34):4885-7.

36. Lin J, Zhou X, Wang J, Jiang P, Tang K. Purification and characterization of curcin, a toxic lectin from the seed of *Jatropha curcas*. Prep Biochem Biotech. 2010;40(2):107 - 18.

37. Wei Q, Ming H, Ying XX, XinShen Z, Fang C. Expression of a ribosome inactivating protein (curcin 2) in *Jatropha curcas* is induced by stress. J Biosciences 2005;30(3):351-7.

38. Lin J, Li Y, Zhou X, Tang K, Chen F. Cloning and characterization of a curcin gene encoding a ribosome inactivating protein from *Jatropha curcas*. DNA Sequence. 2003;14(4):311-7.

39. Luo M, Liu W, Yang X, Xu Y, Yan F, Huang P, et al. Cloning, expression, and antitumor activity of recombinant protein of curcin. Russ J Plant Physl+. 2007;54(2):202-6.

40. Lin J, Yan F, Tang L, Chen F. Antitumor effects of curcin from seeds of *Jatropha curcas*. Acta Pharmacol Sin. 2003;24(3):241-6. Epub 2003/03/06.

41. Dimitrijevic SM, Humer U, Shehadeh M, Ryves WJ, Hassan NM, Evans FJ. Analysis and purification of phorbol esters using normal phase HPLC and photodiode-array detection. J Pharmaceut Biomed. 1996;15(3):393-401.

42. Goel G, Makkar HPS, Francis G, Becker K. Phorbol esters: structure, biological activity, and toxicity in animals. Int J Toxicol. 2007;26(4):279-88.

43. Clemens M, Trayner I, Menaya J. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci. 1992;103(4):881-7.

44. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science. 1992;258(5082):607-14.

45. Segal A, Van Duuren BL, Mate U. The identification of phorbol myristate acetate as a new metabolite in mouse skin. Cancer Res. 1975;35(8):2154-9.

46. Aregheore EM, Makkar HPS, Becker K. Assessment of lectin activity in a toxic and a non-toxic variety of *Jatropha curcas* using latex agglutination and haemagglutination methods and inactivation of lectin by heat treatments. J Sci Food Agr. 1998;77(3):349-52.

47. Chivandi E, Mtimuni JP, Read JS, Makuza SM. Effect of processing method on phorbol esters concentration, total phenolics, trypsin inhibitor activity and the proximate composition of the Zimbabwean *Jatropha curcas* provenance: A potential livestock feed. Pak J Biol Sci. 2004;7(6):1001-5.

48. Devappa RK, Swamylingappa B. Biochemical and nutritional evaluation of *Jatropha* protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J Sci Food Agr. 2008;88(5):911-9.

49. Wirasutisna K, Artri, Elfahmi. Reducing of phorbol ester content in callus cultures of physic nut (*Jatropha curcas* L.) using manganese chloride and n-ethylmaeimid. Pharmacog J. 2011;3(20):42-6.

50. Makkar HPS, Becker K. Nutritional studies on rats and fish (carp *Cyprinus carpio*) fed diets containing unheated and heated

Jatropha curcas meal of a non-toxic provenance. Plant Food Hum Nutr 1999;53(3):183-92.

51. Makkar HPS, Becker K, inventors; Method for detoxifying plant constituents. European patent EP 2 397 240 A1. 2010 22 September 2010.

52. Brooker J, inventor; Methods for detoxifying oil seed crops. Great Britain patent 2466353. 2010 24 June 2010.

53. He Y, inventor; Method for removing phorbol ester form organic material, method for producing organic material having high protein content, organic material having high protein content, method for producing feed, and feed. European patent EP 2397240A13. 2011 21 December 2011.

54. Xiao J, Zhang H, Niu L, Wang X, Lu X. Evaluation of detoxification methods on toxic and antinutritional composition and nutritional quality of proteins in *Jatropha curcas* Meal. J Agr Food Chem. 2011;59(8):4040-4.

55. Francis G, Makkar HP, Becker K. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 2001;199(3/4):197-227.

56. White C, Campbell D, Combs G. Effect of moisture and processing temperature on activities of trypsin inhibitor and urease in soybeans fed swine. Huisman J, Van der Poel T, Liener I, editors. Wageningen: Pudoc; 1989.

57. Reddy NR, Pierson MD. Reduction in antinutritional and toxic components in plant foods by fermentation. Food Res Int. 1994;27(3):281-90.

58. Richardson NL, Higgs DA, Beames RM, McBride JR. Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (*Oncorhynchus tshawytscha*). J Nutr. 1985;115(5):553-67.

59. Fagbenro-Beyioku AF, Oyibo WA, Anuforom BC. Disinfectant/antiparasitic activities of *Jatropha curcas*. E Afr Med J. 1998;75(9):508-11. Epub 1999/09/24.

60. Rug M, Ruppel A. Toxic activities of the plant *Jatropha curcas* against intermediate snail hosts and larvae of schistosomes. Trop Med Int Health. 2000;5(6):423-30.

61. Matsuse IT, Lim YA, Hattori M, Correa M, Gupta MP. A search for anti-viral properties in Panamanian medicinal plants.:

The effects on HIV and its essential enzymes. J Ethnopharmacol. 1998;64(1):15-22.

62. Sauerwin M, Sporer F, Wink M. Insect toxicity of phorbol esters from *Jatropha curcas* seed oil. Planta Med. 1993;59(7):686.

63. Wink M, C. Koschmieder, M. Sauerwein, Sporer F. Phorbol esters of *Jatropha curcas* - biological and potential applications. GM Gübitz, M Trabi, Mittelbach M, editors. Graz: DBV; 1997.

64. Jing L, Fang Y, Ying X, Wenxing H, Meng X, Syed M N, et al. Toxic impact of ingested Jatropherol-I on selected enzymatic activities and the ultrastructure of midgut cells in silkworm, *Bombyx mori* L. J Appl Entomol. 2005;129(2):98-104.

65. Yasuraoka K, Hashiguchi J, Blas BL, editors. Laboratory assessment of the molluscicidal activity of the plant *Jatropha curcas* against *Oncomelania* snail. Philippine-Japan joint conference on schistosomiasis research and control; 1980; Manila.

66. Adam SEI. Toxic effects of *Jatropha curcas* in mice. Toxicology. 1974;2(1):67-76.

67. Oluwole FS, Bolarinwa AF. *Jatropha curcas* extract causes anaemia in rat. Phytother Res. 1997;11(7):538-9.

68. Li C-Y, Devappa RK, Liu J-X, Lv J-M, Makkar HPS, Becker K. Toxicity of *Jatropha curcas* phorbol esters in mice. Food Chem Toxicol. 2010;48(2):620-5.

69. Goonasekera MM, Gunawardana VK, Jayasena K, Mohammed SG, Balasubramaniam S. Pregnancy terminating effect of *Jatropha curcas* in rats. J Ethnopharmacol. 1995;47:117-23.

70. Adam SEI, Magzoub M. Toxicity of *Jatropha curcas* for goats. Toxicology. 1975;4(3):388-9.

71. Abdel Gadir WS, Onsa TO, Ali WEM, El Badwi SMA, Adam SEI. Comparative toxicity of *Croton macrostachys, Jatropha curcas and Piper abyssinica* seeds in Nubian goats. Small Ruminant Res. 2003;48(1):61-7.

CHAPTER 3

Development of tandem mass spectrometry for dehydroxy phorbol esters and phorbol myristate acetate analysis

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Abstract

Two stereoisomers of Phorbol Myristate Acetate (α and β) (PMA) were used as standards in Dehydroxy Phorbol Esters (DHPEs) analysis. Both PMA have different pharmacological effect and analytical profile. The aim of this research was to develop a new LC-MS/MS method for discrimination of both PMA and DHPEs, for improved qualitative and quantitative determination and pharmacological assessment. Precursor ion scan, product ion scan and multiple reaction monitoring (MRM) MS experiments have been applied to analyze DHPE, α and β PMA. The two stereoisomers have different retention times in reversed phase HPLC (R_t α= 23.0, β=23.2 min) while DHPEs were eluted between 22 and 23 min, MS/MS fragmentation yields major product ions at *m/z* 311 and 293 for α, β PMA. The relative intensity of both ions is different (*m/z* 311: β>α, *m/z* 293: β<α). In addition, the $UV_{\lambda max}$ differs between both stereoisomers (β: 239 nm, α: 234 nm). An LC-MS/MS method was developed for explicit discrimination between phorbol myristic acetate stereoisomers and DHPEs which may aid in identification in complex plant extracts of *Jatropha curcas* L.

3.1 Introduction

Jatropha curcas L. is known as the rich source of diterpenes. There are about 20 compounds were reported from different part of *J. curcas*[1-6]. Among these diterpenes there is one group which became an interesting subject for researcher called phorbol esters (PEs). They act as cocarcinogen since they can activate protein kinase C (PKC) that is important for signal transduction leading to cell differentiation and cell growth regulation. The reaction will not be stopped because these PEs cannot be metabolized by the enzyme [7-9]. Six phorbol esters were isolated from the seed of *J. curcas* L.[10]. Chemically they have tigliane skeletons which C12 and C13 were esterified with fatty acid [11]. Phorbol esters from *J.curcas* L. which have a 12-deoxy-16-hydroxyphorbol skeleton have been investigated as mouse skin irritants with weak tumour promoting activity [12, 13]. Many attempts were made to analyze and quantify PEs in different samples like seed, oil, kernel, stem and bark. The most widely used method for quantifying PEs is HPLC with diode array detector using phorbol myristic acetate (PMA) as standard since its first detection in 1967 [14-16].

Due to their complex structure, close structural relationship and high diversity this has been an ongoing challenge. One stereoisomer of PMA, namely β-PMA (Fig.3.1), has been used as analytical standard for UV and HPLC

detection ignoring the possibility of different analytical profiles of different stereochemical structures. This matters for biological assays where both isomers have significantly different pharmacological activities [17]. Herein we suggest a more sophisticated LC-MS/MS method for direct differentiation of both isomers based on multiple reactions monitoring to allow simultaneous quantification.

3.2 Experimental

3.2.1 Chemicals

α- and β-phorbol myristate acetate were obtained from Sigma Aldrich. Identity and purity have been checked before use by ^{1}H NMR. All stock solutions (1 mg/mL) have been stored at 4° C for a maximum of 4 weeks and have been replaced. All other chemicals were obtained from Biosolve B.V., Valkenswaard, The Netherlands.

3.2.2 High Performance Liquid Chromatography (UV detector)

Low pressure gradient chromatography was performed using a Phenomenex Kinetex C18 column 100A, (100 x 4.6 mm, 2.6 µm). The injection volume was 20 µL and the flow rate was set at 0.8 mL/min at 22°C. The mobile phase comprised 100% acetonitrile (0.1% formic acid) (solvent A) and acetonitrile 5% in water (0.1 % formic acid) (solvent B).

3.2.3 High Performance Liquid Chromatography (MS detector)

The mass spectrometer was interfaced to a Phenomenex Kinetex C18 column (100A, 100 x 4.6 mm, 2.6 µm). The mobile phase comprised 5% acetonitril in H_2O (solvent A) and 100% acetonitrile (solvent B) with a flow rate 0.5 mL/min. Post column addition was used for ionization with 75% AcN + 2 mM ammonium acetate and the flow rate was 0.2 mL/min. The separation was done using 70% solvent B for 25 min, followed by gradient eluent 75 to 95 % B in 5 min, 95% B in 5 min, 95%-70% B in 2 min, and 70% B for the last 4 min. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 500^{o}C (SCIEX API 3000 triple quadrupole mass spectrometer). Data processing was performed using Analyst version 1.5.1 software (MDS Sciex, Concord, Canada).

3.3 Result & Discussion

Although α-PMA has the same molecular weight and chemical formula as β-PMA (Fig. 1), both compounds show different retention times at 23.0 min and 23.2 min for α-PMA and β-PMA (Fig. 2), respectively.α-PMA and β-PMA both give MS peaks at *m/z* 617, 634, and 677 corresponding to the protoned, ammoniated ion and protonated acetate adduct forms, respectively.Analysing the MS/MS spectra of the *m/z* 617 and

634 precursorions it is obvious that both stereoisomers show different paths of fragmentation (Fig. 3).

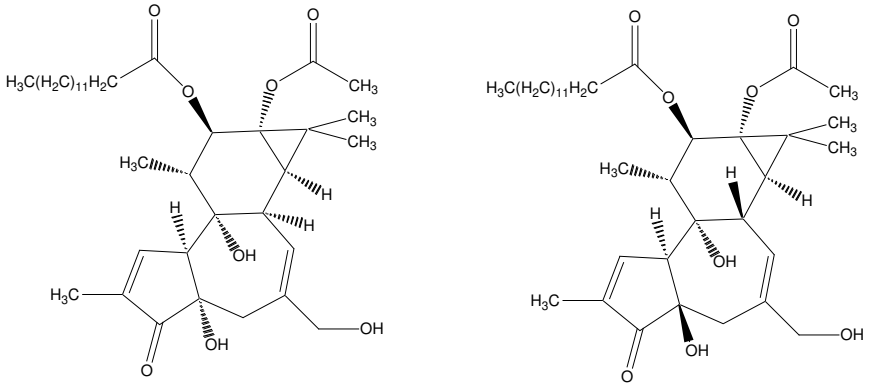


Figure 1. Structure of α-PMA (a) and β-PMA (b)

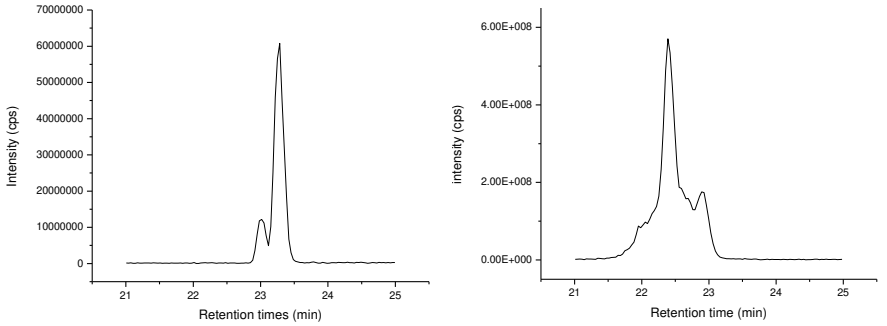
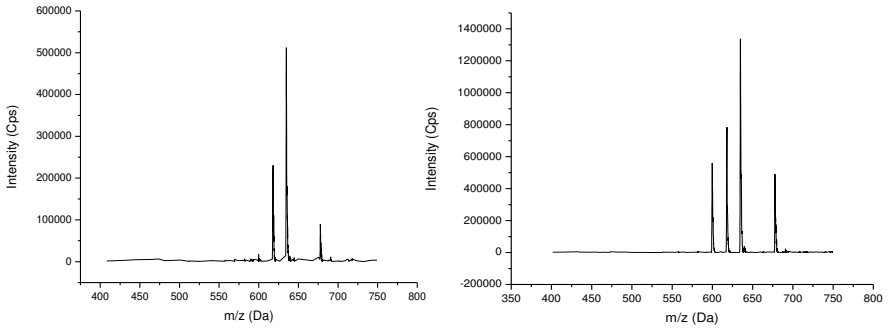


Figure 2. Representative LC-MS chromatogram for α-PMA and β-PMA (a), DHPEs (b).

The difference in intensity of the *m/z* 311 and 293 fragment peaks is of high importance for selective discrimination of β- and

α-PMA. For β-PMA a precursor ion scan of *m/z* 311 showed peaks at *m/z* 599.8, 618.0, 634.8, and 677 and for *m/z* 293 at 599.8, 617.9, 634.8, and 677 (Fig. 3a). In contrast, α-PMA showed only two precursor peaks for *m/z* 311 at 618.0, 634.8, and 677 and for *m/z* 293 at 617.9, 634.8, and 677 (Fig. 3b). Both stereoisomers are fragmented by eliminating their ester groups as free acids (X-OH) resulting in a diterpene nucleus of *m/z* 311, which can subsequently lose water to form the *m/z* 293 ion. The presence of the precursor ion at *m/z* 600 can be explained by the more facile loss of H_2O during ionization and entrance of the mass analyser (up-front fragmentation) of β-PMA.



a **b**

Figure 3. Precursor ion scan of the *m/z* 311 product ion of α-PMA (a) and β-PMA (b)

By calculation the 3d structural the close neighbourhood of the proton H at C1 and the hydroxyl group at C8 in *cis* position

favours splitting of H_2O, which is less favourable in *trans* position for β-PMA. The relative intensity of the *m/z* 311 and 293 ions is different for α-PMA and β-PMA in the MRM mode and can be used as a diagnostic criteria for stereoisomer discrimination. The maximum ion intensities in an LC-MS/MS MRM experiment using an equimolar mixture of α-PMA and β-PMA were 4.4 x 10^5 cps and 3 x 10^5 cps for the 634 to 293 MRM transition, respectively, and 1.0 10^6 cps and 2.3 x 10^6 cps for 634 to 311, respectively (Fig. 4a). The 311 to 293 fragment ratio of α-PMA was 2.3 while for β-PMA the ratio was 7.6 (Fig. 4b)

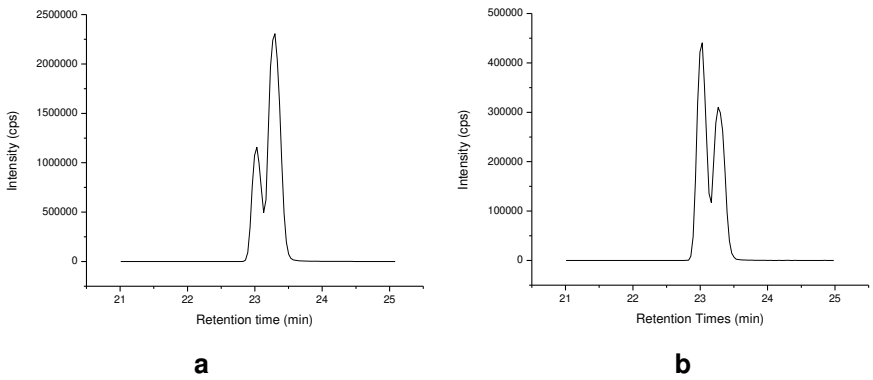


Figure 4. MRM chromatograms of precursor-product pair 634 -> 311 (a) and 634 -> 293 (b) of a mixture of α-PMA and β-PMA

These mass spectrometry results are in agreement with previous reports [18] and show that both stereoisomers show diagnostic ratio of fragment peaks *m/z* 311, and 293 using MRM mode.

Both stereoisomers can be distinguished by their UV maxima as well. α-PMA showed a UV maximum at $[a]_{MeOH}^{20°C}$ 234 nm and β-PMA at $[a]_{MeOH}^{20°C}$ 239 nm (Fig. 5).

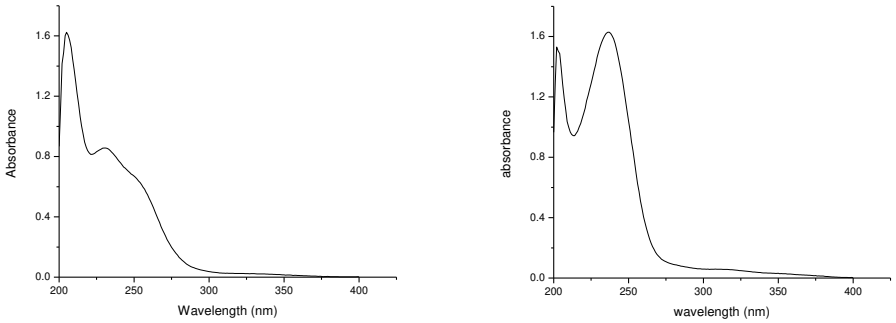


Figure 5. UV absorption spectrum of α-PMA (a) and β-PMA (b)

Because of their different physical characteristics, it is not surprising that both isomers show different intensities in their tumour promoting action. We can confirm that the β-isomeric structure has a significantly stronger tumour promoter and skin irritant activity while the α-isomer does not show any activity at all [17].

3.4 Conclusion

Tandem mass spectrometer can be used to distinguish α, β PMA and DHPEs. α and β PMA cannot be used as standards for analysing DHPEs as they have different fragmentation pattern.

References

1. Ravindranath N, Reddy MR, Mahender G, Ramu R, Kumar KR, Das B. Deoxypreussomerins from *Jatropha curcas*: are they also plant metabolites? Phytochemistry. 2004;65(16):2387-90. Epub 2004/09/24.

2. Chianese G, Fattorusso E, Aiyelaagbe OO, Luciano P, Schroỳder HC, Muỳller WEG, et al. Spirocurcasone, a diterpenoid with a novel carbon skeleton from *Jatropha curcas*. Org Lett. 2011;13(2):316-9.

3. Naengchomnong W, Thebtaranonth Y, Wiriyachitra P, Okamoto KT, Clardy J. Isolation and structure determination of four novel diterpenes from *Jatropha curcas*. Tetrahedron Lett. 1986;27(22):2439-42.

4. Naengchomnong W, Thebtaranonth Y, Wiriyachitra P, Okamoto KT, Clardy J. Isolation and structure determination of two novel lathyrenes from *Jatropha curcas*. Tetrahedron Lett. 1986;27(47):5675-8.

5. Pletsch M, Charlwood BV. Accumulation of diterpenoids in cell and root-organ cultures of *Jatropha* species. J Plant Physiol 1997;150(1-2):37-45.

6. Wang X-C, Zheng Z-P, Gan X-W, Hu L-H. Jatrophalactam, A Novel Diterpenoid Lactam Isolated from Jatropha curcas. Organic Lett. 2009;11(23):5522-4.

7. Clemens M, Trayner I, Menaya J. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci. 1992;103(4):881-7.

8. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science. 1992;258(5082):607-14.

9. Segal A, Van Duuren BL, Mate U. The identification of phorbol myristate acetate as a new metabolite in mouse skin. Cancer Res. 1975;35(8):2154-9.

10. Haas W, Sterk H, Mittelbach M. Novel 12-Deoxy-16-hydroxyphorbol Diesters Isolated from the Seed Oil of Jatropha curcas. Journal of Natural Products. 2002;65(10):1434-40.

11. Goel G, Makkar HPS, Francis G, Becker K. Phorbol esters: structure, biological activity, and toxicity in animals. International Journal of Toxicology. 2007;26(4):279-88.

12. Adolf W, Opferkuch HJ, Hecker E. Irritant phorbol derivatives from four *Jatropha* species. Phytochemistry. 1984;23(1):129-32.

13. Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wongchai V, et al. A new tumor promoter from the seed oil of Jatropha curcas L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. Cancer Res. 1988;48(20):5800-4. Epub 1988/10/15.

14. Makkar HPS, Becker K. Jatropha curcas, a promising crop for the generation of biodiesel and value-added coproducts. European Journal of Lipid Science and Technology. 2009;111(8):773-87.

15. Martínez-Herrera J, Siddhuraju P, Francis G, Dávila-Ortíz G, Becker K. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chem. 2006;96(1):80-9.

16. Hecker E, Bartsch H, Bresch H, Gschwendt M, Härle B, Kreibich G, et al. Structure and stereochemistry of the tetracyclic diterpene phorbol from croton tiglium L. Tetrahedron Letters. 1967;8(33):3165-70.

17. Van Duuren BL, Tseng S-S, Segal A, Smith AC, Melchionne S, Seidman I. Effects of Structural Changes on the Tumor-promoting Activity of Phorbol Myristate Acetate on Mouse Skin. Cancer Research. 1979;39(7 Part 1):2644-6.

18. Solomon JJ, Van Duuren BL, Tseng SS. Chemical ionization mass spectrometry of the tumor promoter related 4aα-phorbol esters. Biomedical Mass Spectrometry. 1978;5(2):164-9.

CHAPTER 4

Curcacycline a and b – new pharmacological insights to an old drug

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Abstract

Two cyclic peptides were isolated from the latex of *Jatropha curcas* L., namely curcacycline A and B. In the past both compounds have not been fully structure elucidated, today modern structure elucidation has been applied by more sophisticated bioanalytical techniques. Additionally, for the first time the structure was confirmed by a full synthetic approach and biological assays have been carried out to shed light on the pharmacological activity of both curcacyclines. Screening of antibiotic activity of curcacycline A was done against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Eschericia coli* ATCC 8939, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231 using paper disk diffussion method. Curcacycline A inhibited the growth of *B. subtilis* and *P. aeruginosa* with inhibition zone was ranged between 6.5 – 10.3 mm. Cytotoxic test show that it decreased the level of OVCAR 3 (ovarium cancer cell) at a concentration of 1 mg/mL, while no effect was found on Colo205 (human colon cancer cell). Curcacycline B did not have an effect on both cell lines. Brine shrimp cytotoxicity assay showed LD_{50} of curcacycline A and B at 49.85 µg/mL and 15.84 µg/mL, respectively. Both curcacyclines did not have a mutagenic effect on *Salmonella typhimurium* TA-98 and TA-mix (TA 7001, TA 7002, TA 7003, TA 7004, TA 7005, TA 7006). This study provides data on the safety as well the efficacy for curcacycline

A and B. Curcacyline A possesses antimicrobial and cytotoxic activities. Based on AMES II assay, curcacycline A and B were proven not to be mutagenic. Both curcacyclines also can be synthesized in the lab. These data will be essential to develop curcacyclines as a new drug candidate for managing infectious and cancer diseases.

4.1 Introduction

Cyclic peptides are formed into a ring via amide ester or disulfide bonds [1]. Because of no exposure of C,N-terminal groups to exopeptides, they are quite stable to enzymatic degradation [2]. Since they have less zwitterionic character, they can cross membranes more readily than linear peptides. Finally, their rigid structure leads them to exhibit higher affinity and selectivity for binding with protein [3]. Cyclic peptides show a wide range of activities such as as antibiotic (gramicidin), anthelmintic (yunnanin F), antineoplastic (dolastatin 3), phytotoxic (tentoxin, HC-toxin), insecticidal (destruxin family) and serve as cytostatic agent (chlamydocin) or antiviral agent (sansalvamide A) [4-11]. Naturally cyclic peptides are non ribosomely biosynthesized by complex multi enzyme in the cytosol. These peptides have been found in organisms, like fungi, bacteria, tunicates, mollusks, coelenterates, sponges and crustaceans as well as in animals. In higher plants cyclic peptides have been found only in 26 families, 65 genera and 120 species [1, 12-14].

A rich source of cyclic peptides is the family of Euphorbiaceae, especially the genus of *Jatropha*. A number of cyclic peptides have been characterized from this genus. Podacycline A, B and C have been isolated from *Jatropha podagrica* while chevalierin A, B and C from *J. chevalieri*.

Labaditin and biobollein isolated from *Jatropha multifida* show selective inhibition of the classical pathway of the human complement system. Integerrimide A and B, isolated from *Jatropha integerrimide* inhibit basic cytoskeleton-dependent cellular processes. Pohlianin A, B and C from *Jatropha pohliana* show antimalarial effect. Mahafacyclin A, B and C have been extracted from *Jatropha mahafalensis* while cyclogossine A and B have been extracted from *Jatropha gossypifolia*. Originally curcacycline A and B have been isolated from *Jatropha curcas* latex (Fig. 1a and 1b). Curcacycline A is an octapeptide with moderate dose depending inhibitory activity on human T-cell proliferation and the classical pathway complement system. Curcacycline B, a cyclic nonapeptide, enhances rotamase activity of cyclophillin [15-23].

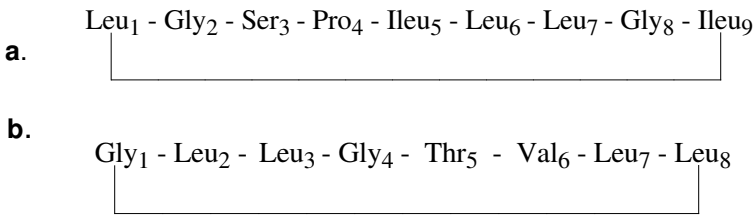


Figure 1. Structure of curcacycline A (a) and curcacycline B (b)

Although the structure of curcacycline A and B have already been established for almost twenty years, there is still a lack of information regarding their pharmacological effects. The objectives of the research are to gain more information on the structure of both curcacyclines with today latest bioanalytical techniques and to broaden up our understanding about pharmacological activity of both compounds.

4.2 Material and Methods

4.2.1 Plant Material

Jatropha curcas L. was growing in the region of Subang, Ujungberung, Bandung, West Java, Indonesia. It was collected in January 2007 and identified in Herbarium Bandungense, School of Life Sciences and Technology, ITB. The specimen is deposit in Pharmacognosy Laboratorium, School of Pharmacy, ITB with voucher speciment number 433-2007. Crude latex

was collected early in the morning (7.00-10.00 am) by cutting off leaf stalks and adding few drops of methanol (MeOH) to prevent the latex from extra foaming. The latex was kept at -20°C before use.

4.2.2 Extraction and isolation by MPLC

To 250 mL of latex 250 mL of deionized water was added, the suspension was extracted with 3 x 500 mL of ethyl acetate. Ethyl acetate was removed using rotary evaporator. The viscous extract was dissolved in 5 mL MeOH and fractionated on Sephadex LH-20 (Pharmacia, 4 x 25 cm) using MeOH as an eluent at a flow rate 10 mL/min. An amount of 50 mL for each fractions was colleted and monitored for cyclic peptides by thin layer chromatography, 20 µL were spotted onto silica gel 60 GF_{254} (Merck), the plate was developed with $CHCl_3$: MeOH (85:15 v/v), and cyclic peptides were visualized by Cl_2/*o*-tolidine. Fractions containing cyclic peptides were further separated by column chromatography using a Büchi C-690 series borosilicate glass column (40x 450 mm) pack with sillica gel 60, (Merck). Methanol and chloroform were used as mobile phase. The solvent flow was set at 30 mL/min, and every 15 mL of fractions were collected. Thin layer chromatography analysis was conducted on silica gel plates Merck F_{254} and use o-tolidine/Cl_2 as visualizing agent.

4.2.3 HPLC

Low pressure gradient chromatography was performed using a LiChrospher-100 C_{18} column (250 x 4.6 mm with 5µm packing). The injection volume was 20 µL and the flow rate was set at 1 mL/min at room temperature. The mobile phase comprised 100% H_2O + 0.1% TFA (solvent A) and 100% Acetonitrile + 0.1% TFA (solvent B) The separation was commenced at 30% solvent B for 5 min, followed by gradient eluent 30 to 60% B in 10 min, in 5 min 60% B, in further 4 min to 95%B, and for 6 min 95% B.

4.2.4 HPLC/MS

The mass spectrometer was interfaced to a Vydac C_{18} column (150 x 2.1 mm, 5 µm particle size). The mobile phase comprised 100% H_2O + 0.1% formic acid (solvent A) and 100% acetonitrile + 0.1% Formic acid. The separation was done using 30% solvent B for 5 min, followed by gradient eluent 30 to 60% B in 10 min, 60% B in 5 min, 95% B in 4 min, and 95% B for the last 6 min. The injection volume was 20 µL and the flow rate 1mL/min at room temperature. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 500^{o}C. Data processing was performed using Analyst version 1.42 software (MDS Sciex, Concord, Canada).

4.2.5 NMR

Curcacycline A and B were dissolved in (deuterated DMSO, d6). ^{1}H NMR and ^{13}C NMR were recorded by 400 MHz Varian NMR, while 2D NMR was recorded by 500 MHz Varian. Tetra Methyl Silan was used as internal standard. Data processing was performed by MestReNova 6.02.

Curcacycline A white crystalline, Rf = 0.7 (See extraction and isolation); UV λ_{max} (methanol) : 220 nm; IR γ_{max} : 3290, 2960, 2873, 1640, 1520, 1260, 1157, 1126 ; ESI MS m/z (rel. int.) : 767 (2.2 x 10^3), 655 (1.3 x 10^5), 542 (1.8 x 10^5), 485 (1.0 x 10^5), 372 (1.2 x 10^5), 259 (3.2 x 10^4), 160 (5.0 x 10^3) ; ^{1}H NMR (400 MHz, dmso) δ 8.67 (t, *J* = 5.7 Hz, 1H), 8.63 – 8.54 (m, 1H), 8.51 (s, 1H), 8.17 (d, *J* = 8.5 Hz, 1H), 8.03 (s, 1H), 7.73 (d, *J* = 7.5 Hz, 1H), 7.53 (d, *J* = 8.9 Hz, 1H), 7.39 (d, *J* = 9.9 Hz, 1H), 5.34 (d, *J* = 10.8 Hz, 1H), 4.76 (dd, *J* = 9.9, 3.7 Hz, 1H), 4.60 (dd, *J* = 14.2, 8.3 Hz, 1H), 4.37 – 4.22 (m, 2H), 4.12 – 4.05 (m, 1H), 3.96 – 3.79 (m, 4H), 3.45 (d, *J* = 5.2 Hz, 1H), 3.40 (d, *J* = 4.9 Hz, 1H), 3.15 (d, *J* = 5.2 Hz, 1H), 2.50 – 2.46 (m, 1H), 2.32 (td, *J* = 13.8, 6.9 Hz, 1H), 1.69 – 1.18 (m, 13H), 1.08 – 0.69 (m, 32H) ; ^{13}C NMR (101 MHz, dmso) δ 172.94, 172.90, 172.86, 170.99, 170.48, 169.42, 169.20, 79.59, 69.13, 58.70, 56.90, 53.36, 52.93, 51.00, 42.90, 40.55, 40.34, 40.14, 39.93, 39.72, 39.51, 39.30, 31.11, 28.73, 24.69, 24.55, 24.29, 23.50, 23.26, 22.99, 22.78, 22.62, 22.10, 21.83, 19.61, 18.91, 17.08.

Curcacycline B white crystal, Rf : 0.66 (see extraction and isolation) ; UV λ_{max} (methanol) : 220 nm; IR γ_{max} : 3290, 2960, 2873, 1644, 1520, 1259, 1027, 800. ESI MS m/z (rel. int.) : 864.4 (4.9 x 10^4), 777.4 (1.3 x 10^4), 720.4 (1.37 x 10^4), 607 (3.6 x 10^4), 494 (3,1 x 10^4), 437 (4.1 x 10^4), 324 (3.2 x 10^4), 211.4 (2.9 x 10^4) ; ^{1}H NMR (500 MHz, dmso) δ 8.72 (s, 1H), 8.63 (s, 1H), 8.54 (s, 1H), 8.32 (s, 4H), 8.25 (s, 1H), 8.10 (s, 1H), 8.02 (s, 1H), 7.69 (d, *J* = 7.1 Hz, 1H), 7.46 (d, *J* = 9.5 Hz, 1H), 7.36 (d, *J* = 8.3 Hz, 1H), 6.88 (d, *J* = 9.0 Hz, 1H), 6.54 (s, 1H), 6.11 – 6.07 (m, 1H), 5.76 (s, 2H), 4.63 (s, 1H), 4.45 (s, 1H), 4.39 – 4.24 (m, 3H), 4.20 – 4.08 (m, 4H), 4.04 (dd, *J* = 14.2, 7.1 Hz, 3H), 3.98 – 3.76 (m, 6H), 3.60 (s, 1H), 3.52 (d, *J* = 5.7 Hz, 1H), 3.48 – 3.41 (m, 2H), 3.36 (s, 49H), 3.18 (d, *J* = 5.1 Hz, 4H), 2.57 – 2.45 (m, 4H), 2.14 – 2.06 (m, 2H), 2.00 (s, 3H), 1.87 (d, *J* = 9.3 Hz, 2H), 1.71 (s, 1H), 1.66 – 1.57 (m, 3H), 1.54 (d, *J* = 7.4 Hz, 2H), 1.49 – 1.35 (m, 4H), 1.25 (s, 1H), 1.23 – 1.11 (m, 4H), 1.23 – 0.98 (m, 7H), 1.23 – 0.73 (m, 26H).

4.2.6 Cyclic Peptides synthesis

Peptides were synthesized by ALMAC Sciences.Each peptide was assembled on 0.3 mmol scale using pre-loaded chlorotityl chloride resin and an automated peptide synthesizer. Each amino acid was incorporated as an active ester in a single

couple cycle. The resin bound peptide was treated with 10% TFE in DCM, 10 mL for two hours. The solvent was evaporated under vacuum and the residue was lyophilized from water/acetonitrile (1:1) to yield the crude protected linear peptide. The crude linear peptide (50 mg) was dissolved in DMF (50ml). HOBt (3eq), PyBOP (3eq) and DIEA (3eq) were added to solution. The mixture was stirred at room temperature for 72 hours. The solvent was evaporated under vacuum and the residue lyphilised from water/acetonitrile 1:1 to yield the crude protected peptide. The crude protected peptide was dissolves in DMF (2 mL) and diluted with water and lyophilized to yield crude product. The crude product was dissolved in DMF (2mL) and diluted with water. The resulting precipitate was triturated and isolated by filtration to provide final product.

4.2.7 Cytotoxicity test with Human ovarian cancer cell-line OVCAR3

The cancer cells were routinely cultured in DMEM (Gibco®), supplemented with 10% of heat-inactivated fetal bovine serum (Gibco®) and 1% penicillin/streptomycin (Gibco®), at 37°C in the presence of 5% CO_2. The addition of paclitaxel reference compound (plant and fungal extracts as well as pure components thereof) dissolved in 1 % DMSO. The solution was sterillized by filtration (0.2 µm) and diluted with cell culture

medium, in consecutive 2-fold dilutions. It was preceded by seeding the test cells into 96-well plates (5000 cells/well) for 24 h incubation. Cell viability was assessed 72 h later by means of the MTS assay (CellTiter 96® AQ_{ueous}Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA), following the instructions of the manufacturer [24].

4.2.8 Cytotoxicity test with Human colon cancer cell-line Colo205

The cancer cells were routinely cultured in RPMI (Invitrogen®), supplemented with 10% of heat inactivated fetal bovine serum (Invitrogen®) and 1% penicillin/streptomycin (Invitrogen®), at 37°C in the presence of 5% CO_2. The addition of TRAIL and paclitaxel reference compounds dissolved in 1% DMSO. The solution was sterillized by filtration (0.2 µm) and diluted with cell culture medium, in consecutive 2-fold dilutions. It was preceded by seeding the test cells into 96-well plates (5000 cells/well) for 24 h incubation. Cell viability was assesed 24 h later by means of the MTS assay (CellTiter 96® AQ_{ueous}Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA), following the instructions of the manufacturer. All assays were performed in triplicate [24].

4.2.9 Brine Shrimp assay

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Hundred microliters of suspension of nauplii containing about (9-12) larvae was added into 96-well microtiter plate. Curcacyclines were dissolved in sea salt water with concentration ranging between 1-1000 µL/mL. Into each well curcacyclines were added 50 µL and incubated for 24 h (22-28^{o}C). The plates were examined under a microscope (12.5x) and the number of dead nauplii in each well counted. Then 100 µL of methanol was added to each well. After 10 min, total number of shrimp in each well were counted and recorded. Calculation of LC_{50} values for each assay was done by taking average of three experiments using a Finney Probit analysis [25-28].

4.2.10 AMES II Test

Carcinogenicity test was determined by AMES kit from Xenomatric Article number E10-213 and E10-213-S1. Two bacteria were used, *Salmonella typhimurium* TA-98 and

S.typhimurium TA-Mix (TA 7001, TA 7002, TA 7003, TA 7004, TA 7005, TA 7006). These bacteria were exposed to six concentrations (1000; 500; 250; 125; 62.5; 31.25; 15.63; 7.82; 3.9 μg/mL) of samples, as well as positive and negative control, for 90 min in medium containing histidine to support approximately two cell divisions. After 90 min, the exposure cultures were diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of 384 well plates. Within two days, cells which have undergone the reversion to His will grow into colonies. Metabolism by the bacterial colonies reduces the pH of the medium, changing colour of reagent in the well (Xenometric®) [29].

4.2.11 Antibacterial Activity

The antibacterial activity of the curcacycline A was evaluated by the paper-disk agar diffusion method against the two Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and *Bacilus subtilis* (ATCC 6633), two gram-negative bacteria *Escherichia coli* (ATCC8939) and *Pseudomonas aeruginosa* (ATCC9027), one fungi *Candida albicans* (ATCC10231). Organisms were maintained on Müller-Hinton agar (MH) (BIORAD). Inoculum were prepared by diluting overnight (24 h at 37°C) cultures in Muller Hinton Broth medium to approximately 10^6 CFU/mL. Absorbent disks (Whatman disc No.

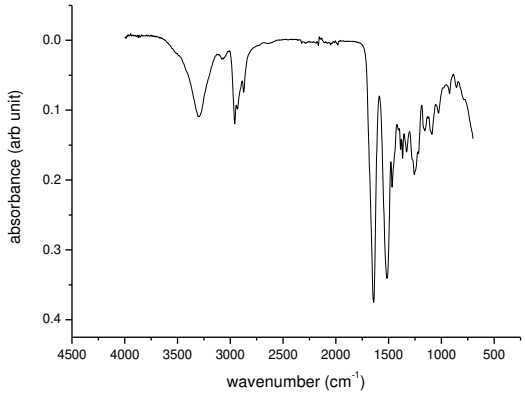
3, 6 mm diameter) were impregnated with 10 µL of curcacycline A and then placed on the surface of inoculated Petri dishes (90 mm). Positive control discs of tetracycline (10 µg/disc) were included in each essay. Diameters of growth inhibition zones were measured after incubation at 37°C for 24 h.

4.3 Result and Discussion

4.3.1 Structure elucidation

After extraction and synthesis, curcacycline A and B were obtained as white crystals. They were UV active with a maximum wavelength (λ_{max}) 220 nm, but no absorption was found at 260 and 280 nm. HPLC chromatogram showed their retention time at 15.02 and 16.93 for curcacycline A and B, respectively. They showed positive reaction with Cl_2/*o*-tolidineon tlc spots. No reactions were detected with nynhidrine reagent. These results proved that both compounds were expected cyclopeptides without C and N terminal. The Infra-Red spectra of these compounds exhibited typical bands at functional group area which were 3290, 2960, 2873, 1640, 1520 cm^{-1} indicating the present of (-NH), (-OH) and (C=O), respectively. Because of different amino acid sequences both curcacyclines showed different finger print area (Fig. 2a and 2b).

a.



b.

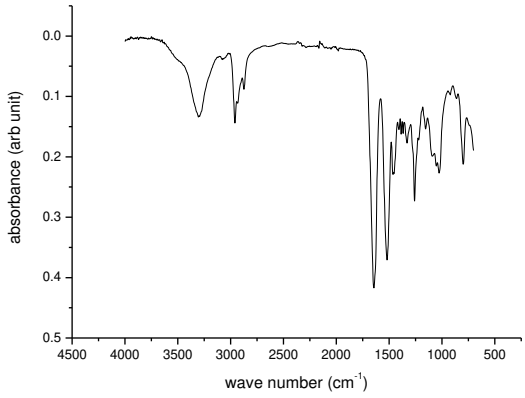
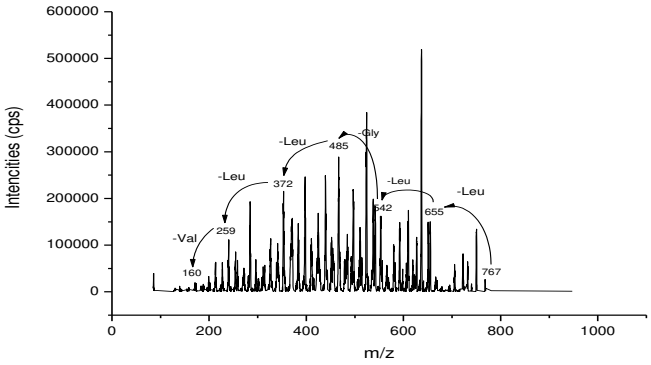


Figure 2. Infra-red spectrum of curcacycline A(a) and curcacycline B(b)

ESI-MS of curcacycline A indicated a molecular ion peak at m/z 767 corresponding to $C_{37}H_{67}N_8O_9$ and with fragments m/z 655 (M^+, -Leu), 542 (M^+, -Leu), 485 (M^+, -Gly), 372 (M^+, -Leu), 259 (M^+, Leu), 160 (M^+, -Val) (Fig. 3a).^{1}HNMR revealed eight protons at δ 8.67, 8.63, 8.51, 8.17, 8.03, 7.53, and 7.39 indicating amide protons.

Conformation with ^{13}C NMR and COSY spectra (Table 1) revealed that this compound was identical to previous compound reported by van den Berg *et al*., 1995.

a.



b.

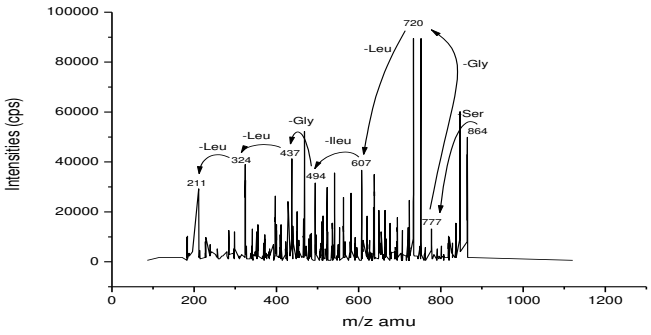


Figure 3. Fragmentation pattern of curcacycline A (a) and curcacyline B (b) by ESI-MS

Table 1. COSY spectrum data of curcacycline A (500 MHz varian NMR, d6, DMSO, TMS as internal standard)

Amino acid	NH	αH	βH	Other	
Gly_1	8.63	3.92 3.40	- -	- -	- -
Leu_2	7.56	4.64	1.4 1.34	CH_3	0.85
Leu_3	8.54	3.87	1.55 1.38	CH_3	0.87 0.84
Gly_4	8.71	3.91 3.43	- -	- -	- -
Thr_5	7.42	4.79	4.32	CH_3 OH	0.95 5.38
Val_6	7.76	4.1	2.34	CH_3	0.85 0.85
Leu_7	8.22	4.36	1.66 1.25	CH_3	1.54
Leu_8	8.07	3.94	1.43	CH_3	0.86

Fragmentation pattern of curcacycline B showed m/z 864 and a molecular ion peak at m/z 777 (M^+, -Ser), 720 (M^+, -Gly), 607 (M^+, -Leu), 494 (M^+, -Ileu), 437 (M^+, -Gly), 324 (M^+,-Leu), 211(M^+, -Leu) corresponding to $C_{42}H_{73}N_9O_{10}$ (Fig. 3b). ^1H NMR revealed only eight amide protons at δ 8.72, 8.59 7.69, 7.46, 7.35, 8.00, 8.25, 6.88 while proline had no amide proton. Comparing to COSY spectra of this compound (Tab. 2)

indicated that this compound is similar to curcacycline B reported by Auvin *et al.,*1997.

Table 2. COSY data of curcacycline B (500 MHz varian NMR, d6, DMSO, TMS as internal standard)

Amino	NH	αH	βH	Other	
Leu_1	8.72	3.78	1.54	-	-
	-	-	1.44	-	-
Gly_2	8.59	3.98	-	-	-
	-	3.37	-	-	-
Ser_3	7.69	4.63	4.13	OH	6.03
	-	-	3.51	-	-
Pro_4	-	-	-	-	-
	-	-	-	-	-
Ile_5	7.46	4.15	1.71	-	-
	-	-	-	-	-
Leu_7	8.00	3.96	1.62	-	-
	-	-	1.45	-	-
Gly_8	8.25	3.83	-	-	-
	-	3.44	-	-	-
$Ileu_9$	6.88	4.25	1.90	-	-

The yield of the synthesized compounds was low. Based on ESI-MS chromatogram and ^{1}H-NMR spectra both synthethic curcacyclines have similar characteristic with isolated curcacyclines.

4.3.2 Biological assays

Curcacycline A and B were tested for their toxicity against brine shrimp using modified brine shrimp lethality assay with microwell [28]. Both curcacyclines show significant toxicity with LC_{50} values 49.85 μg/mL for curcacycline A and 15.85 μg/mL for curcacycline B (Tab. 3).

Table 3. Effects of various concentrations of curcacyclines on brine shrimp

Compounds	Concentration (μg/mL)	Percent of mortality (%)	LD_{50} (μg/mL)
Curcacycline A	1	35.45 ± 5.06	49.85
	10	30.00 ± 10.00	
	50	36.67 ± 11.55	
	100	50.00 ± 4.50	
	1000	81.67 ± 7.64	
Curcacycline B	1	38.33 ± 12.58	15.85
	10	33.33 ± 11.55	
	50	46.67 ± 5.77	
	100	70.00 ± 0.00	
	1000	93.30 ± 5.77	

Mutagenic activity was determined using *Salmonella typhimurium* TA-98 and TA-Mix (AMES II assay). The results are depicted in Fig. 5a and 5b. All concentrations of curcacyclines A and B did not cause mutation on TA-98 and TA-mix because only 10 wells (out of 48) gave positives reaction while the positive control 2-nitrofluorene and 4-nitroquinoline *N*-oxide (2-NF/4-NQO) had 46 positive wells (out of 48), which were statistically not significant. Cytotoxic properties of these peptides were further investigated using MTS assay. Human ovarium cancer cell (OVCAR3) and human colo cancer cell (Colo 205) were used. The results are summarized in Fig. 6a and 6b. To ensure proliferation arrest, TRAIL (TNF-related apoptosis-inducing ligand) and paclitaxel was used as positive control. TRAIL is a protein that induced apoptosis while paclitaxel stabilize microtubule polymer which block mitosis progression [24, 30, 31].

Ovarian cancer cells were treated with paclitaxel (IC_{50} = 4.89 ng/ml) and human colon cancer cells were treated with TRAIL (IC_{50} = 4.89 ng/mL). We found that IC_{50} of curcacycline A on OVCAR3 was 724.44 µg/mL while IC_{50} of curcaycline B was above 1000 µg/ml. Both curcacyclines did not have any effect on Colo 205 (IC_{50}> 1000 µg/mL).

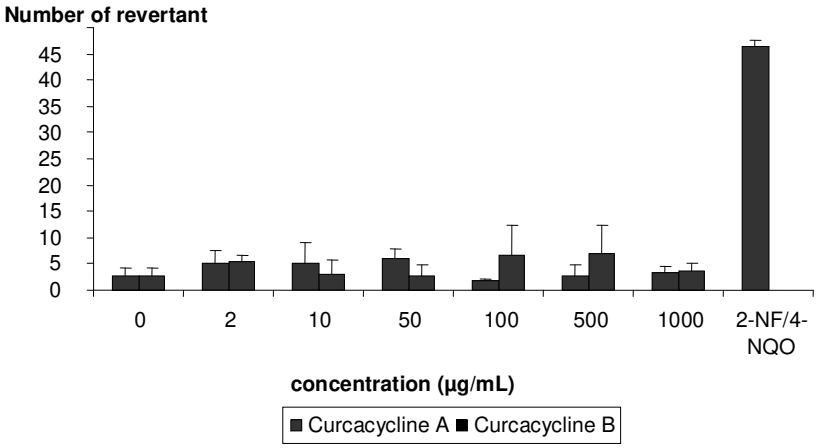


Figure 4a. Mutagenic shifts of *Salmonella typhimurium* TA-98 after incubation with curcacycline A and B

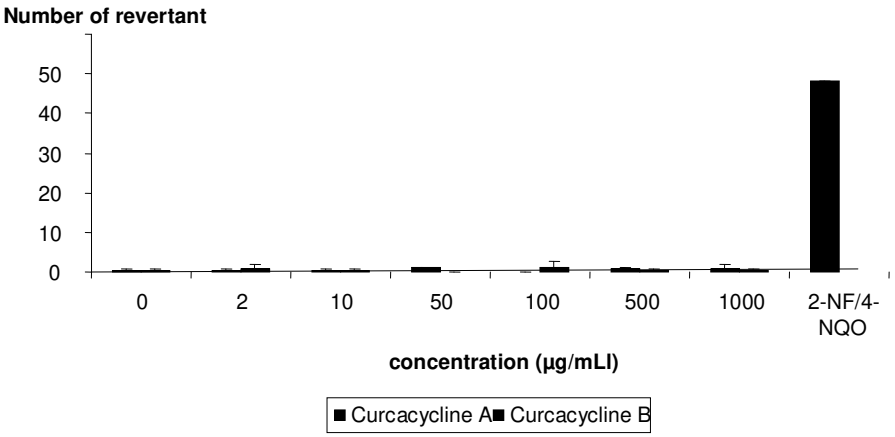


Figure 4b. Mutagenic shifts of *Salmonella typhimurium* TA-Mix after incubation with curcacycline A and B

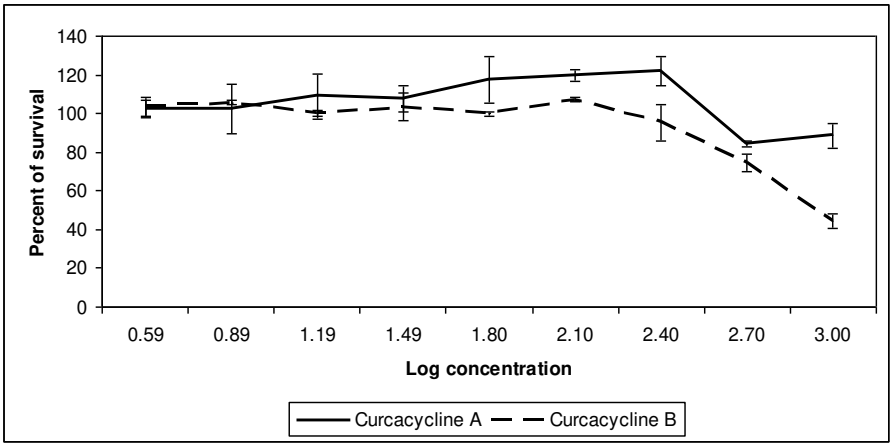


Figure 5a. Percentage survival of human Colo 205 cells after incubation with curcacycline A and B at various concentrations.

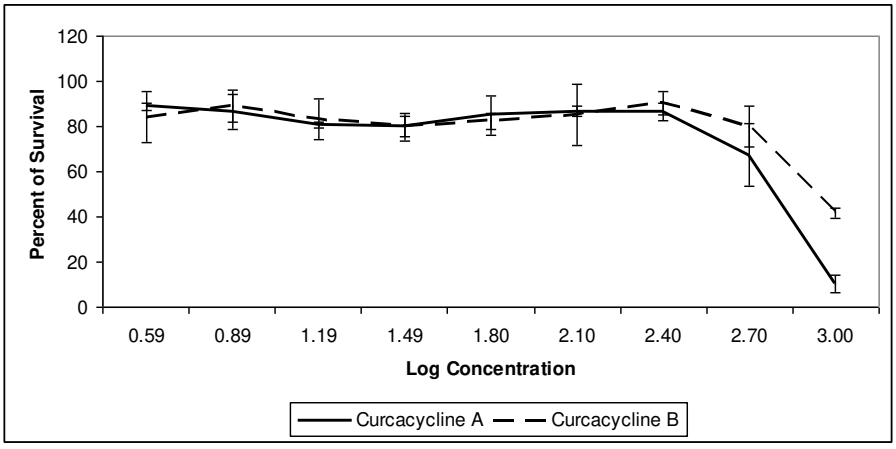


Figure 5b. Percentage survival of OVCAR3 cells after incubation with curcacycline A and B at various concentrations.

Curcacycline A was tested for its antibacterial activity against five different strains, and results were summarized in Table 3. Based on the inhibition zone diameter (mm), results were classified as: not sensitive (-) for diameter equal to 8 mm or below; sensitive (+) for diameter between 8 and 14 mm; very sensitive (++) for diameter 14 and 20 mm and extremely sensitive (+++) for diameter equal or larger than 20 mm. According to this classification curcacycline A was active against *Bacillus subtilis*, *Pseudomonas aeruginosa* at (8.6 $\pm$ 0.1 mm) and (8.3 $\pm$ 0.8) respectively. Curcacycline A did not have significant activity against *Escherichia coli, Staphylococcus aureus* and *Candida albicans*. Due to lack of sample amount, the activity profile could not be determined for curcacycline B. This study strengthens previous data that *Jatropha curcas* latex had a strong antibacterial activity against positive and negative strains of gram bacteria [32]. Inhibition zone of the latex against *B. subtilis, E. coli, P. aeruginosa, S. aureus* and *C. albicans* ranged between 24-26 mm [33]. It is reported that antimicrobial activity of latex was stronger because latex does not only contain cyclic peptides but also tannins, flavonoids and saponins having synergistic effect [34].

Table 4. Antibacterial activity of curcacycline A against *E. coli, P. aeruginosa, B. subtilis, S. aureus, C. albicans*.

Compound	Conc. (µg/mL)	Diameter Inhibition (mm ± SD)				
		E. coli	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***C. albicans***
Curcacycline A	1000	-	8.3 ± 0.8	10.3 ± 0.4	-	-
	500	-	7.1 ± 0.9	9.7 ± 0.5	-	-
	300	-	6.5 ± 0.5	8.6 ± 0.1	-	-
	100	-	-	8.0 ± 0.0	-	-
	50	-	-	8.0 ± 0.1	-	-
	10	-	-	7.6 ± 0.2	-	-
	1	-	-	-	-	-
	0.5	-	-	-	-	-
	0.1	-	-	-	-	-
Tetracycline	100	23.8 ± 0.3	11.6 ± 0.6	13.9 ± 0.3	24.4 ± 0.3	-
	10	11.3 ± 0.2	8.8 ± 0.3	8.3 ± 0.6	11.5 ± 0.3	-
	1	-	-	7.9 ± 1.2	-	-
Ketekonazole	100	-	-	-	-	13.9 ± 0.1
	10	-	-	-	-	-
	1	-	-	-	-	-

They have different mechanism for inhibiting bacterial activity. Tannins and saponins influence the bacterial cell wall while flavonoids inhibit bacterial enzymes [35, 36]. Although the mechanistic action of curcacyline A is still unclear, based on previous research [3] cyclic peptides exhibit antibacterial activity because of their ability to cross membranes easily and their higher selective affinity to bind with certain protein.

The Ames II test revealed no mutagenic activity for both compounds without S9 mixture. At all concentration the mutagenic conversion was as low as the negative control. This is the first report about mutagenic activity studies of cyclic peptides obtain from *Jatropha curcas* L. latex.

4.4 Conclusion

From ethnopharmacological point, *Jatropha curcas* L. latex has been traditionally used for treating dental problems in Indonesia because of antimicrobial activity. This claim is proven by previous research which showed antimicrobial activity of latex against gram positive and negative bacteria. Curcacycline A and B were isolated over twenty years ago. Isolated curcacycline A and B were subjected to latest analytical techniques like NMR-spectroscopy and HPLC/MS to confirm amino acid sequences. Furthermore, both structures were confirmed by an organic synthesis approach. Here we report for the first time about

antimicrobial, mutagenic, cytotoxic and ecotoxic activity. The results showed that curcacycline A has antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* and cytotoxic activity against OVCAR 3. No mutagenic effects were found on curcacycline A and B against *Salmonella typhimurium* TA-98 and TA-100. These data show that curcacycline A would be potential for new and safe antimicrobial candidate.

References

1. Pomilio AB, Battista ME, Vitale AA. Naturally-Occurring Cyclopeptides: Structures and Bioactivity. Current Organic Chemistry: Bentham Science Publishers Ltd.; 2006. p. 2075-121.

2. Wu L, Lu Y, Zheng Q-T, Tan N-H, Li C-M, Zhou J. Study on the spatial structure of annomuricatin A, a cyclohexapeptide from the seeds of Annona muricata. Journal of Molecular Structure. 2007;827(1-3):145-8.

3. Sakai R, Rinehart KL, Kishore V, Kundu B, Faircloth G, Gloer JB, et al. Structure and Activity Relationships of the Didemnins1,2. Journal of Medicinal Chemistry. 1996;39(14):2819-34.

4. Izumiya N, Kato T, Waki M. Synthesis of biologically active cyclic peptides. Biopolymers. 1981;20(9):1785-91.

5. Païs M, Das BC, Ferron P. Depsipeptides from Metarhizium anisopliae. Phytochemistry. 1981;20(4):715-23.

6. Schmidt U, Utz R, Lieberknecht A, Griesser H, Potzolli B, Bahr J, et al. Amino Acids and Peptides; 60.1 Synthesis of Biologically Active Cyclopeptides; 10. Synthesis of 16 Structural Isomers of Dolastatin 3; II:2 Synthesis of the Linear Educts and the Cyclopeptides. Synthesis. 1987;1987(03):236-41.

7. Kawai M, Gardner JH, Rich DH. Stereoselective synthesis and absolute configuration of epoxyketones in chlamydocin and related cyclic tetrapeptides. Tetrahedron Letters. 1986;27(17):1877-80.

8. Jacquier R, Verducci J. Nouvelle synthese de la tentoxine. Tetrahedron Letters. 1984;25(26):2775-8.

9. Poojary B, Belagali SL. Synthetic studies on cyclic octapeptides: Yunnanin F and Hymenistatin. European Journal of Medicinal Chemistry. 2005;40(4):407-12.

10. Hwang Y, Rowley D, Rhodes D, Gertsch J, Fenical W, Bushman F. Mechanism of Inhibition of a Poxvirus Topoisomerase by the Marine Natural Product Sansalvamide A. Molecular Pharmacology. 1999;55:1049.

11. Rich DH, Gardner JH. Synthesis of the Cytostatic Cyclic Tetrapeptide, Chlamydocin. Tetrahedron Letters. 1983;24(48):5305-8.

12. Cavelier-Frontin F, Achmad S, Verducci J, Jacquier R, Pèpe G. How to perform small peptide cyclizations. Journal of Molecular Structure: THEOCHEM. 1993;286:125-30.

13. Tang Y-Q, Yuan J. A Cyclic Antimicrobial Peptide Produced in Primate Leukocytes by the Litigation of Two Truncated. Science. 1999;286(5439):498.

14. Kelecom A. Secondary metabolites from marine microorganisms. Anais da Academia Brasileira de Ciências. 2002;74:151-70.

15. Baraguey C, Auvin-Guette C, Blond A, Cavelier F, Lezenven F, Pousset J-L, et al. Isolation, structure and synthesis of chevalierins A, B and C, cyclic peptides from the latex of Jatropha chevalieri. Journal of the Chemical Society, Perkin Transactions 1. 1998(18):3033-40.

16. Kosasi S, Hart LA, van Dijk H, Labadie RP. Inhibitory activity of Jatropha multifida latex on classical complement pathway activity in human serum mediated by a calcium-binding proanthocyanidin. Journal of ethnopharmacology. 1989;27(1-2):81-9.

17. Auvin-Guette C, Baraguey C, Blond A, Pousset J-L, Bodo B. Cyclogossine B, a Cyclic Octapeptide from Jatropha gossypifolia. Journal of Natural Products. 1997;60(11):1155-7.

18. Auvin C, Baraguey C, Blond A, Lezenven F, Pousset J-L, Bodo B. Curcacycline B, a cyclic nonapeptide from Jatropha curcas enhancing rotamase activity of cyclophilin. Tetrahedron Letters. 1997;38(16):2845-8.

19. Baraguey C, Blond A, Correia I, Pousset J-L, Bodo B, Auvin-Guette C. Mahafacyclin A, a cyclic heptapeptide from Jatropha

mahafalensis exhibiting [beta]-bulge conformation. Tetrahedron Letters. 2000;41(3):325-9.

20. Horsten SFAJ, van den Berg AJJ, Kettenes-van den Bosch JJ, Leeflang BR, Labadie RP. Cyclogossine A: A Novel Cyclic Heptapeptide Isolated from the Latex of Jatropha gossypifolia. Planta Med. 1996;62(01):46,50.

21. Mongkolvisut W, Sutthivaiyakit S, Leutbecher H, Mika S, Klaiber I, Moller W, et al. Integerrimides A and B, Cyclic Heptapeptides from the Latex of Jatropha integerrima. Journal of Natural Products. 2006;69(10):1435-41.

22. van den Berg AJ, Horsten SF, Kettenes-van den Bosch JJ, Kroes BH, Beukelman CJ, Leeflang BR, et al. Curcacycline A--a novel cyclic octapeptide isolated from the latex of Jatropha curcas L. FEBS Lett. 1995;358(3):215-8. Epub 1995/01/30.

23. Schmidt VU, Beuttler T, Lieberknecht A, Griesser H. Aminosäuren und peptide - XXXXII. Synthese von Chlamydocin + epi-Chlamydocin. Tetrahedron Letters. 1983;24(34):3573-6.

24. Staniek A, Woerdenbag HJ, Kayser O. Screening the endophytic flora of Wollemia nobilis for alternative paclitaxel sources. Journal of Plant Interactions. 2010;5(3):189-95.

25. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine Shrimp: A Convenient General

Bioassay for Active Plant Constituents. Planta Med. 1982;45(05):31,4.

26. Krishnaraju AV, Rao, T.V.N., Sundararaju, D., Vanisree, M., Tsay, H.S., and Subbaraju, G.V.,. Assessment of Bioactivity of Indian Medicinal Plants Using Brine Shrimp (Artemia salina) Lethality Assay. International Journal of Applied Science and Engineering. 2005;3(2):125-34.

27. Wanyoike GN, Chhabra SC, Lang'at-Thoruwa CC, Omar SA. Brine shrimp toxicity and antiplasmodial activity of five Kenyan medicinal plants. Journal of ethnopharmacology. 2004;90(1):129-33.

28. Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. A microwell cytotoxicity assay using Artemia salina (brine shrimp). Planta Medica. 1993;59(3):250-2.

29. Fluckiger-Isler S, Baumeister M, Braun K, Gervais V, Hasler-Nguyen N, Reimann R, et al. Assessment of the performance of the Ames II assay: a collaborative study with 19 coded compounds. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2004;558(1-2):181-97.

30. Wiley S, Schooley K, Smolak P, Din W, Huang C, Nicholl J, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995;3(6):673-82.

31. Bharadwaj R, Yu H. The spindle checkpoint, aneuploidy, and cancer. Oncogene. 2004;23(11):2016-27.

32. Fagbenro-Beyioku AF, Oyibo WA, Anuforom BC. Disinfectant/antiparasitic activities of *Jatropha curcas*. E Afr Med J. 1998;75(9):508-11. Epub 1999/09/24.

33. Oyi AR, Onaolapo J, Haruna AK, Morah CO. Antimicrobial screening and stabaility studies of the crude extract of *Jatropha curcas* L. latex (Euphorbiaceae). Nigerian Journal of Pharmaceutical Sciences. 2007;6(2):14-20.

34. Levens M, Vandan-Berghe DA, Marten J, Vihen T, Lomiveas EC. Screening of higher plants for biological activity. Planta Medica. 1979;36:311-2.

35. Iwu M, Igboko OA, Okeniyi CD, Tempesta MS. Inhibition of the enzyme activity of aldosoreductase of some flavonoids by some flavonoids. J Pharm Pharmacology. 1990;42:290-2.

36. Pathak D, Pathak L, Singla AK. Inhibition of Xanthine-oxidase activity by some flavonoids. Fitoterapia. 2002;62:371-85.

CHAPTER 5

Validation of detoxification process for *Jatropha curcas* L. kernel meal for use as animal feed

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Abstract

Processed and detoxified *Jatropha curcas* L. kernel meal (JKM) has been tested for its potential as an animal feedstock. The detoxification process has been validated by determining cytotoxicity, mutagenicity and ecotoxicity of fractions along the down streaming process. Cytotoxic activity was tested against Colo205 and OVCAR3 cells indicating high toxicity for unprocessed *Jatropha* kernel material but no detectable cytotoxicity for final processed JKM. Mutagenic activity was investigated by AMES II assay using *Salmonella typhimurium* TA-98 and TA-Mix. No mutagenic activity was detected in both *Salmonella* strains after incubation with all processed material. Ecotoxicity conducted by the brine shrimp lethality assay did not show significant toxicity for all processed fractions either. This report concludes that the detoxification process opens a way for the valorization of JKM as a feed constituent.

5.1 Introduction

Jatropha curcas L. is a tropical plant, widely known as physic nut or purging nut. It is drought resistant and can be cultivated in a wide variety of soils. The plant is mainly cultivated for the high oil content of its seed (up to 40%). This oil is traditionally used for soap or candle manufacture, lighting or as lubricant, but in recent time it is introduced as alternative source as second generation of biodiesel [1-3]. *J. curcas* L. oil received considerable economic potential for fulfilling local energy requirements in rural areas, but today it is well introduced for running trucks or even aircrafts. The seed also contains a high level of protein (up to 28%), which composition is excellent to use it as an animal feed [3-5]. However, the presence of anti-nutritional factors and toxic components make the seed kernel unsuitable for direct use in an animal's diet. Some seed constitutents affect protein digestion like trypsin inhibitors (TIs) and lectins) or mineral utilization as known for phytates being presence [6]. Others, including saponins, curcin and phorbol esters (PEs) have direct or indirect toxic effects on cellular functions and membrane transport. Phorbol esters (especially phorbol myristate acetate (PMA)), have been studied extensively in medical research because of their known tumor promoting activity [7]. Previous research reported six diterpenoid esters from the seed of this plant, all of them containing a 12-deoxy-16-hydroxyphorbol (DHPE) skeleton [8]. Phorbol esters act as skin irritants [9] with weak tumour

promoting activity [10]. The targets of phorbol esters are membrane receptors, which activate protein kinase c (PKC), an important step in signal transduction, cell differentiation and regulation of cell growth. PKC is activated by diacylglycerol (DAG) and phorbol esters are known to be agonists of DAG, which bind stronger to the complex than DAG, thereby pushing the cell to proliferation.

Many attempts have been made to eliminate the anti-nutritional and toxic components (trypsin inhibitors, phorbol esters, curcin, lectins, phytate) from Jatropha kernel meal. In Mexico *Jatropha* genotypes that exist do not contain phorbol esters in the seed, but the other anti-nutritional factors are present. These genotypes are being used as traditional food after roasting, indicating that all anti-nutritional and toxic components can be inactivated by heat.

Various approaches were used to eliminate the phorbol esters from the kernels. Double solvent extraction (petroleum ether and ethanol) combined with a chemical treatment using 0.07% $NaHCO_3$ eliminated 95.8% of the phorbol ester content [11]. This treatment was also suggested to be the best to reduce lectin activity. Protein extraction of *Jatropha* curcas L. meal under alkaline conditions and isoelectric precipitation before steam injection and washing treatment decreased the level of trypsin inhibitor, phytate, and saponins by more than 90%, and toxic phorbol esters were not detected anymore in the meal [12].

The company Quinvita, formerly D1 Oil, has recently developed and patented (WO2010070264) a one-step solvent extraction using an azeotrope mixture of ethyl acetate and methanol combined with a standard heat treatment to produce industrial quantities of detoxified *Jatropha curcas* L. kernel meal for use in animal feeds [13]. However, before commercial quantities of feed can be produced and incorporated into animal feed, it is essential that the meal is assayed exhaustively for the absence of any traces of potentially toxic components. This paper describes the analyses of various processed and raw meal samples for the presence of phorbol esters and potential cytotoxic and mutagenic agents.

5.2 Materials and Methods

5.2.1 Materials

All of the sample meals were obtained from D1 oil company, Innovation centre, innovation way, York, YO105DG, [13]. *Jatropha curcas* L. seeds were obtained from plants grown under standardized conditions in Cape Verde, in 2008.

5.2.2 Seed processing and detoxification

About 400 kg *Jatropha curcas* L. seeds were shipped to CREOL, Pessac, France. The seeds were cracked and dehulled using standard mechanical seed processing equipment. The kernels

were flaked and stored until use. 80 kg of flakes were solvent extracted essentially according to in a Guedu 500 L batch extractor with a mixture of ethylacetate and methanol at 60°C six times [13]. The remaining 35 kg of meal was desolventised and heat treated at 130°C for 20 min. Phorbol ester (PE) fractions were prepared from the seed of toxic and non-toxic varieties of *J. curcas* (stored at room temperature). The kernel was milled, batch extracted with hexane then soxhlet extracted with hexane for 3 hours. The defatted meal contained 0.46% oil. This meal was than separated into 5 samples of 3.5 g each that were soxhlet extracted for 1 hour with methanol and filtered through a 0.2 µm syringe filter. Crude *J. curcas* oil was batch extracted into an excess of methanol at 60°C. The methanol fraction was separated from the oil by gravity. Methanol was removed under vacuum using a rotary evaporator to the point of dryness. The remainder containing PE was dissolved in methanol and the residual oil removed by back-extracting the methanol phase into hexane. The methanolic solution was reduced in volume and analyzed for absence of PE. *Jatropha curcas* kernel from two varieties (toxic and non-toxic), and processed JKM were methanol soxhlet extracted for 3 hours. The methanolic solution was filtered and dried by rotary evaporation at 30°C. The residue was dissolved in methanol to 1000 µg/mL for further analysis.

5.2.3 Soxhlet Extraction

Meal was transferred into a thimble, covered with cotton and placed in a soxhlet. The soxhlet was equipped with a condenser and placed on a round bottom flask (500 mL), which was filled with Methanol (300 mL), boiling chips and glass pearls. The MeOH was refluxed for 4 h on a steam bath with which an average of 9 cycles was obtained. After cooling in an ice bath the flask was kept at 4ºC in the fridge in anticipation of further procedures. The methanol extracts of *Jatropha* kernel, defatted, oil,

5.2.4 Cytoxicity test with OVCAR3 cell-line

The cancer cells were routinely cultured in DMEM (Gibco®), suplemented with 10% of heat-inactivated fetal bovine serum (Gibco®) and 1% penicillin/streptomycin (Gibco®), at 37ºC in the presence of 5% CO_2. The addition of paclitaxel reference compound (plant and fungal extracts as well as pure components thereof) dissolved in 1% DMSO. The solution was sterillized by filtration (0.2 µm) and diluted with cell culture medium, in consecutive 2-fold dilutions. It was preceded by seeding the test cells into 96-well plates (5000 cells/well) for 24 h incubation. Cell viability was assessed 72 h later by means of the MTS assay (CellTiter 96® AQ_{ueous}Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA), following the instructions of the manufacturer [14].

5.2.5 Cytotoxicity test with Colo205 cell-line

The cancer cells were routinely cultured in RPMI (Invitrogen®), supplemented with 10% of heat inactivated fetal bovine serum (Invitrogen®) and 1% penicillin/streptomycin (Invitrogen®), at 37°C in the presence of 5% CO_2. The addition of TRAIL and paclitaxel reference compounds dissolved in 1% DMSO. The solution was sterillized by filtration (0.2 µm) and diluted with cell culture medium, in consecutive 2-fold dilutions. It was preceded by seeding the test cells into 96-well plates (5000 cells/well) for 24 h incubation. Cell viability was assesed 24 h later by means of the MTS assay (CellTiter 96® AQ_{ueous}Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA), following the instructions of the manufacturer. All assays were performed in triplicate [14].

5.2.6 Toxicity assay

Cell viability was evaluated by the MTS cell proliferation assay. The conversion of MTS into aqueous, soluble formazan by dehydrogenase enzymes in mitochondria of metabolically active cells is measured by colorimetric method. Cells were washed using PBS. An amount of 500 µL trypsin was added to the flask. The cells was incubated for 2 min. at 37°C and deactivated by trypsine with 5 mL medium which contain 10% FBS. Cell number and viability was determined by tryphan blue and it was suspended to final concentration of $1x10^5$ cells/mL in cultivating

medium. To all wells of the plate 100µL of the cell suspension (10,000 cells) were dispensed. They were incubated for 24 hours at 37°C in a humidified, 5% CO_2 atmosphere. In the next day, 20 µL of MTS solution was added. The plate was incubated for 1–4 hours at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance was recorded at 490 nm using ELISA plate reader.

5.2.7 Mutagenicity assay

Mutagenicity test was determined by AMES kit from Xenomatric. Two bacteria were used, *Salmonella typhimurium* TA-98 and *S. typhimurium* TA-Mix (TA 7001, TA 7002, TA 7003, TA 7004, TA 7005, TA 7006). These bacteria were exposed to six concentrations (1000; 500; 250; 125; 62.5; 31.25; 15.63; 7.82; 3.9 µg/mL) of samples, as well as positive and negative control, for 90 min in medium containing histidine to support approximately two cell divisions. After 90 min, the exposure cultures were diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of 384 well plates. Within two days, cells which have undergone the reversion to His will grow into colonies. Metabolism by the bacterial colonies reduces the pH of the medium, changing colour of reagent in the well (Xenometric®) [15].

5.2.8 Brine shrimp assay

Brine shrimp (*Artemia salina*) eggs were hatched in a conical shaped vessel of 1 liter, filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1 N NaOH) under constant aeration for 48 hours. After hatching, active nauplii free from egg shells were collected from a bright portion of the hatching chamber and used for the assay.

5.2.9 HPLC-MS analysis

The mass spectrometer was interfaced to a Phenomenex Kinetex C18 column (100A, 100 x 4.6 mm, 2.6 µm). The mobile phase comprised 5% acetonitril in H_2O (solvent A) and 100% acetonitrile (solvent B) with a flow rate 0.5 mL/min. Post column addition was used for ionization with 75% acetonitrile + 2 mM ammonium acetate and the flow rate was 0.2 mL/min. The separation was done using 70% solvent B for 25 min, followed by gradient eluent 75 to 95% B in 5 min, 95% B in 5 min, 95%-70% B in 2 min, and 70% B for the last 4 min. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 500°C (SCIEX API 3000 triple quadrupole mass spectrometer). Data processing was performed using Analyst version 1.5.1 software (MDS Sciex, Concord, Canada).

5.2.10 Statistics

The results of experiment were analized by *t-test*.

5.3 Result and discussion

All processed fractions of the *Jatropha curcas* L. meal detoxification process were tested for cytotoxicity, ecotoxicity and mutagenicity. Depending on the step of detoxification reduced toxicity was determined up to no cytotoxicity or ecotoxicity in the final fractions that are considered to be feed stock. Phorbolesters (PE) as main responsible constitutents for toxicity but also mutagenicity were not detected in the final processed fraction by LC-MS (Fig. 1). This result may correlate with absence of any toxicity and mutagenicity, but still authors are aware that other constituents may cause toxic effects (e.g. lectines).

The presence of phorbol esters were observed by HPLC MS using precursor ion scan 311 and multiple reactions monitoring (Fig. 1). Based on testing of fractions from the detoxification process, there was a signal of PE in kernel, defatted meal the oil from toxic genotypes. While no phorbol esters were detected in nontoxic genotypes and processed *Jatropha* mealAs first committed step towards process validation acute cytoxicity was determined with COLO205 and OVCAR-3 cell.

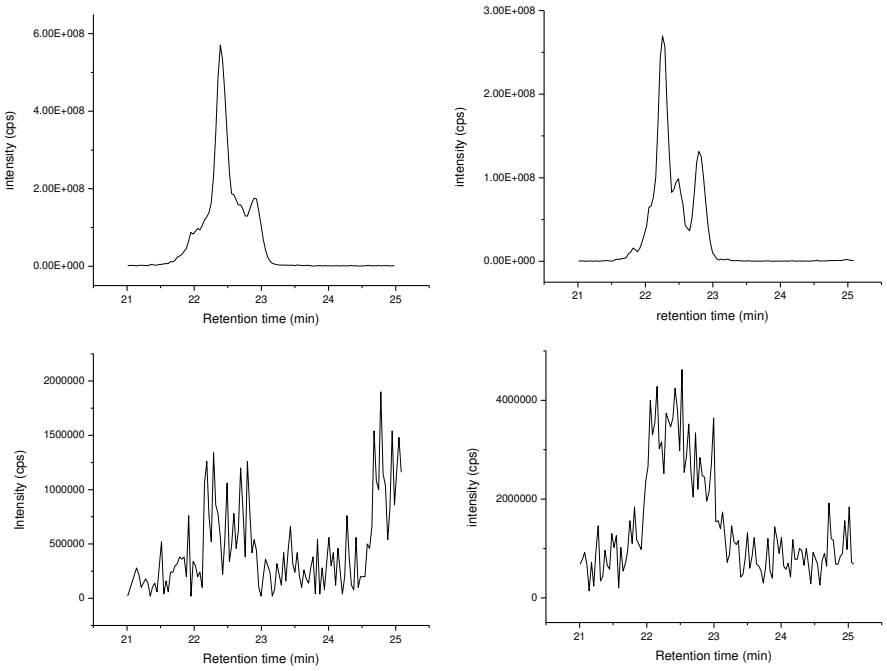

Figure 1. Chromatogram of various extract of *J. curcas*. a. MeOH extract of *J. curcas* kernel; b. MeOH extract of *J. curcas* oil ; c. MeOH extract of non-toxic *J. Curcas*; d. MeOH extract of processed *J. curcas* meal

Both cell cell lines were chosen for the high sensitivity against PE. α and β PMA were used as positive control for the toxicity in Colo205 cells (Tab. 1). The IC_{50} values were respectively 72.4 and 17.4 µg/mL against Colo205 and OVCAR3, respectively. Colo205 and OVCAR-3 cells were incubated with various concentrations of extracted *Jatrophacurcas* L. seed fractions and IC_{50} values were calculated (Tab. 1 and 2). Tested fractions of *J. curcas* L. showed for defatted meal and obtained oil moderate

activity against Colo 205 cell with 524 and 426 µg/mL, respectively. Interestingly, Ovcar-3 cell did not show any cytotoxicity indicted by IC_{50} values over 1000 µg/mL. Detoxified seed kernel in the last process step did not show any cytotoxicity with 1000 µg/mL for both cell lines documenting its efficacy. As control the detoxification process was carried out with a *Jatropha curcas* L. variety from Mexico that does not form toxic PE or the concentration of PE is below LOD and it is considered as the non-toxic genotype [5]. In contrast to the assumption that cytotoxicity is correlated with PE content, methanol fraction of non-toxic genotype showed also moderate cytotoxicity at 537 µg/mL in the MeOH fraction (Tab. 1).

Table 1 IC_{50} (µg/mL) of processed extracts and fractions of *J. curcas* L. against Colo 205 and OVCAR-3 cell lines

Extracts	**Colo 205**	**Ovcar 3**
	IC_{50} (µg/mL)	IC_{50} (µg/mL)
PE defatted meal	524	> 1000 µg/ml
PE from oil	426	> 1000 µg/ml
PE inactive meal	> 1000	> 1000 µg/ml
PE defatted meal non-toxic	> 1000	> 1000 µg/ml
MeOH extract non-toxic genotype	537	> 1000 µg/ml
MeOH extract toxic genotype	> 1000	> 1000 µg/ml
α-PMA	72.44	n.d
β-PMA	17.37	n.d

n.d : not determined

Table 2. Brine Shrimp letality Assay of untreated and processed extracts from *J. curcas* L.

Extracts	**LC_{50} (95 interval)**
Shell	> 1000 µg/mL
Seed	234 µg/mL (112-280)
inactive meal	> 1000 µg/mL
Methanol	10 %

This may indicate that besides of PE other toxic constituents are present in the seed kernel and detoxification have to be addressed as well in the process design.

The mutagenic potential of *Jatropha curcas* L. seed kernel material and its fractions from the detoxification process were tested with the AMES assay. In comparison to the positive control 2-nitrofluorene and 4-nitroquinoline N-oxide with a revertant rate of 50 the number of revertants of *Salmonella typhimurium* TA-98 and TA-mix strains was with about 1-3 less than 5%, that can statistically be accepted as not mutagenic. These results are in accordance with the fact that phorbol esters are known to be tumor promoting agents and not tumor inducers. This long term assay was not in the scope of this report because in vivo animal tests are required.

Finally, ecotoxicity was tested in the brine shrimp assay. All extracts of the seed kernel and shell material were tested in the

brine shrimp assay. In contrast to the seeds (IC_{50} = 234 µg/mL) no ecotoxicity was found for either the shells or the final detoxified fraction (IC_{50}> 1000 µg/mL).

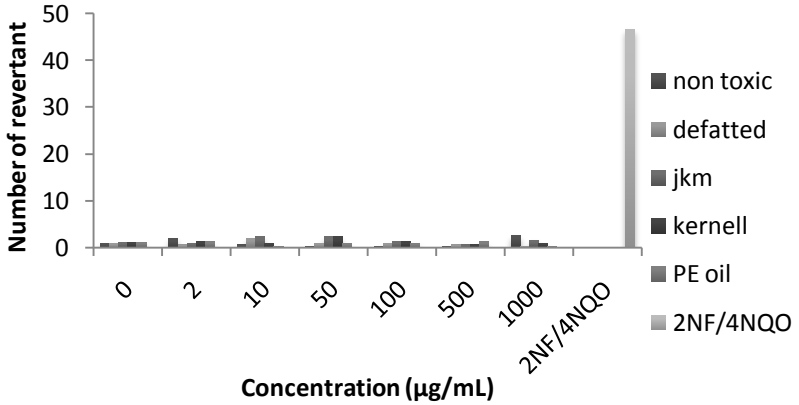

Figure 2. Mutagenic potential of processed extracts and fractions of *J. curcas* L. against*Salmonella typhimurium* TA98

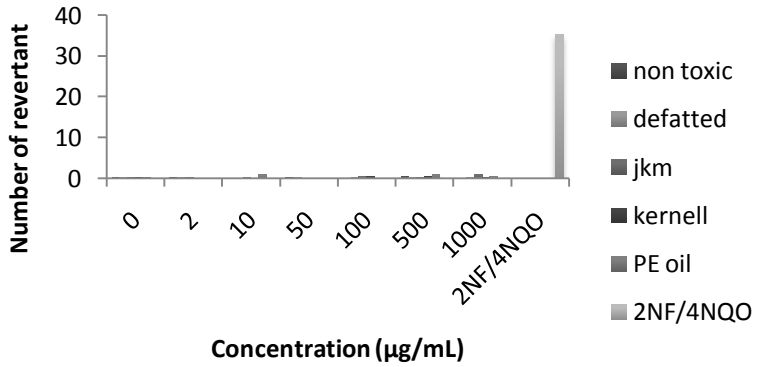

Figure 3. Mutagenic potential of processed extracts and fractions of *J. curcas* L. against*Salmonella typhimurium* TA-Mix

5.4 Conclusion

The proposed detoxification process is efficient to separate toxic principles from the Jatropha curcas seed kernel to obtain a protein extract being safe to be used as feed stock. No final fraction showed any mutagenic potential and none showed any ecotoxicity or cytotoxicity in cell culture testing. By functional biological assay, only intermediate fractions of the raw kernel were toxic. These combined results confirm the effectiveness of the extraction procedure and demonstrate that the processed meal product can be valorized as potential feed stock. The applied technical detoxification process may lead to increase technical capabilities for biorefining of *Jatropha curcas* L. waste material and to give new resources for cattle and chicken production in areas where land resources are limited and fed supply is critical to raise productive livestock.

References

1. Gandhi VM, Cherian KM, Mulky MJ. Toxicological studies on ratanjyot oil. Food Chem Toxicol. 1995;33(1):39-42. Epub 1995/01/01.

2. Makkar HPS, Becker K, Sporer F, Wink M. Studies on Nutritive Potential and Toxic Constituents of Different Provenances of Jatropha curcas. Journal of Agricultural and Food Chemistry. 1997;45(8):3152-7.

3. Asseleih LMC, Plumbley RA, Hylands PJ. Purification and Partial Characterization Of a Hemagglutinin From Seeds of *Jatropha Curcas*. Journal of Food Biochemistry. 1989;13(1):1-20.

4. Aderibigbe AO, Johnson COLE, Makkar HPS, Becker K, Foidl N. Chemical composition and effect of heat on organic matter and nitrogen degradability and some antinutritional components of *Jatropha* meal. Animal Feed Science and Technology. 1997;67(2-3):223-43.

5. Makkar HPS, Aderibigbe AO, Becker K. Comparative evaluation of non-toxic and toxic varieties of Jatropha curcas for chemical composition, digestibility, protein degradability and toxic factors. Food Chemistry. 1998;62(2):207-15.

6. Francis G, Makkar HP, Becker K. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 2001;199(3/4):197-227.

7. Baird WM, Boutwell RK. Tumor-promoting Activity of Phorbol and Four Diesters of Phorbol in Mouse Skin. Cancer Research. 1971;31(8):1074-9.

8. Haas W, Sterk H, Mittelbach M. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of Jatropha curcas. J Nat Prod. 2002;65(10):1434-40. Epub 2002/10/26.

9. Adolf W, Opferkuch HJ, Hecker E. Irritant phorbol derivatives from four Jatropha species. Phytochemistry. 1984;23(1):129-32.

10. Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wongchai V, et al. A new tumor promoter from the seed oil of Jatropha curcas L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. Cancer Res. 1988;48(20):5800-4. Epub 1988/10/15.

11. Martínez-Herrera J, Siddhuraju P, Francis G, Dávila-Ortíz G, Becker K. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in

four provenances of Jatropha curcas L. from Mexico. Food Chemistry. 2006;96(1):80-9.

12. Devappa RK, Swamylingappa B. Biochemical and nutritional evaluation of *Jatropha* protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J Sci Food Agr. 2008;88(5):911-9.

13. Brooker J, inventor; Methods for detoxifying oil seed crops. Great Britain patent 2466353. 2010 24 June 2010.

14. Staniek A, Woerdenbag HJ, Kayser O. Screening the endophytic flora of Wollemia nobilis for alternative paclitaxel sources. Journal of Plant Interactions. 2010;5(3):189-95.

15. Fluckiger-Isler S, Baumeister M, Braun K, Gervais V, Hasler-Nguyen N, Reimann R, et al. Assessment of the performance of the Ames II assay: a collaborative study with 19 coded compounds. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2004;558(1-2):181-97.

CHAPTER 6

Discussion, Summary and Perspective

6.1 General discussion

The process of finding renewable energy sources is still very interesting issue. The increase of crude oil price every year make the Indonesian government budget increased, because they should provide subsidy to reduce the price of the fuel. At the first time *J. curcas* provided a new of hope as an alternative energy sources, because of the low production costs. Unfortunately this influence on the low selling price, thus making the profit margins of the farmers were low, it made them look for other plant to crop. This situation was not expected considering *J. curcas* L. has other potential and it can provide a lot of benefits.

Naturally, *Jatropha curcas* L. produced different kinds of secondary metabolites, i.e alkaloid, diterpenes, flavonoid, cyclopeptides and phorbol esters. The two last groups were studied in this thesis. Cyclopeptides were usually found in marine organism. In higher plant, almost all member of *Jatropha* genus produce these compounds. They can be found mostly in latex. Cyclopeptides were grouped as oligopeptide because they were constructed from 3-10 amino acids. They were unique, because they cannot be visualized by nynhydrin and H_2SO_4 reagents since they do not have C and N terminal. They also have different pharmacological activities, like anticancer, antifungi, and antimicrobial effect. Two cyclopeptides were isolated from *J. curcas* latex, they were curcacycline A and B. In this thesis we found that curcacycline A had antimicrobial effect

against *Bacillus subtillis* and *Pseudomonas aeruginosa* which represent positive and negative gram bacteria. So it is potential to be developed as new antimicrobial. Phorbol esters (PEs) became an interesting topics although they were toxic and potential for promoting tumour. Curcin, a ribosome inactivating protein is a potential lead compound as anticancer and immunosuppressive drug. Curcusone B isolated from the root also has good prospects to be used for its antiproliferative effect, since it inhibits the growth of cancer cell lines. Jatropherol I has insecticidal activity, because it induces apathological condition in endoplasmic reticulum, chromatin, lysosome, mitchondria and microvilli of insects. Phorbol ester has become a major issue regarding *Jatropha curcas* L. oil and toxicity. Since it is dissolved well in the oil, intoxication risk for workers in the oil producing industry is high. It was reported that multiple steps in the oil refining process like degumming, deodorization, neutralization (with alkali hydroxides), bleaching and stripping processes reduce the level of phorbol ester significantly. Detoxified oil can be used as a candidate ingredient for ointment and dermal application preparations.

Jatropha curcas L. pressed cake contains a high protein yield. It can be used as animal feed, but the utilization is limited due to its toxic ingredients. A strategy to develop a detoxification process of the pressed cake has become of high interest. Physical and chemical treatments have been investigated in

toxic removal. For safety reason, biosafety assays have been observed for this detoxified product to various *in vitro* and *in vivo* studies. The results will show more evidence whether detoxification of pressed cake is possible and safe for further use. In analyzing DHPEs content, HPLC with Dioda Array Detector was used with β-phorbol myristic acetate as standard. Based on our findings, this was not correct and can lead to quantification error. We proved using MRM in tandem mass spectrometer that both compounds had different fragmentation patterns. This method gave more advantage because we were able to see the signal of the sample in smaller concentration.

Developing method for seed detoxification play an important role for giving *J. curcas* more valuable, seed of *J. curcas* without its toxic contents can be used for animal feed and fertilizer. In order to test the safety of *J. curcas* meal, we can use *in vitro* and *in vivo* methods. Several animals have been used for this assay, they were mice, rat, goat and fish. The meal were given repeatedly, and some biochemical change were evaluated to see the influence of it to the animal. In this study, we used *in vitro* assays to validate the effectiveness of detoxification processes. *J. curcas* meals were applied to the cell culture (for the carcinogenicity test), *Salmonellatyphimurium* TA 98 and TA mix, the results showed that all processed meal were not cytotoxic and mutagenic.

From the data above investigation on latex and *J. curcas* meal can be applied for new pharmaceutical product and food application. These conditions will give more additional economic values to the farmer who want to crop this plant.

6.2 Summary

Jatropha curcas L. has a great potential and value for being cultivated as economic crop for biodiesel production. It is not only a source of non-edible renewable oil, but it also contains secondary metabolites with interesting biological activities. As discussed **(chapter 2)**, these secondary natural compounds were isolated from different parts of *Jatropha curcas* L. and characterized. Some experiments have been conducted in the past for validating pharmacological uses to know their efficacies.

In phorbol ester studies, α dan β Phorbol myristic acetate (PMA) were used as standards. Although α and β are stereoisomers, they showed different physicochemical characteristics **(chapter 3).** Their maximum wavelengths and retention times are different. Based on our result using tandem mass spectrometry, we can confirm that the α dan β PMA also have different fragmentation pattern with dehydroxy phorbol ester (DHPE) in *J. curcas*. Because of that, the use of α dan β PMA in DHPE analysis is not suitable.

From ethnopharmacological point of view, *Jatropha curcas* L. latex has been traditionally used for treating dental

problems in Indonesia, because of its antimicrobial activity. This claim is proven by previous research which showed antimicrobial activity of latex against gram positive and gram negative bacteria. Curcacycline A and B were isolated over twenty years ago. Isolated curcacycline A and B have now been subjected to the latest analytical techniques like NMR-spectroscopy and HPLC/MS to confirm the amino acid sequences. Furthermore, both structures were confirmed by an organic synthesis approach. Here we report for the first time about antimicrobial, mutagenic, cytotoxic and ecotoxic activity of curcacycline A and B **(chapter 4)**. The results showed that curcacycline A has antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* and cytotoxic activity against OVCAR 3. No mutagenic effects were found on curcacycline A and B against *Salmonella typhimurium* TA-98 and TA-100. These data showed that curcacycline A would be potentially a new and safe antimicrobial candidate.

Different techniques of seedcake detoxification were developed. To validate the detoxification results, two assays were done, mutagenicity and cytotoxicity (**chapter 5)**. None of the extract showed any mutagenic potential and none showed any cytotoxicity in cell culture testing. By biological assay, only extracts of raw kernel were toxic. All other extracts had no significant effect on the survival of brine shrimps. These combined results confirm the effectiveness of the extraction

procedure and demonstrate that the processed meal product has no mutagenic potential and no detrimental effects on cells in culture, or in biological assay.

6.3 Perspective

Because *J. curcas* L. produced many natural products with interesting pharmacological activities. More efforts should be done to prove their efficacies (in vitro and in vivo). Not only their efficacies but their safeties should be observed. Beside doing toxicity tests by cell culture and brine shrimp test, in vivo studies in higher animal can be an option. The tests include acute toxicity test to know LD_{50} of each compounds and subchronic test to know the safety of those compounds if they were given in repeated doses. Beside general toxicity test, we have done special test for mutagenicity, additional work with s-9 fraction can be done for additional information if the compounds were metabolized by liver. Further investigation is still needed for oil safety, because direct contact of remaining phorbol ester with skin has to be avoided in all cases. Studies about indoor pollution by *Jatropha curcas* L. oil should be conducted, in order to give a clear distinction from combustion products from the oil and related toxicity.

In analytical work, validation method for analyzing phorbol esters should be done using HPLC/MS. This validation work should cover accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), specificity, and robustness. So the development of this analytical method can be accepted by others.

Recent progress on *Jatropha curcas* L. studies indicates that all parts of this plant are valuable. Utilization of all plant parts and ingredients can help to increase the economic potential of *Jatropha curcas* L. and it might increase the economic value of huge plantations.

APPENDIX

Nederlandse en Indonesische samenvatting

List of Publication

Acknowledgements

Nederlandse samenvatting

Jatropha curcas L. heeft als gewas een groot potentieel en economische waardevoorde productie van biodiesel. Het isnietalleen een hernieuwbare bron voor olie, maar bevat ooksecundairemetabolieten met interessante biologische activiteiten. **Hoofdstuk 2** van dit proefschrift beschrijft de eigenschappen van deze natuurlijke stoffen afkomstig uit de verschillende delen van het *Jatropha curcas* L. plant. In het verleden zijn deze stoffen gebruikt als referentiestof bij de analyse van farmacologische actieve verbindingen.

In studies naar werking en toxiciteit van phorbol ester worden α en β*p*horbol *m*yristic *a*cetaat (PMA) gebruikt als standaards. Hoewel α en β stereo-isomeren zijn, vertonen ze verschillende fysisch-chemische karakteristieken (**hoofdstuk 3**). Zo zijn de maximale golflengte in de spectrofotometer en de retentie tijden bij chromatografie verschillend. Door onze resultaten met tandem massa spectrometrie kunnen we bevestigen dat de α en β PMA ook verschillende fragmentatie patronen hebben in vergelijking tot dehydroxyphorbol ester (DPHE) in *J. curcas* L. Daarhalve is het gebruik van α of β PMA als referentiestof in DHPE analyse niet geschikt.

Vanuit de ethnopharmacologie is bekend dat *Jatropha curcas* L. latex gebruikt kan worden voor de behandeling van tandheelkundige problemen in Indonesië, vanwege de antimicrobiële activiteit. Deze bevinding is in eerder onderzoek gedaan, waarin antimicrobiële activiteit van de latex tegen gram positieve en gram negatieve bacteriën aangetoond werd. Curcacycline A en B werden ruim twintig jaar geleden geïsoleerd uit *J. curcas* L.. Geïsoleerd curcacycline A en B zijn nu onderworpen aan de meest recente analytische technieken zoals NMR-spectroscopie en HPLC/MS om de aminozuurvolgorde bevestigen. Bovendien werden beide structuren bevestigd door een organische synthese benadering. Hier rapporteren we voor het eerst over de antimicrobiële, mutagene, cytotoxische en ecotoxische activiteit van curcacycline A en B (**hoofdstuk 4**). De resultaten laten zien dat curcacycline A antimicrobiële werking heeft tegen *Bacillus subtilis* en *Pseudomonas aeruginosa* en cytotoxische activiteit tegen OVCAR3 cellen. Geen mutagene effecten werden gevonden van curcacycline A en B op *Salmonella typhimurium TA-98* en *TA-100*. Deze gegevens laten zien dat curcacycline A een potentieel nieuwe en veilige antimicrobiële verbinding is.

Verschillende technieken van detoxificatie van oliezaadkoek zijn ontwikkeld. Om de detoxificatie resultaten te valideren werden twee testen gedaan, mutageniteit en cytotoxiciteit (**hoofdstuk 5**).

Geen van de extracten toonde mutagene activiteit en ook werd geen cytotoxiciteit in celcultuur gemeten. Uit biologische testen bleek dat alleen het extract uit ruwe kernel giftig is. Alle andere extracten hadden geen significant effect op de overleving van pekelkreeftjes. Deze gecombineerde resultaten bevestigen de doeltreffendheid van de extractie procedure en laten zien dat de verwerkte koek product geen mutagene werking heeft. Ook zijn er geen aanwijzingen voor toxische effecten op cellijnen of in een eenvoudig diermodel.

Kesimpulan

Jatropha curcas L. memiliki potensi dan nilai ekonomi yang besar sebagai produsen biodiesel. Bukan hanya sebagai sumber bahan bakar terbarukan, tetapi juga memiliki kandungan metabolit kimia dengan aktivitas biologis yang menarik. Seperti yang telah dibahas sebelumnya **(chapter 2)**, metabolit sekunder tersebut telah diisolasi dan dikarakterisasi dari berbagai bagian tubuh *Jatropha curcas* L. Beberapa penelitian telah dilakukan dalam upaya pemastian efek farmakologinya. Curcin, suatu senyawa penginaktivasi ribosom protein adalah senyawa potensial sebagai antikanker dan penekan sistem imun tubuh. Curcusone B yang diisolasi dari akar juga memiliki prospek yang baik untuk digunakan sebagai antiproliperatif, karena kemampuannya dalam menghambat pertumbuhan sell kanker.

Jathoperol I memiliki aktivitas sebagai insektisida, karena dapat menginduksi patologi pada reticulum endoplasmik, kromatin, lisosom, mitokondria dan mikrovilli serangga. Curcacycline A menunjukkan inhibisi pada proliferasi sel T sedangkan curcacycline B dapat mengikat cyclophilin manusia sehingga dapat meningkatkan peptidyl-prolil *cis trans* isomerase.

Phorbol ester menjadi permasalahan utama pada pengolahan minyak jarak pagar. Karena kelarutannya baik dalam minyak, resiko intoksikasi pada pekerja pengolahan minyak jarak sangatlah besar. Untuk itu diperlukan beberapa penelitian untuk menelaah bahaya tersebut. Telah dilaporkan bahwa proses pengolahan bertingkat pada pengolahan minyak seperti *degumming, deodorization, neutralization* (dengan alkali hidroksida), *bleaching,* dan *stripping* dapat mereduksi kadar phorbol ester secara signifikan. Hasil pengolahan minyak tersebut dapat digunakan sebagai kandidat bahan dasar salep atau sediaan kulit lainnya. Penelitian lanjut diperlukan untuk melihat keamanan minyak, karena kontak langsung phorbol ester dengan kulit harus dihindari. Studi tentang polusi akibat pembakaran pun harus dilakukan, sehingga dapat memberikan penjelasan apakah pengaruh tersebut akibat minyak atau proses pembakarannya.

Limbah pengolahan minyak *Jatropha curcas* L. (seedcake) diketahui memiliki kandungan protein yang tinggi.

Limbah tersebut dapat digunakan sebagai bahan makanan hewan, akan tetapi penggunaannya terbatas oleh kandungan senyawa toksiknya. Strategi untuk upaya detoksifikasi menjadi fokus utama. Upaya yang telah dilakukan adalah proses fisika dan kimia. Untuk alasan keamanan, uji toksisitas telah dilakukan baik secara *in vitro* dan *in vivo*. Hasilnya dapat digunakan sebagai bukti apakah hasil proses detoksifikasi tersebut aman digunakan untuk proses selanjutnya.

Dalam penelitian phorbol ester, senyawa yang sering digunakan sebagai standard adalah α dan β phorbol miristik asetat (PMA), walaupun kedua senyawa tersebut adalah stereoisomer akan tetapi memperlihatkan karakteristik fisikokimia yang berbeda **(chapter 3)**, tidak heran kedua senyawa tersebut memiliki aktivitas promoter tumor yang berbeda. Kedua senyawa tersebut juga memiliki karakteristik fragmentasi ionisas yang berbeda dengan senyawa dehidroksi phorbol ester (DHPE) phorbol ester yang terdapat pada *Jatropha curcas* L. Sehingga pada penelitian ini disimpulkan bahwa penggunaan α dan β PMA sebagai standard tidaklah tepat untuk analisis DHPE.

Dari sudut pandang etnofarmakologi, getah *J. curcas* telah digunakan secara tradisional oleh masyarakat untuk mengatasi permasalahan gigi. Terutama sebagai antimikroba. Klaim khasiat tersebut telah dibuktikan dari beberapa penelitian

sebelumnya, yang menunjukkan aktivitas getah pohon jarak pagar terhadap beberapa bakteri gram positif dan gram negatif. Curcacyline A dan B telah diisolasi lebih dari 20 tahun yang lalu. Isolasi dan karakterisasi curcacycline A dan B pada penelitian ini menggunakan spektroskopi NMR dan HPLC/MS untuk mengkonfirmasi urutan asam amino yang ada. Selain itu, upaya sintesis organiknya pun telah berhasil dilakukan. Pada penelitian ini dilaporkan untuk pertama kali mengenai aktivitas antimikroba, mutagenisitas, sitotoksisitas, dan ekotoksisitas dari curcacycline A dan B (**chapter 4)**. Hasil penelitian menunjukkan bahwa curcacycline A memiliki aktivitas antimikroba terhadap *Bacillus subtilis* dan *Pseudomonas aeruginosa* dan aktivitas sitotoksik terhadap sel OVCAR 3. Tidak ditemukan efek mutagenik terhadap *Salmonella typhimurium* TA-98 dan TA-mix. Hasil tersebut menunjukkan bahwa curcacycline A potensial dikembangkan sebagai antimikroba.

Beberapa teknik telah dikembangkan untuk detoksifikasi ampas *J. curcas*. Untuk memvalidasi proses detoksifikasi, tiga metode digunakan. Uji mutagenik menggunakan metode AMES test II, uji sitotoksik menggunakan sel kanker, dan uji ekotoksisitas menggunakan brine shrimp. Hasil penelitian menunjukkan tidak ada ekstrak yang memiliki efek sitotoksik ataupun mutagenik. Uji ekotoksisitas menunjukkan bahwa ekstrak dari biji *J. curcas* yang belum diolah toksik terhadap larva *Artemia salina.* Hal ini membuktikan bahwa upaya

detoksifikasi yang telah dilakukan tidak menunjukkan efek yang berarti sehingga dapat dikatakan proses tersebut telah efektif.

Berdasarkan penelitian terhadap tanaman *J. curcas* mengindikasikan bahwa seluruh bagian tanaman sangat berharga. Penggunaan seluruh bagian tanaman akan dapat meningkatkan nilai ekonomi *J. curcas* dan memberikan keuntungan bagi petani yang menanamnya.

List of Publications

1. Muhamad Insanu, Jana Anggadireja, Oliver Kayser, 2010, Isolation of curcacycline A from latex of Jatropha curcas and its antibiotic and cytotoxic effect, Planta Medica, 76 (12), P-435
2. Muhamad Insanu, Jana Anggadireja, Oliver Kayser, 2012, Curcacycline a and b – new pharmacological insights to an old drug,International Journal of Applied Research in Natural Products, 5 (2), 26-34
3. Muhamad Insanu, Chryssa Dimaki, Richard Wilkins, John Brooker, Piet van der Linde, Oliver Kayser, 2013, Rational use of *Jatropha curcas* L. In food and medicine : from toxicity problems to safe applications, Phytochemistry reviews, 12 (1), 107-119

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