UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
MESTRADO EM BIOQUÍMICA E FISIOLOGIA

Purificação, caracterização e avaliação de atividades biológicas da proteína tipo vicilina de *Cratylia mollis*

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RECIFE, 2008

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Aprovada por: _____
Profª Dra Patrícia Maria Guedes Paiva

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Ata da defesa de dissertação da Mestranda **Ana Luiza Germano Ramos,** realizada em 29 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 09:20 horas, do dia vinte e nove de fevereiro de 2008, foi aberto, no Auditório Prof. Marcionilo Lins – Depto. de Bioquímica, do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco, o ato de defesa de dissertação da mestranda **Ana Luiza Germano Ramos**, aluna do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Patrícia Maria Guedes Paiva fez a apresentação da aluna, de sua orientadora, ela própria, bem como da Banca Examinadora composta pelos professores doutores: Patrícia Maria Guedes Paiva, na qualidade de Presidente, Maria Tereza dos Santos Correia, Ranilson de Souza Bezerra, ambos do Depto. de Bioquímica/UFPE e Maria Inês Sucupira Maciel, do Depto. de Ciências Domésticas, Alimentos, Nutrição e Saúde/UFRPE. Após as apresentações, a Profa. Dra. Patrícia Maria Guedes Paiva convidou a aluna para a apresentação de sua dissertação intitulada: "**Purificação, Caracterização e Avaliação de Atividades Biológicas da Proteína Tipo Vicilina de *Cratylia mollis***", e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pela aluna para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu à explanação e comentários acerca do tema em **20 (vinte) minutos**. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Profa. Dra. Maria Inês Sucupira Maciel que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para o Prof. Dr. Ranilson de Souza Bezerra, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Logo após, a Sra. Presidente passou a palavra para a Profa. Dra. Maria Tereza dos Santos Correia, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente, na qualidade de orientadora, usou da palavra para tecer alguns comentários a respeito do trabalho da aluna, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 29 de fevereiro de 2008.

Maria Inês Sucupira Maciel

Patrícia Maria Guedes Paiva

Tereza Correia

SUMÁRIO

AGRADECIMENTOS

Agradeço a Deus, por todas as bênçãos derramadas em minha vida, pela capacitação e cuidado não só nesses dois anos, mas em todos os anos de minha vida, pois sei que sem Ti nada posso fazer.

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RESUMO

Uma proteina sem atividade hemaglutinante ou inibitória de tripsina foi isolada de sementes de *Cratylia mollis* por fracionamento com sulfato de amônio e cromatografia em Sephadex G-75 e denominada cratilina. SDS-PAGE na ausência de agente redutor revelou a presença de polipeptídeos de M_r 205, 150 e 50 kDa e em condições redutoras como peptídeos de M_r 50, 26, 24 e 22 kDa. Cratilina aquecida (100ºC) foi resolvida como peptídeos de 26, 24 e 22 kDa. Cratilina (10–1000 ppm) foi tóxica para *Artemia salina* com LC_{50} de 558.7 µg/ml. Atividade antitumoral de cratilina 11,5 mg/kg, 23 mg/kg ou cratilina aquecida 23 mg/kg foi avaliada sobre carcinoma de Ehrlich implantado em camundongo (*Mus muscullus*) e foi determinada atividade de inibição do tumor de 63%, 73% e 34%, respectivamente. Atividade anti-hiperlipidêmica de cratilina (10 ou 20 mg/kg/day) foi testada usando camundongo hiperlipidêmico e mudanças no colesterol e triglicerídeos do soro não foram detectadas. Atividade artemicida de cratilina é uma indicação da propriedade de inibição tumoral.

Palavras-chave: *Artemia salina;* Atividade antitumoral; *Cratylia mollis*; Carcinoma de Ehrlich; Hiperlipidemia; Vicilina.

ABSTRACT

Protein without hemagglutinating or trypsin inhibitory activities was isolated from *Cratylia mollis* seeds by ammonium sulphate protein fractionation and Sephadex G-75 chromatography and named cratylin. SDS-PAGE at absence of reducing agent revealead the presence of polypeptides of M_r 205, 150 e 50 kDa and under reducing conditions as peptides of M_r 50, 26, 24 e 22 kDa. Heated (100ºC) cratylin was resolved as 26, 24 and 22 kDa peptides. Cratylin (1000–10 ppm) was toxic to *Artemia salina* with LC_{50} of 558.7 µg/ml. Antitumoral activity of cratylin 11.5 mg/kg, 23 mg/kg or heated (100ºC) cratylin 23 mg/kg were evaluated on Ehrlich carcinoma implanted in Swiss albino mice (*Mus muscullus*) and activities of tumor-weight inhibition were achieved of 63%, 73% and 34%, respectively. Anti-hyperlipidemic activity of cratylin (10 or 20 mg/kg/day) was assayed using hyperlipemic Swiss albino mice and changes in serum cholesterol and triglycerides were not detected. Artemicide activity of cratylin is an indication of tumor inhibitory property.

Keywords: *Artemia salina; A*ntitumoral activity; *Cratylia mollis*; Ehrlich carcinoma; Hyperlipidemia; Vicilin.

1. INTRODUÇÃO

1.1. Proteínas de leguminosas

As proteínas têm a capacidade de formarem complexos estáveis específicos que são fundamentais para os processos biológicos. A presença, na estrutura protéica, de diferentes superfícies associativas que interagem com pequenos ligantes, tais como o oxigênio e íons ou moléculas maiores, tais como ácidos nucléicos, outras proteínas e carboidratos (Peters *et al.,* 1996) possibilita a formação dos complexos.

As proteínas compreendem uma classe versátil de biomoléculas que participam de atividade celular como enzimas, inibidores de enzimas, hormônios, proteínas de transporte, proteínas contráteis, proteínas de reserva, proteínas estruturais, entre outros.

Proteínas também constituem a dieta humana e sementes de cereais e leguminosas têm um importante papel nutricional, por representarem uma fonte protéica de baixo custo (Zhao *et al.,* 2008)**.** O conhecimento da estrutura das proteínas presentes na semente é pré-requisito para manipulação genética visando valorizar seu conteúdo nutricional (Shutov *et al.,* 1998).

As sementes de leguminosas contêm grandes quantidades de proteínas importantes para os processos biológicos e que também podem ser usadas como ferramentas biotecnológicas no estudo de vários fenômenos biológicos.

Proteínas de reserva são definidas como aquelas acumuladas em quantidades significativas em um estágio do ciclo do desenvolvimento da semente e cuja degradação, em um estágio metabólico mais ativo, fornecerá carbono, enxofre e nitrogênio para os processos celulares (Milisavljevicä *et al.,* 2004).

Tavano e Neves (2007) descreveram a extração e purificação da fração globulina tipo vicilina de *Cicer arietinum* L. As proteínas da farinha foram extraída com solução

0,5 M NaCl (1:10 p/v) e as frações de albumina e globulina foram separardas por diálise exaustiva (72 h) contra água destilada desde que as globulinas precipitam e as albuminas permanecem no sobrenadante. A vicilina foi então isolada por cromatografia da preparação globulina em coluna de Shepharose CL-6B. A massa molecular da proteína foi determinada por cromatografia de gel filtração.

A caracterização das proteínas de reserva é feita pela determinação da composição de aminoácidos, conteúdo de carboidratos, identificação de suas subunidades constituintes e determinação da estrutura tridimensional (Sebastiani *et al.,* 1990; Lawrence *et al.,* 1994; Múren *et al.,* 1996).

As proteínas de sementes são classificadas, de acordo com a solubilidade, em albuminas solúveis em água, globulinas solúveis em soluções salinas diluídas, glutelinas solúveis em soluções fracas de ácidos ou bases e prolaminas solúveis em álcool (Shewry *et al*., 1995).

As globulinas apresentam coeficiente de sedimentação de 7S a 12 S, são geralmente de elevado peso molecular e ricas em arginina, glutamina, leucina e asparagina (Khorshid *et al*., 2007). Em legumes são as principais proteínas de reserva (Duranti & Gius, 1997).

As globulinas são geralmente separadas em duas frações denominadas legumina e vicilina, definindo leguminas como proteínas menos solúveis em soluções salinas diluídas e que não coagulam pelo aquecimento a 95°C, e vicilinas como proteínas solúveis que coagulam a 95° C.

As vicilinas (proteína 7 S) podem ser glicoproteínas ou não; em geral têm massas moleculares de 105 a 330.000 e suas subunidades, na maioria dos casos, não são ligadas por pontes de sulfeto. As vicilinas são pobres em histidina e em aminoácidos

contendo enxofre, faltam triptófano e cisteína e conseguentemente não podem constituir ponte dissulfetos (Shewry *et al.,* 1995).

A fração 7S de proteína de soja contém α-, β- e τ-conglicinina. β -conglcinina (180 kDa) é o principal componente desta fração e existe como trimero de subunidades. Estas subunidades são ricas em asparagina, aspartato, glutamato/glutamina, leucina, arginina, e pobres em aminoácidos contendo enxofre (Khorshid *et al.,* 2007).

As vicilinas apresentram atividade inseticida e antifúngica (Sales *et al*., 2001; Wang *et al*., 2001; Becker-Ritt *et al.,* 2007) e também são associadas a efeitos antigênicos (Seabra *et al.,* 2001) e alergia alimentar (Sanchez-Monge *et al*., 2004).

1.2. *Artemia salina*

Artemia salina Leach (Figura 1), usualmente conhecida como "artemia", pertence à classe *Brachiopoda*, subclasse *Sasostroca* e ordem *Anostroca.* É um crustáceo de água salgada encontrado em todo o mundo. Muito fértil reproduz-se com muita facilidade e rapidez e é utilizado como alimento vivo para peixes, sendo seus ovos facilmente encontrados em lojas de artigos para animais (Koutsaftis & Aoyama, 2007).

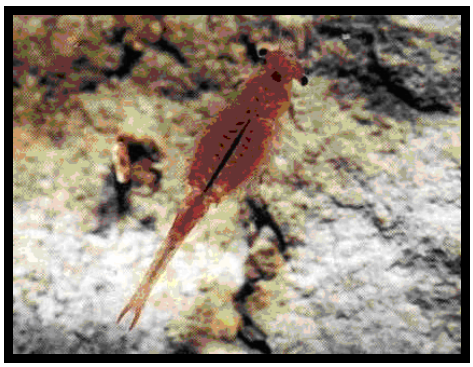

Figura 1: *Artemia salina*.
Fonte: http://www.vialattea.net/spaw/image/biologia/May2005/a_salina500.jpg
(endereço eletrônico acessado dia 15 de fevereiro de 2008).

A. salina por ser um organismo sensível que pode sentir o efeito de qualquer substância biologicamente ativa e apresentar facilidade na obtenção de sua larva para avaliar o efeito de agentes sobre taxa de sobrevivência é considerada um biensaio ideal (Santos Pimenta *et al.,* 2003). Esse biensaio é utilizado para monitorar fracionamento de extratos de plantas com atividade citotóxica, farmacológica ou pesticida (Pelka *et al.,* 2000; McLaughlin *et al.,* 1998). A letalidade de organismos simples tem sido utilizada para monitoramento rápido e simples de resposta biológica, onde existe apenas um parâmetro envolvido: morte ou vida. O ensaio de letalidade permite a avaliação da toxicidade geral e, portanto, é considerado como ensaio preliminar no estudo de compostos com potencial atividade biológica (Taylor *et al.,* 2005), sendo bastante útil como teste de apoio para os demais testes de atividade específica.

1.3. Carcinoma de Ehrlich

O tumor de Ehrlich, um tipo de tumor transplantável utilizado para avaliações experimentais, é uma neoplasia de origem epitelial maligna, correspondente ao adenocarcinoma mamário de camundongos fêmea (Dagli *et al*., 1992). Cresce em várias linhagens dessa espécie animal na forma ascítica, quando inoculado intraperitonealmente, e na forma sólida, quando inoculado no subcutâneo (Ehrlich, 1906) (Figura 2). O tumor de Ehrlich tem sido utilizado no estudo da ação de componentes químicos, físicos e biológicos sobre o crescimento, patogênese, imunologia, citogenética e terapêutica de células tumorais (Segura *et al*., 2000, Palermo-Neto *et at*., 2003; Nascimento *et al.,* 2006; Sakai *et al.,* 2006; Ferreira *et al.,* 2007). A vantagem da utilização de neoplasias transplantáveis, em comparação com as não transplantadas, recai sobre o conhecimento prévio da quantidade e das características iniciais das células tumorais a serem inoculadas e sobre o

desenvolvimento rápido da neoplasia, que reduz o tempo de estudo (Stewart *et al*., 1959). O carcinoma de Ehrlich, por ser um dos primeiros tumores transplantáveis conhecido, é um dos mais extensamente utilizados para experimentação animal (Dagli *et al.*, 1992).

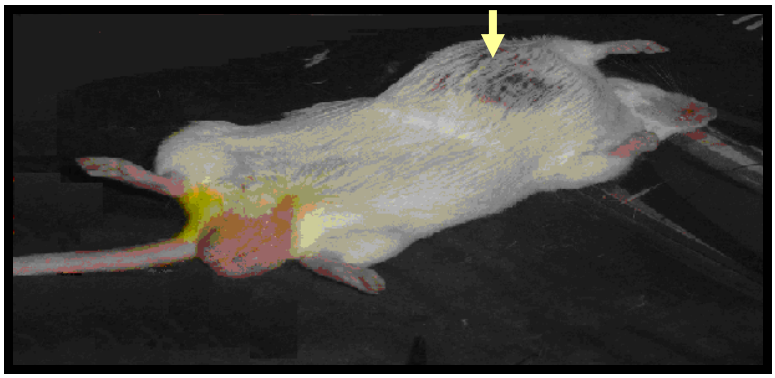

Figura 2: Camundongo com tumor sólido implantado no subcutâneo.
Foto: Ramos, A.L.G.

1.4. Hiperlipidemia

Em países em desenvolvimento, as doenças relacionadas ao estilo de vida, tais como diabetes, hiperlipidemia e hipertensão têm aumentado gradualmente nas últimas décadas. Várias terapias têm sido desenvolvidas para essas doenças, que muitas vezes são efetivos em pessoas sintomáticas. Entretanto uma terapia dietética é importante e deve ser considerada como primeira escolha de tratamento, ou pelo menos mais importante que o tratamento médico (Moriyama *et al.,* 2004). Este e outros fatores têm motivado o desenvolvimento de pesquisas envolvendo modificações dietéticas e o uso de diferentes drogas hipolipidêmicas, que diminuem os níveis de lipídios plasmáticos (Srinivasan, 2005).

Há situações em que alguns pacientes apresentam resistência às medidas terapêuticas, tais como, manipulação dietética, redução no peso e exercício. Isto também tem levado ao uso de drogas hipolipidêmicas, as quais parecem beneficiar os níveis de lipídeos, deixando-os nos valores homeostáticos. Contudo, a utilização de

drogas comerciais tem seu uso restrito devido à ocorrência de hepatotoxidade, distúrbios cardiovasculares e gástricos ou por não reduzir de forma efetiva ambos colesterol e triglicerídeos (Hall *et al.*, 1983). Além disso, algumas drogas que têm sido freqüentemente usadas, incluindo clofibrato, ciprofibrato e colestirina, requerem doses que são geralmente altas (Hérnandez-Mijares *et al.,* 2000). Isto tem motivado contínuas investigações por novos agentes hipolipidêmicos (Labarrios *et al.,* 1999).

Um agente natural capaz de promover efeitos hipolipidêmicos significativos é a β-conglicinina, principal globulina 7 S da soja, que tem recebido atenção por mostrar efeito regulatório de receptores LDL de alta afinidade no fígado (Adams *et al.,* 2004). Essa proteína de reserva também tem demonstrado ser capaz de reduzir os níveis de triglicerídeos e de colesterol em camundongos (Duranti *et al.,* 2004, Fukui *et al.,* 2004). Devido ao restrito número de drogas hipolipidêmicas disponíveis, faz-se necessárias contínuas investigações por novos agentes com o objetivo de se obter fármacos cada vez mais eficazes e que apresentem menos efeitos colaterais.

Para desenvolver um trabalho com esta finalidade, é de suma importância a existência de estudos "*in vivo*". Isto, porém, poderia colocar em risco a saúde e a vida de seres humanos. Faz-se, então, necessário o desenvolvimento de modelos animais que possam ser estudados em laboratório, sob condições extremamente controladas. Os eventos de hiperlipidemia têm sido bem elucidados por estudos em modelos animais incluindo coelhos, galinhas (García-Fuentes *et al.*, 2002) porcos (Hill *et al.*, 2001), roedores (Lusis, 2000) e o sagüi (*Callitrix jacchus*) (Lima *et al.,* 1998). Entretanto, o uso de camundongo em pesquisas tem diversas vantagens sobre outras espécies (Daugherty, 2002).

A não disponibilização em nível nacional de uma dieta comercial hiperlipidêmica para animais, além do alto custo de importação do colesterol puro

comercial necessário para desenvolver hiperlipidemia em camundongos, como modelo experimental, torna-se importante o estabelecimento de uma dieta preparada com alimento de origem local, capaz de promover hipercolesterolemia e hipertrigliceridemia em camundongos. Trabalho realizado no Laboratório de Química e Metabolismo Lipídico do Departamento de Bioquímica da UFPE, para o desenvolvimento de dietas hiperlipidêmicas, utilizando o miolo de boi como fonte de colesterol, que é citado na literatura como o alimento mais rico em colesterol (Franco, 1998), foi capaz de desenvolver hiperlipidemia em roedores (LEITE *et al.*, 2004).

A dieta hiperlipidêmica, cuja composição é demonstrada na tabela 1. Para a preparação da dieta hiperlipidêmica, miolo de boi foi inicialmente picado e submetido à desidratação em estufa a 65°C durante 48 horas. Em seguida uma mistura foi feita com miolo de boi, manteiga e substâncias que ajudam a desenvolver um quadro de hiperlipidemia como o Colato de Sódio e o Citrato de Colina. A mistura foi adicionada à ração comercial (LABINA® Purina Brasil) e a dieta armazenada a - 20°C.

Tabela 1. Composicão centesimal da dieta hiperlipidêmica

	Componentes da Dieta (g%)						
Ingredientes	**Quantidade em 100g**	**Proteínas**	**Gorduras**	**Sais Minerais**	**Colesterol**	**Fibras (Carboidratos)**	**Outros**
Labina	43	10,0	1,6	4,3	-	2,15	56,05
Manteiga	14,6	-	14,6	-	-	-	
Citrato de Colina	0,40	-	-	-	-	-	
Colato de sódio	2,0	-	-	-	-	-	
Miolo desidratado	40,0	-	-	-	11,3	-	
Total	100	10,0	16,2	4,3	11,3	2,15	56,05

1.5. *Cratylia mollis*

Cratylia mollis, feijão camaratu ou camaratuba é uma leguminosa da região Semi-Árida do Estado de Pernambuco que tem sido investigada no Laboratório de Glicoproteínas do Departamento de Bioquímica da Universidade de Pernambuco, desde 1983.

Por constituir uma espécie nativa e resistente à escassez de recursos hídricos, o feijão camaratu é amplamente utilizado na dieta de bovinos e caprinos. Sementes de *C. mollis* (Figura 3) foram avaliadas quanto ao seu valor nutricional. Inicialmente as sementes foram utilizadas para determinação de proteína, fibra, carboidrato, nitrogênio não protéico, taninos e minerais e em seguida ratos Wistar foram alimentados com uma ração contendo a farinha da semente. Os resultados revelaram uma composição centesimal semelhante a outras leguminosas comestíveis e o ensaio biológico indicou que a farinha suplementada com metionina é eficiente na promoção do crescimento dos ratos (Santos *et al.,* 1996).

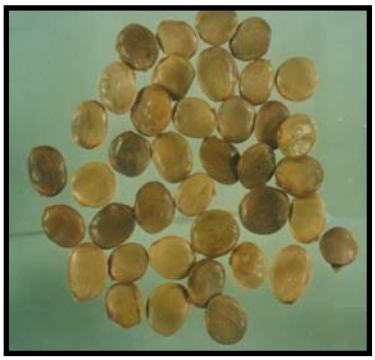

Figura 3: Sementes de *Cratylia mollis.*
Fonte: Laboratório de Glicoproteínas - UFPE

Três proteínas com atividade hemaglutinante (AH), lectina, foram isoladas e caracterizadas (Paiva & Coelho, 1992; Correia & Coelho, 1995) e têm sido amplamente investigadas quanto as suas características estruturais (Santos *et al.,* 2004), atividades

biológicas (Andrade *et al.,* 2004) e potencial biotecnológico (Paiva *et al.,* 2003). Das sementes de *C. mollis* também foram isolados dois isoinibidores da enzima tripsina (Paiva *et al.,* 2006). Uma proteína solúvel em NaCl 0,15 M que não apresenta AH nem inibitória de tripsina tem sido detectada. A purificação e caracterização dessa proteína tipo vicilina denominada cratilina é fundamental para avaliação do potêncial uso biotecnológico das sementes de *C. mollis*.

2. OBJETIVOS

2.1. Geral

Purificar, caracterizar estruturalmente e avaliar atividades biológicas de uma proteína, tipo vicilina, presente em sementes de *C. mollis*.

2.2. Específicos

- Purificar a proteína tipo vicilina.
- Caracterizar estruturalmente esta proteína pela determinação da natureza glicoprotéica e da massa molecular.
- Verificar a citotoxicidade *in vitro* desta proteína pela determinação da concentração letal (CL_{50}) da proteína frente ao microcrustáceo *Artemia salina*.
- Avaliar o efeito antitumoral sobre Carcinoma de Ehrlich.
- Desenvolver hiperlipidemia, em camundongo, com dieta hiperlipidêmica rica em colesterol, preparada a partir de miolo de boi, e administrada aos animais durante 91 dias.
- Realizar tratamento intraperitoneal e oral em camundongos com a proteína purificada.
- Avaliar os perfis lipídicos dos animais tratados, analisando os níveis de colesterol sérico e triglicerídeos.

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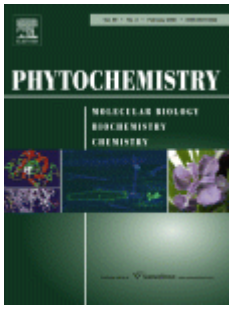
4. ARTIGO - ANTITUMORAL ACTIVITY OF VICILIN FROM *Cratylia mollis* SEEDS

Artigo a ser submetido ao periódico "Phytochemistry**"**



ANTITUMORAL ACTIVITY OF VICILIN FROM *Cratylia mollis* SEEDS

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4.1. Abstract

Protein without hemagglutinating or trypsin inhibitory activities was isolated from *Cratylia mollis* seeds by ammonium sulphate protein fractionation and Sephadex G-75 chromatography and named cratylin. SDS-PAGE at absence of β-mercaptoethanol resolved cratylin as polypeptides of M_r 205, 150 e 50 kDa and under reducing conditions as peptides of M_r 50, 26, 24 e 22 kDa. Heated (100ºC) cratylin was resolved as 26, 24 and 22 kDa peptides. Cratylin (10–1000 ppm) was toxic to *Artemia salina* with LC_{50} of 558.7 µg/ml. Antitumoral activity of cratylin 11.5 mg/kg, 23 mg/kg or heated (100ºC) cratylin 23 mg/kg were evaluated on Ehrlich carcinoma implanted in Swiss albino mice (*Mus muscullus*) and activities of tumor-weight inhibition were achieved of 63%, 73% and 34%, respectively. Anti-hyperlipidemic activity of cratylin (10 or 20 mg/kg/day) is assayed using hyperlipemic Swiss albino mice and changes in serum cholesterol and triglycerides were not detected. Artemicide activity of cratylin was an indication of tumor inhibitory property.

Keywords: *Artemia salina; A*ntitumoral activity; *Cratylia mollis*; Ehrlich carcinoma; Hyperlipidemia; Vicilin.

4.2. Introduction

Seeds of Leguminosae are rich sources of storage proteins including vicilins (Sales et al., 2001). Analysis of the pattern of storage protein mobilization during germination showed that vicilin represented the initial source of amino acids for early growth and differentiation processes in *Vicia sativa* (Tiedemann et al., 2000). Vicilins showed insecticidal or antifungal activities (Sales et al., 2001; Wang et al., 2001; Becker-Ritt et al., 2007) and are also associated to antigenic effects (Seabra et al., 2001) and food allergenecity (Ogawa et al., 2000).

Vicilin type proteins are resolved by SDS-PAGE as multiple proteins due the co-existence of aggregated and non-aggregated protein (Okuda et al., 2001). Vicilins from different species showed similar structural characteristics as demonstrated occur between β-conglycinin, phaseolin and canavalin (Okuda et al., 2001). A comparative study of lentil vicilin gene indicated similarity with others legume species (Mieira et al., 1998).

Artemia salina leach, commonly known as "brine shrimp," is a salt-water crustacean found in the world used as live food for fish, and eggs easily found in shops of animals (Koutsaftis and Aoyama, 2007). *A. salina* is a simple body that may feel the effect of substance biologically active. The mortality of this microcrustacean is widely used to investigate toxicity of synthetic compounds (Almeida et al., 2002) as well as plant products (Silva et al., 2007). The ratio of the number of dead larvae and the number of larvae live compared to a negative control is used to estimate toxicity of solutions (McLaughlin et al., 1998). Additionally, the bioassay can indicate potential antitumor agents (Santos Pimenta et al., 2003).

The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma. It can be said that the Ehrlich's carcinoma is known as one of the first transplantable tumors known (Dagli et al., 1992). Nowadays, this experimental model of tumor is frequently used on studies concerning tumor growth (Palermo-Neto et al., 2003, Sakai et al., 2006).

In developing countries, diseases related to lifestyle, such as diabetes, hypertension and hyperlipidemia has gradually increased in recent decades. Due to the limited number of hypolipidemic drugs available investigations by new agents have been made and was described that the main globulin 7 S of soybean, β-conglycinin, was able to promote hypolipidemic effects by its regulatory effect of LDL receptors in the liver (Adams et al., 2004). β-conglicinin reduced the levels of triglycerides and cholesterol in mice (Fukui et al., 2004, Duranti et al., 2004).

Cratylia mollis, or camaratu bean, is a Leguminosae that is native to the semi-arid Northeast region of Brazil and is highly resistant to desiccation. The seeds have been an alternative source of nutrition in livestock feed, contributing to regional development and a better quality of life for the local inhabitants. In this study, we describe the purification process for, molecular mass of, as well as the investigation of artemicide, antitumoral and hypolipidemic activities of storage protein from *C. mollis* seeds, named Cratylin.

4.3. Results and Discussion

Leguminosae seeds contain multiple proteins that shown as structural characteristics sites for specific interaction with other molecules or cells that results in biological properties. Hemagglutinating proteins (lectins) and storage proteins such as vicilins, are examples of biactive molecules explored as biothecnologic tools (Andrade *et al.,* 2004, Ohara et al., 2007). Three isolectins and two trypsin isoinhibitors have already been isolated from *C. mollis* seeds (Paiva and Coelho, 1992, Correia. and Coelho, 1995; Paiva et al., 2006). Another protein detected during lectin and trypsin inhibitor purification processes and named cratylin was now purified and assayed for biological activities.

The 10% seed extract containing 108 mg of protein was used in the protocol of isolation of cratylin. Precipitation of protein present in the extract with ammonium sulfate gave a fraction (supernatant 60) rich in proteins (25 mg). The fraction containing soluble proteins at 60% ammonium sulphate saturation (supernatant 60) was selected as source of cratylin because protein vicilins are high salt-soluble proteins. Chromatography of supernatant 60 on Sepahadex G-75 isolated cratylin (16 mg) in the non-adsorbed fractions separating of adsorbed protein eluted by 0.3 M glucose and 1M NaCl (Fig. 1).

The chromatography of supernatant 60 on Sepahadex G-75 isolated non-adsorbed cratylin of hemagglutinating activity (HA) adsorbed. The gel filtration chromatography efficiently eliminated the contaminant due to lectin binding to polysaccharide matrix similarly occurred during purification of lectin by affinity chromatography (Trindade et al., 2006). Hydrolytic activity of bovine trypsin towards N-α-benzoyl-DL-arginyl-ρ-nitroanilide was the same (A405 nm=0.462) at presence or

absence of cratylin. The protein not display trypsin inhibitory activity and therefore the preparation of cratylin from Sephadex G-75 column was not contaminated with the inhibitor that exists in *C. mollis* seeds.

SDS-PAGE at absence of β-mercaptoethanol (Fig. 2B) revealed cratylin as polypeptides of M_r 205, 150 e 50 kDa. When heated cratylin was treated with the reducing agent β-mercaptoethanol three subunits of molecular masses 26, 24 and 22 kDa were detected (Fig. 2D). Reduced cratylin showed four peptides of molecular masses 50, 26, 24 and 22 kDa (Fig. 2C). Cratylin of M_r 205, 150 and 50 kDa corresponds probably to arrangements of 26, 24 and 22 subunits similar to canavalin that exist as molecular aggregates (Barcellos et al., 1993). The cratylin subunits were negative for glycoprotein detection, indicating that the protein did not contain carbohydrate moiety. Cratylin was chromatographed on C-18 column and the profile showed two peaks eluted with the acetonitrile gradient which retention times were 40.39 and 46.025 min. SDS-PAGE of HPLC peaks revealed that the chromatographic process was able to separate the 26 and 22 kDa peptides in the peaks eluted at 40.39 and 46.025 min, respectively. The subunits separation by C-18 column probably was due to low pH of chromatographic medium. It has been reported that temperature and pH interfere in the structural organization of storage protein. Heating at 90ºC of globulin isolated from *Phaseolus vulgaris* promoted subunit dissociation that was irreversible at pH greater than 10,0 (Sun et al., 1974).

Cratylin showed toxicity on *A. salina* at 500 and 1000 ppm (Table 1). LC_{50} of 558.713 µg/ml showed good artemicide activity. According to Meyer et al. (1982) crude extracts and pure substances with LC_{50} value < 1000 µg/ml and LC_{50} value >1000 µg/ml are toxic and non-toxic, respectively. LC_{50} values determined for extracts and fractions from Annonaceous Brazilian plants ranged from 0.2 to 1,333 µg/ml (Santos

Pimenta et al., 2003). *A. salina* bioassay has indicated potential antitumoral agents due to good correlation artemicide activity-cytotoxic activity (Santos Pimenta et al., 2003). The toxicity determined for cratylin by this bioassay can indicate the possible antitumor activity of protein (Maclaughlin, 1995).

Antitumoral activities of cratylin 11.5 mg/Kg, 23 mg/Kg or heated cratylin 23 mg/Kg were evaluated on Ehrlich carcinoma implanted in Swiss albino mice (Fig. 3). The results showed that cratylin at concentration of 23 mg/Kg promoted tumor inhibition higher (73%) than when half the concentration was used (63%). Antitumoral activity was already detected for lectin isolated from *C. mollis* seeds and encapsulated into liposomes (Andrade et al., 2004) that promoted shrinkage of Sarcoma 180 (71%). Low TWI% of 34% was determined for the group treated with heated cratylin. The later results revealed that heating promoted inactivation of cratylin since according to national of cancer institute TWI less than 58% for treated tumors comparing to the control tumors, is not considered significant (Geran et al., 1972). The no effect of heated cratylin on tumor inhibition may indicate that aggregate form of protein is important for antitumoral activity. The purification protocol yielded cratylin without lectin contamination since HA was not detected and thus *C. mollis* seeds contain at least two proteins with antitumor activity.

Administration of the cratylin (10 and 20 mg/kg/day) for 16 days not significantly alters the levels of serum cholesterol and triglycerides in hyperlipidemic mice (Table 2). Several physical properties of lipid bilayer membranes are altered by cholesterol. Cholesterol and triglycerides are transported in the circulation by lipoproteins. By interacting with cell membranes, alterations on the circulating lipid composition have an adverse effect upon the process of reverse cholesterol transport, and induce cellular dysfunction indirectly by altering membrane lipid composition,

which modulate cellular ion transport, enzymatic properties (Imai et al., 1992). Thus, changes in the concentration of cholesterol will greatly affect the fluidity of cell membrane and thereby can bring about abnormal changes in the membrane property and function (Krasilnikov et al., 2007). Many herbs and plant products have been shown to alter lipids concentration (Bahramikia and Yazdanparast, 2008; Ogawa et al., 2007) and hypolipidemic properties of plant extracts or storage protein have been documented (Yao et al., 2008; Ji and Gong, 2007; Fukui et al., 2004; Duranti et al., 2004). However, in this study the cholesterol and triglyceride compositions in serum of mice treated with similar doses of cratylin used for tumor analysis were only slightly different from those of untreated mice. Serum cholesterol it is very important in order to maintain the animal cells membrane fluidity, integrity and function.

4.4. Conclusions

Cratylin, oligomeric protein isolated from seeds of *C. mollis* shows antitumor activity on Ehrlich carcinoma. The effect of cratylin on survival of *A. salina* and its inefficiency change in the levels of cholesterol and triglycerides indicates that the reduction of the tumor was due to the toxic effect and not the change in the metabolism of lipids.

4.5. Experimental

4.5.1. Reagents, protease, substrate, markers and columns

Bovine trypsin (EC 3.4.21.4) and DL-Bz-Arg-pNan were purchased from Sigma Chemical Company (St. Louis, MO, USA). Molecular mass standard mixture and Sephadex G-75 were also from Sigma. C_{18} column was from Waters (USA). Biochemical parameters determination (serum cholesterol and triglycerides) was measured by enzymatic methods (Labtest Diagnostica, Brazil/SA).

4.5.2. Cratylin purification

Mature *Cratylia mollis* Mart. seeds were manually harvested in the Northest region of Brazil (Pernambuco, PE) and were ground in a multiprocessador. The crude 0.15 M NaCl extract (10% w/v) was obtained as a supernatant fraction after centrifugation at 5000 *g* for 15 min. Ammonium sulfate was added to the seed extract to a final saturation of 60%, constant magnetic stirring for 4 h was performed and the supernatant fraction was obtained after centrifugation at 5000 *g* for 15 min. The 30 ml of supernatant 60 (25 mg of protein) was loaded onto a Sephadex G-75 column (2.0 x 60 cm), previously equilibrated with 0.15 M NaCl at 36 ml/h flow rate. Following extensive washing with 0.15 M NaCl (cratylin fraction), the column was eluted with 0.3 M glucose. The column was monitored by protein (A280 nm) and HA. The protein concentration of cratylin and adsorbed fractions was determined according to Lowry et al. (1951). Cratylin (250 μg) was applied onto a C_{18} reverse-phase column using an HPLC system equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. Separation was achieved using an acetonitrile gradient (0-100%, 70 min) in 0.1% (v/v) TFA. Cratylin and cratylin heating to 100ºC by 45 min were furthermore evaluated.

4.5.3. Polyacrylamide gel electrophoresis

SDS polyacrylamide gel (10%) electrophoresis at absence or presence of β-mercaptoethanol was carried out according to Laemmli, 1970. The gels were stained with Coomassie Brilliant Blue or submitted to glycoprotein staining with Schiff´s reagent (Pharmacia Fine Chemicals, 1980).

4.5.4. Hemagglutinating activity

Hemagglutination assay was performed in microtiter plate in a final volume of 100 μl containing 50 μl of a 2.5% suspension of glutaraldehyde-treated rabbit erythrocytes (Bing et al, 1967) and 50 μl of *C. mollis* sample (each serially diluted with 2-fold increments). Hemagglutinating activity was recorded visually after 45 min at 25°C as the lowest sample dilution which showed hemagglutination.

4.5.5. Trypsin inhibiory activity

The assay (Kakade et al., 1969) was made with bovine trypsin (10 μg ml^{-1} in 0.1 M Tris-HCl, pH 8.0; 20 μl); pre-incubation (37°C, 10 min) of enzyme and cratylin (30, 40 and 60 μl) was followed by addition (30 μl) of 4 mM N-α-benzoyl-DL-arginyl-ρ-nitroanilide dissolved in dimethyl sulfoxide and diluted with tris buffer. After 30 min at 37°C, 10% (v/v) acetic acid was added (300 μl). The substrate hydrolysis was followed by measurement of absorbance at 405 nm, and the molar extinction coefficient for ρ-nitroanilide used for concentration calculation was 9100 (Oliva et al., 2000). The inhibitory activity evaluated the remaining hydrolytic activity towards N-α-benzoyl-DL-arginyl-ρ-nitroanilide.

4.5.6. Brine Shrimp lethality activity

The assay was according to Meyer et al. (1982) modified. *A. salina* eggs (25 mg) were hatched in seawater natural at temperatures of 20 to 30 ºC. The pH was adjusted between 8.0 and 9.0 using Na_2CO_3 to avoid risk of death to the larvae by decrease of pH during incubation (Lewan et al., 1992). The eggs were placed in container with two compartments. The compartment with the eggs was covered in order to keep the eggs in a dark ambient. The other compartment of the container was illuminated in order to attract *A. salina* through perforations at the boundary plate. After 24 h, the phototropic brine shrimp, which went to the illuminated compartment, were collected by Pasteur pipette. Stock solution (10,000 ppm) was prepared in seawater. *A. salina* bioassay used a 10,000 ppm cratylin stock solution prepared in seawater; stock solution was diluted (1,000–10 ppm) in vials containing 5 ml of seawater and 15 *A. salina*. The control group was set with vehicle used for dilutions and the time of exposition of the target organisms was of 24 h. Survival rate was determined and used to estimate LC_{50}. The assays were carried out in triplicate. The collected data were computerized and the LC_{50} value estimated using the statistical method of probity (Finney, 1971).

4.5.7. Antitumoral activity

Male Swiss albino mice (*Mus musculus*) with 60 days of age, weighing 30±5g were obtained from the Centro de Pesquisa Aggeu Magalhães - Fiocruz. They were maintained in cage (n=5), maintained under controlled environmental conditions of temperature, humidity and under clear and dark cycles of 12 hours. The mice were fed on diet of the animal house (LABINA® Purina Brasil) and water *ad libidum*.

Ascytic tumor cells were inoculated subcutaneous in the axillar region of the healthy animals previously weighed and they were divided into four grups (N 5) (Stock

and Sugiura, 1954, Komiyama and Funayama. 1992). After 24 h of the implantation, the treatment was started. The control group received 0.9% NaCl and the test groups received doses intraperitoneal of cratylin (11.5 or 23 mg/kg) or heated (100ºC by 45 min) cratylin (23 mg/kg) for seven consecutive days. After treatments animals were sacrificed; tumoral mass was removed and weighted. The efficacy of treatment was calculated for tumor-weight inhibition (TWI%).

4.5.8. Anti-hyperlipidemic activity

Male Swiss albino mice (35-45g) were purchased from Centro de Pesquisa Aggeu Magalhães – Fiocruz . Animals were kept in an environmentally controlled room (22±3°C; 12/12h light/dark cycle) and divided into 4 groups of 6 animals each, which were submitted to the administration of hiperlipidemic diet and water *ad libitum* for 91 days. To evaluate the anti-hyperlipidemic effect, hyperlipidemic animal model was established. A hyperlipidemic mouse model obtained after 75 days of hyperlipidemic diet was treated during 16 days with saline (control), cratylin 10mg/kg/dia (CT10) or cratylin 20mg/kg/dia (CT20).

Blood (0.1 mL) of mice was collected before the diet, every 15 days of diet and during treatment with cratylin (each 8 days) after fasting for 12 h through the puncture plexus retro orbital with capillary tubes. Then the serum was obtained by centrifugation (2,500 x g for 15 min) and used for biochemical parameters determination (cholesterol and triglycerides) by enzymatic methods (Labtest Diagnostica, Brazil/SA). All experiments were approved by the Animal Care and Use Committee at Federal University of Pernambuco and are in accordance with guidelines for Care and Use of Laboratory Animals.

4.5.9. Statistical analysis

The experimental results were expressed as mean±SEM. Data were assessed by the method of analysis of ANOVA followed by *t*-test. $P<0.05$ was considered as statistically significant.

4.6. Acknowledgements

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4.7. Figures

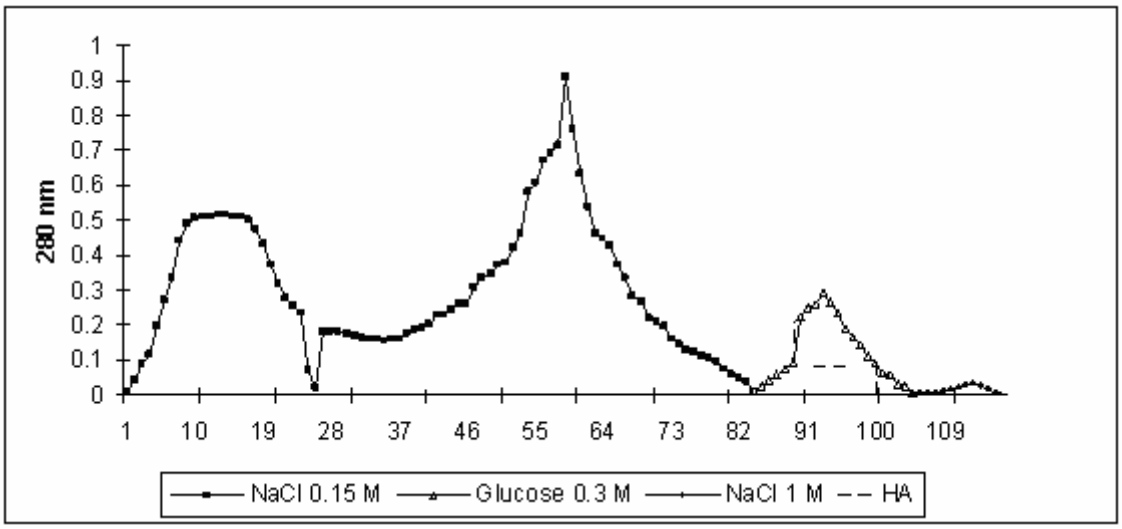

Fig. 1. Isolation of cratylin by Sephadex G-75 chromatography.

A sample of supernatant 60 (25 mg in 0.15 M NaCl) was applied to the column (2 x 60 cm) equilibrated with 0.15 M NaCl. Fractions of 2 ml were collected.

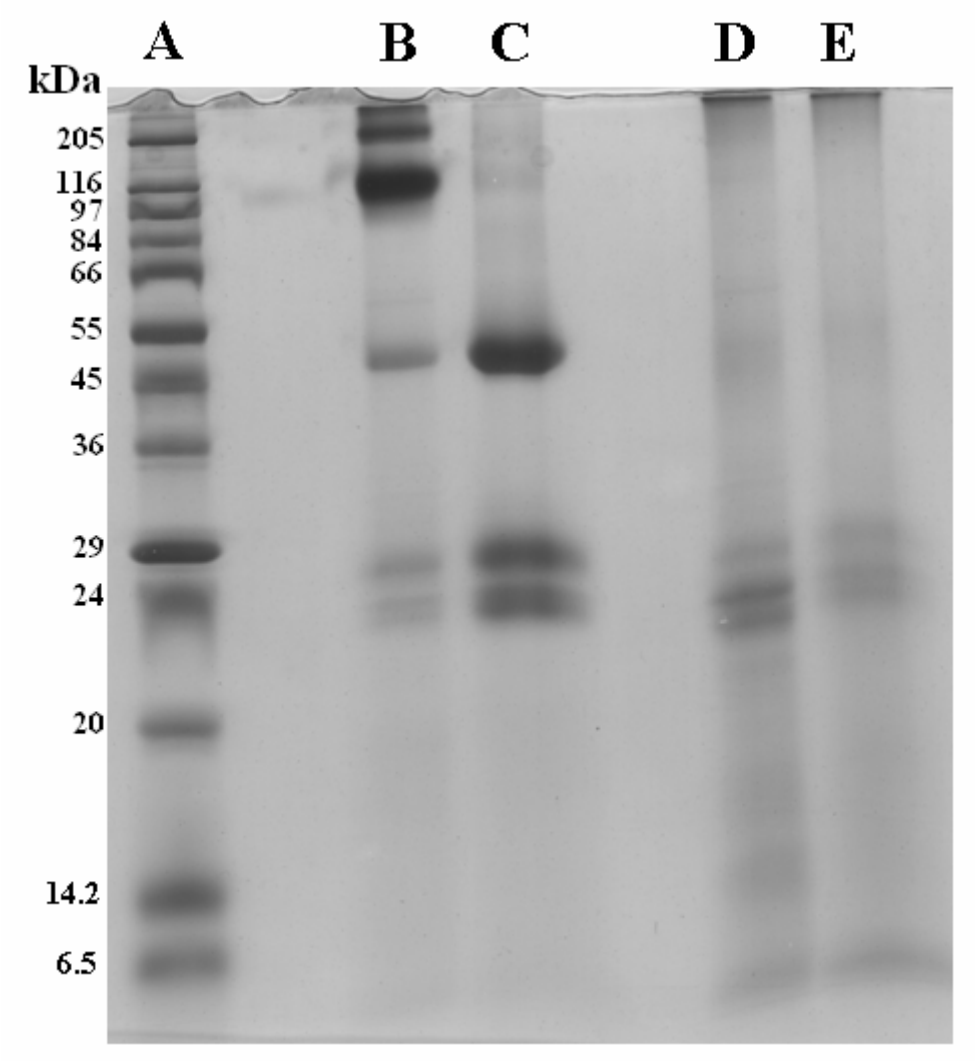

Fig. 2. Electrophoretic patterns of cratylin

Molecular weight markers (kDa): Myosin (205), β-galactoctosidade (116), phosphorilase B (97), fructose- 6-phosphatase (84), albumin (66), glutamic dehydrogenase (55), ovalbumin (45), glyceraldehyde-3-phosphae dehydrogenase (36), carbonic anidrase (29), trypsinogen (24) trypsin inhibitor (20), α-Lactalbumin (14,2), Aprotinin (6,5) (A). SDS-PAGE of cratylin (60 μg) non-reduced (B), reduced (C), heated (D), heated and reduced (E). The cratylin samples and markers were loaded onto a 12 % gel. Peptides were stained with Coomassie Brilliant Blue.

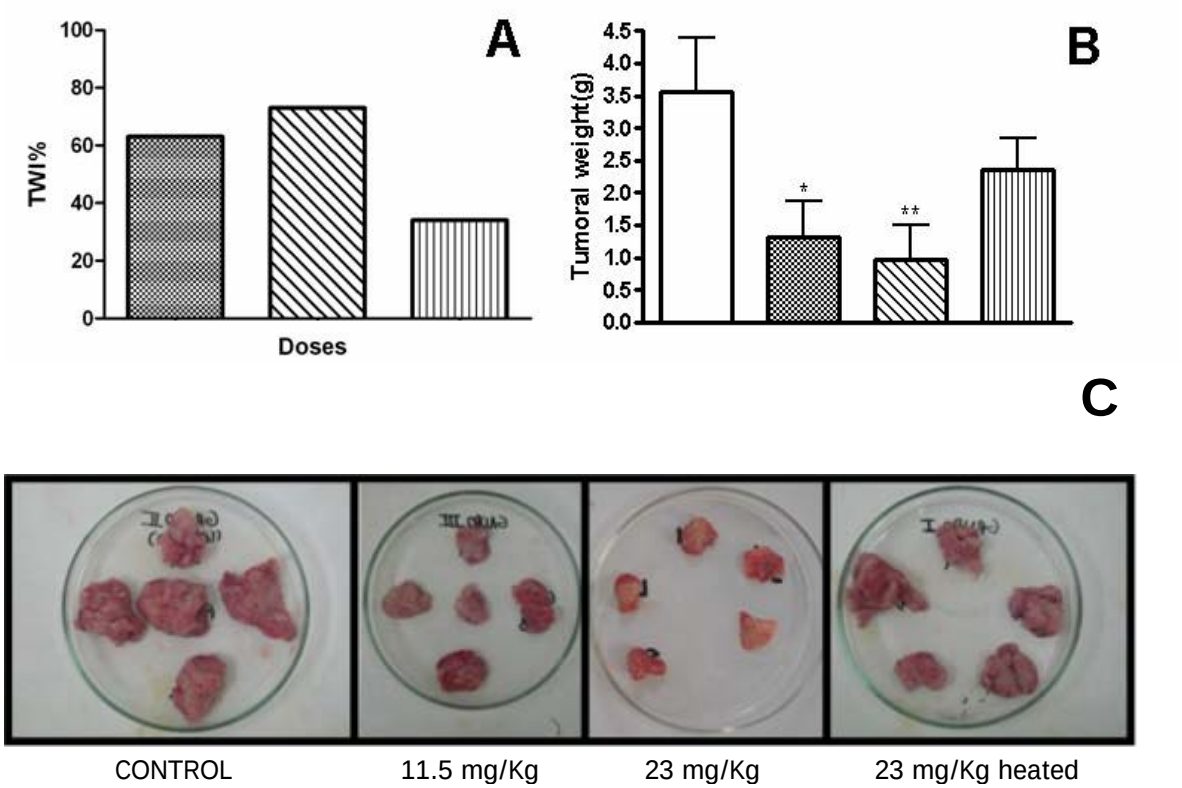

Fig. 3: Effect of cratylin (▩ 11.5 mg/kg/day, ▨ 23 mg/kg/day) heated cratylin (▥ 23 mg/kg/day) on Ehrlich carcinoma growth. Control (□). TWI% (A), tumoral weight (B) and macroscopic aspect of tumor (C). Values are averages ± S.E.M. For statistical analysis was applied one-way ANOVA test $p < 0.05$. * Difference significant at $p < 0.005$, ** $p < 0.003$.

4.8. Tables

Table 1

Artemicide activity of cratylin

Sample (ppm)	Mortality (%)	Lethal concentration (ppm)		
		LC_{10} [IC_{95}]	LC_{50} [IC_{95}]	LC_{90} [IC_{95}]
Cratylin		162.673 [154.967; 170.379]	558.713 [551.007; 566.419]	954.752 [947.047; 926.458]
1000	100			
500	33.3			
100	2.2			
50	0			
10	0			

Table 2

Effect of cratylin under biochemical parameters and body weight of hyperlipidemic mice

	T_0			T_8			T_{16}		
Parameters	**Control**	**T10**	**T20**	**Control**	**T10**	**T20**	**Control**	**T10**	**T20**
Weight (g)	44.5±0.8	42.6±2.0	42±1.3	43.8±0.8	41.3±1.9	40.5±1.4	40.1±0.4	40±1.9	38.5±1.4
Cholesterol (mg/dL)	184.6±5.9	181.6±6.2	182.9±5.1	179.5±6.5	166.8±8.2	175.3±5.2	191.8±8.6	177.1±9.7	165.6±4.6
Triglyceride (mg/dL)	168.5±9.4	183.9±7.4	181.1±5.1	167.7±5.6	156.3±4.4	186.5±8.3	165.5±8.7	163.7±8.6	171.3±5.5

Biochemical measurements made in serum collected before (T_0), after 8 days (T_8) and 16 days (T_{16}) of treatment with cratylin 10 mg/kg/day (T10) and 20 mg/kg/day (T20). Control (0.15 M NaCl). Values are averages ± S.E.M. For statistical analysis was applied one-way ANOVA test.

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5. CONCLUSÕES

Uma proteína solúvel em elevada concentração (60%) de sulfato de amônio foi isolada de sementes de *Cratylia mollis* e denominada cratilina. A avaliação estrutural da proteína por eletroforese em gel de poliacrilamida na ausência e presença de agente redutor definiu a proteína como agregado molecular de Mr 205, 116 e 50 kDa constituído de duas subunidades de diferentes massas moleculares (26 e 22 kDa). A proteína reduzida ainda conserva peptídeo de 50 kDa sendo só totalmente desenovelada em suas subunidades constituintes após aquecimento a 100ºC por 45 min. Cratilina (1000–10 ppm) foi tóxica para *Artemia salina* e apresentou atividade antitumoral (concentrações de 11,5 mg/kg e 23 mg/kg) sobre carcinoma de Ehrlich. Aquecimento de cratilina a 100ºC aboliu sua atividade antitumoral indicando que a agregação das subunidades é necessária para a atividade. Cratilina não apresenta atividade hemaglutinante, inibitória de tripsina e anti-hiperlipidêmica.

6. ANEXOS

6.1. Normas para redação de artigos para a revista "Phytochemistry".

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The manuscript is required to be written in English, with numbered pages, double-spaced, using 10 or 12 point font, and in a suitable word-processing format. Each page should have adequate margins (4 cm) and liberal spaces at top and bottom of the manuscript. All textual elements should begin flush left, with no paragraph indents, and should use the wrap-around end-of-line feature, i.e. no returns at the end of each line. Place two returns after every element such as title, headings, paragraphs, figure and table call-outs. Most formatting codes will be removed or replaced on processing your article. Please do not use options such as automatic word breaking, justified layout, double columns or automatic paragraph numbering (especially for numbered references). However do use bold face, italic, subscripts, superscripts etc. The Editors reserve the right to adjust style to certain standards of uniformity.

If Authors are unfamiliar with Phytochemistry, they should consult a recent copy (or the free online sample copy available from www.elsevier.com/locate/phytochem) to see the conventions currently followed for guidance in preparing submissions.

The content of manuscripts must be arranged as follows: (1) a Graphical Abstract; (2) a Title Page with authors name(s) and address(es); (3) an Abstract, in which contents are briefly stated; (4) a Keyword Index, (5) Introduction, and (6) the Results and Discussion (preferably combined). Although each section may be separated by headings, they should form one continuous narrative and only include details essential to the arguments presented. If a discussion is separately provided, it should not include a repetition of the results, but only indicate conclusions reached on the basis of them, and those from other referred works; (7) Conclusions or Concluding Remarks; (8) the Experimental should include brief details of the methods used such that a competent researcher in the field may be able to repeat the work; (9) Acknowledgments; (10) Figures and Legends, Formulae, Tables and References.

Title page and author names: Titles must be as brief as possible, consistent with clarity, and should not exceed 10 words in length. Uninformative phrases such as "Chemical examination of", "Studies on", "Survey of", "New", "Novel" etc. will be deleted. The taxonomic authority after a plant name must be omitted from the title. If a paper is part of a series, this must not be given in the heading, but referred to in a footnote in the form, jPart 9 in the series "The Alkaloids of Papaver somniferum", followed by a numbered reference to the previous part. Only papers describing new compounds are accepted for publication in the Chemistry section.

Each author should identify himself or herself with one forename, initials of other forenames and surname. This is to enable more exact computerized indexing and information retrieval. An asterisk * should be placed following the name of the author to whom correspondence inquiries should be made, and the corresponding author should include full details of telephone and fax numbers, and e-mail address(es). Full postal addresses must be given for all co-authors. Superscript letters a,b,c should be used to identify authors located at different addresses.

Abstract. The abstract, distinct from the graphical abstract, should briefly describe the results obtained and conclusions reached, not the methods used, or speculations on any other matter. They are not expected to be a complete summary but only an outline of the main findings. The abstract should be contained within 250 words and should be readable without reference to the rest of the paper.

Graphical abstract: At the time the paper is first submitted, authors must supply a suitable (mandatory) text (up to 50 words) and a graphic designed to summarize the content of the paper in a concise, pictorial form to capture the attention of a wide readership. Graphical abstracts have purposefully been given much latitude in their design. For example, the pictorial form could be a chemical structure, a plant form, or an enzymatic step. Graphical abstracts will be typeset by the publisher, but authors should ensure that the manuscript title, author(s), the appropriate graphic and the (optional) text should be kept within an area of 5 cm by 17 cm. The graphical abstract should be supplied as a separate file. For examples of graphical abstracts, please consult a recent issue of the journal or visit

the journal page on ScienceDirect at: http://www.sciencedirect.com/science/journal/00319422 and click 'Sample Issue Online'.

Keyword index: Authors must give 3–10 “keywords” or phrases, which identify the most important subjects covered by the paper. They should be placed at the beginning of the manuscript in the following order: name of plant species examined (Latin binomial); plant family; common epithet (where applicable); type of investigation; class of compound, protein or gene; name of compound(s), protein(s) and gene(s).

Introduction should give the minimum historical data needed to give appropriate context to the author’s investigation and its relationship to other similar research previously or currently being conducted.

Only information essential to the arguments should be presented. Much data can be taken for granted or quoted in abbreviated form.

Specific names (genus, species, authority for the binomial) of all experimental plants must be given at first mention according to the *Index Kewensis* (searchable online at www.ipni.org) or similar authority (The Plant-Book: A Portable Dictionary of the Vascular Plants, by D.J. Mabberley, 2nd ed., June 1997, Cambridge University Press; ISBN: 0521414210), and preferably be in the form recommended by the *International Code of Botanical Nomenclature* (www.bgbm.fu-berlin.de/iapt/nomenclature/code/tokyo-e/default.htm). Named varieties of cultivars are given, e.g. *Lactuca sativa* cv. Grand Rapids. (The official printed version of the *International Code of Botanical Nomenclature* has been published as International Code of Botanical Nomenclature {Tokyo Code}. Regnum Vegetabile 131. Koeltz Scientific Books, Königstein. ISBN 3-87429-367-X or 1-878762-66-4 or 80-901699-l-0.)

Results and Discussion: These sections should be carefully prepared with discussions of the results being compared with existing and/or previous knowledge within the field. Authors are, however, encouraged to combine the *Results and Discussion* sections wherever possible.

Experimental: Subsections on the Experimental Procedures should be *italicized* and inserted as part of the first line of the text to which they apply. *Phytochemistry* encourages an extensive use of abbreviations (these are listed at the back of the Instructions to Authors, or the reader is referred to other sources). The Experimental should begin with a subsection entitled *General Experimental Procedures*. This subsection will typically contain brief details of instruments used, and identification of sources of specialized chemicals, biochemicals and molecular biology kits.

The next subsection describes the source(s) and documentation of biological materials used, whether in reference to whole plants or parts therefrom, crude drugs, or any other plant material from which identifiable chemical substances are obtained for the first time. Documentation must also include a reference to voucher specimen(s) and voucher number(s) of the plants or other material examined. If available, authors should quote the name and address of the authority who identified each non-cultivated plant investigated. Specimens should preferentially be deposited in a major regional herbarium where the

collection is maintained by state or private institution and which permits loan of such materials.

With other microorganisms, the culture collection from which they were either accessed and/or deposited should be included, together with identification of the strain designation code.

The Experimental Procedures employed should be concise but sufficiently detailed that a qualified researcher will be able to repeat the studies undertaken, and these should emphasize either truly new procedures or essential modifications of existing procedures. Experimental details normally *omitted* include: (1) method of preparation of common chemical and biochemical derivatives, (2) excessive details of separation of compounds, proteins and enzymes, e.g. preparation of columns, TLC plates, column and fraction size.

Compound characterization: Physical and spectroscopic data for new compounds must be comprehensive, and follow the order shown below: compound name (and assigned number in text); physical state of compound (e.g. oil, crystal, liquid, etc.), melting and/or boiling point; optical rotation and/or circular dichroism measurements, if optically active; UV; IR, 1H NMR; 13C NMR; MS. For all new compounds, either high-resolution mass spectral or elemental analysis data are required. See later section for method of data presentation.

Nomenclature: Chemical nomenclature, abbreviations and symbols must follow IUPAC rules. Whenever possible, avoid coining new trivial names; every effort should be made to modify an existing name. For example, when a new compound is described, it should be given a full systematic name according to IUPAC nomenclature and this should be cited in the Abstract or in the Experimental section. Isotopically-labeled substances should be written with the correct chemical name of the compound. The symbol for the isotope should be placed in square brackets and should precede that part of the name to which it refers, e.g. sodium [^{14}C]formate.

Optical rotation, optical rotary dispersion, circular dichroism: Data should be presented in the established form, e.g.
[α] Value (+ or –) in ° (c {wt of compound in 100 ml of solvent}, solvent used)
Example: [α] +32° (c 0.3210, EtOH).
ORD curves usually described as a series of values based on [α] or [θ] (molecular rotation) at various wavelengths.
CD values may be expressed as molecular ellipticity values [θ], e.g., [θ]256 +21 780, [θ]307 – 16 113 or as differential dichroic absorption, e.g. δε253 – 1.0 (MeOH; c 0.164).

Ultraviolet–visible spectra: ε values are given as log values in parentheses, e.g.
λ nm (log ε): 203 (4.7), etc.

Infrared spectra: Data should be presented in the established form, e.g.
ν cm – 1: 1740, etc.

Absorption should be expressed only in wave-numbers and structural assignments should be indicated when possible in parentheses after the relevant wave-number, e.g. 1740 (>C=O), etc. The following abbreviations should be used if the intensity of absorption

bands are included: w—weak intensity, m—medium intensity, v—variable intensity, s—strong intensity, vs—very strong intensity.

NMR spectral data should only be presented in full if they have not been published separately elsewhere, in which case only relevant references should be quoted. Data must be specified as 1H NMR or 13C NMR and should indicate the frequency of the instrument, the solvent used and the internal standard. Chemical shifts should be quoted in δ units relative to TMS with indication of whether the signal is a singlet s, doublet d, doublet of doublets dd, triplet t, multiplet m, etc. 13C NMR spectral data should specify the carbon concerned, using the recommended IUPAC numbering (e.g. C-l, C-2), and should be given to one decimal place. 1H NMR spectral data should indicate the number of hydrogens involved and their position of attachment based on the numbering of the carbon atoms, preferably according to IUPAC rules. For example—13C NMR spectral data (25.15 MHz, CDCl3): δ 30.1 (t, C-5), 74.1 (d, C-6), 121.7 (d, C-3), 144.2 (s, C-4), etc. 1H NMR spectral data (100 MHz, CDCl3): δ 0.68 (3H, s, H-18), 0.88 (6H, d, J = 6 Hz, H-26 and H-27), 0.90 (3H, d, J = 5 Hz, H-21), 4.34 (1H, q, J6α,7α = 4.5 Hz, J6α,7β = 2Hz, H-6), 4.21 (1H, m, W1/2 18 Hz, H-3α).

Mass spectral data should only be presented in full if they have not been published separately elsewhere, in which case only relevant references should be quoted. Presentation of mass spectral data should in general follow the recommendations given in Int. J. Mass Spectrom. Ion Processes, 142, 211–240 (1995), and must indicate the method used (EIMS, CIMS, GC–MS, etc.) and the ionizing energy. The data should give only diagnostically important ions, the character of the fragmentation ions in relation to the molecular ion and the intensity relative to the major ion. For example—EIMS (probe) 70 eV, m/z (rel. int.): 386 [M]+ (36), 368 [M – H2O]+ (100), 353 [M – H2O – Me] + (23), 275 [M – 111] + (35), etc. CIMS (iso-butane, probe), 200 eV, m/z (rel. int.): 387 [M + H] + (100), 369 [(M + H) – H2O] + (23), etc. High-resolution spectra can be given in more detail if necessary for [M] + and the more important fragment ions.

X-ray crystallography. Only essential data (e.g. a three-dimensional structural drawing with bond distances) should be included in manuscripts. A complete list of data in CIF (Crystallographic Information File) format should be prepared separately and deposited with the Cambridge Crystallographic Data Centre (see www.ccdc.cam.ac.uk for further information) before the paper is submitted. A footnote indicating this fact is to be included in the manuscript. "CCDC … contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk)". Crystal structures of proteins should be submitted to the Protein Data Bank (see www.rcsb.org/pdb; e-mail: info@rcsb.org). Please submit a copy of the CIF data when you submit your manuscript.

Elemental analysis results for compounds which have been adequately described in the literature must be given in the form: (Found: C, 62.9; H, 5.4. Calc. for C13H13O4N: C, 63.2; H, 5.3%.) New compounds must be indicated by giving analytical results in the form: (Found: C, 62.9; H, 5.4. C13H13O4N requires: C, 63.2; H, 5.3%.)

Thin-layer chromatography

(a) For analytical TLC, dimensions of the plates can be deleted if layer thickness is 0.25 mm.
(b) Abbreviate common adsorbents: (but use silica gel, not SiO2 as this does not describe the material accurately), Al2O3 (alumina).
(c) Preparative forms of the technique should include details of (i) layer thickness (preparative TLC only), (ii) amount of sample applied to the layer, (iii) method of detection used to locate the bands and (iv) the solvent used to recover the compounds from the adsorbent after development.
(d) Special forms of TLC on impregnated adsorbents can be abbreviated, e.g. AgNO3–silica gel (1:9), by wt can be assumed.

Gas chromatography

(a) Detector used should be specified, e.g. dual FID, EC, etc.
(b) Carrier gas and flow rate should be given, e.g. N2 at 30 ml min–1.
(c) Operating conditions, such as injector and detector heater temperatures etc., should be included.
(d) Packed columns, e.g. 6 m x 3 mm (i.d. measurement only) packed with 1% SE-30 (support material and mesh size can be omitted unless unusual).
(e) Capillary columns should be specified, e.g. WCOT (wall coated open tubular), SCOT (support coated open tubular). The split ratio used in the injection system and the injection volume for the sample should also be included.

High performance liquid chromatography

(a) Solvent or solvent gradients used together with flow rate should be given.
(b) Column dimensions (length x i.d. only) and packing used.
(c) Method of detection employed, e.g. UV or refractive index.

Biochemical conventions

Unless a common biochemical term (e.g. ATP, NADH), biochemicals that are abbreviated should be spelled out in full (in brackets) immediately following their first usage in the text. Enzyme names are typically not abbreviated, unless there are accepted abbreviations, such as ATPase. Where possible, E.C. numbers should be used for enzymes, and the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) should be used (see below).

Enzyme characterization

(a) Enzyme activity is expressed in units of katals (symbol kat), the conversion of one mol of substrate per sec. It should be made clear that the measurements were made under specified optimum conditions and were not seriously affected by losses during extraction and analysis.
(b) pH optima should be given together with pH values for half maximal activity.
(c) Kinetic parameters should be expressed as Vmax, Km etc.
(d) Enzyme inhibitors—effectiveness should be expressed as Ki or concentration for half-maximal activity.

(e) Optimal temperature of enzymes should not be given. This should be expressed in terms of "Energy of Activation" and "Energy of Activation for Denaturation".
(f) Enzyme nomenclature is now given in "Enzyme Nomenclature, Recommendations", Academic Press (1992) (www.chem.qmul.ac.uk/iubmb).
(g) Labeling of proteins and nucleic acids—use of labelled precursors in assessing the rate of synthesis of macromolecules must be validated by evidence of real, direct incorporation. The possibility of occlusion or adsorption of isotopic material should be noted and it should be shown that the labeled precursor is incorporated without prior catabolism.

Protein and nucleotide sequences

The Experimental must contain explicit documentation of the ends of nucleotide probes used in the study if previously unpublished, or by appropriate reference to published nucleotide numbers and/or restriction map.

In manuscripts to be published in Phytochemistry, any new protein and/or nucleotide sequence must have been submitted to EMBL, GenBank™ or DNA Data Bank of Japan databases, with designated accession number(s) obtained prior to paper acceptance by the Regional Editor. The Author(s) must ensure access to this database information by timely release of data prior to publication, as well as providing necessary documentation to those already in the databases.

Nucleotide sequence data can be submitted either electronically (e-mail) or in computer-readable format, GenBank™, EMBL and the DNA Data Bank of Japan addresses are: GenBank Submissions, National Center for Biotechnology Information, Building 38A, Room 8N-803, Bethesda, MD 20894. Tel.: +1 301 496-2475; e-mail (submissions): gb-sub@ncbi.nlm.nih.gov; e-mail (information): info@ncbi.nlm.nih.gov; EMBL Nucleotide Sequence Submissions, European Bioinformatics Institute, Hinxton Hall, Hinxton, Cambridge CB10 1SD, UK. Tel.: +44 (0) 1223-494401; fax: +44 (0) 1223-494472; e-mail: datasubs@ebi.ac.uk; world wide web: www.ebi.ac.uk/embl; or DNA Data Bank of Japan, Center for Information Biology, National Institute of Genetics, Mishima, Shizuuoka 411-8540, Japan. Tel.: (+81) 559-81-6853; fax: (+81) 559-81-6849; e-mail: ddbjsub@ddbj.nig.ac.jp (for data submissions); world wide web: www.ddbj.nig.ac.jp.

Contributors must obtain the designated accession number, which will be incorporated into the paper, prior to printing.

Only novel DNA sequences will be published. Sequences that show close similarity to known coding or other sequences such as promoters will not be published and will be cited by accession number. Translated protein sequence information should be published as alignments against other gene family members. Papers containing such information about genes already known in other species should have sufficient novelty and biological significance. Sequence only papers or papers which duplicate work in another species will not be published.

Genes known by three letter names should be written in italics. The corresponding cognate protein should be written in capital, non-italic text.

GenBank/DNA sequence linking

DNA sequences and GenBank accession numbers: Many Elsevier journals cite "gene accession numbers" in their running text footnotes. Gene accession numbers refer to genes

or DNA sequences about which further information can be found in the database at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources should type this information in the following manner.

For each and every accession number cited in an article, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalised (see example 1 below). This combination of letters and format will enable Elsevier's typesetters to recognize the relevant texts as accession numbers and add the required link to GenBank's sequences.

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In the final version of printed article, the accession number text will not appear bold or underlined (see example 2 below).

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In the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases, enabling readers to go directly to that source from the article

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References: All publications cited in the text should be presented in a list of references following the text of the manuscript. In the text refer to the author's name (without initials) and year of publication (e.g. "Since Peterson (1993) has shown that …" or "This is in agreement with results obtained later (Kramer, 1994)". For two authors both authors are to be listed, with "and" separating the two authors. For more than two authors, use the first author's surname followed by et al. The list of references should be arranged alphabetically by authors' names. Journal titles should be abbreviated (e.g. Carbohydr. Res.) following the Chemical Abstracts Service Source Index (CASSI) style (a list of abbreviated journal titles is available online at www.cas.org/sent.html). The manuscript should be carefully checked to ensure that the spelling of authors' names and dates are exactly the same in the text as in the reference list. References should be given in the following form:

Cabello-Hurtado, F., Durst, F., Jorrín, J.V., Werck-Reichhart, D., 1998. Coumarins in Helianthus tuberosus: characterization, induced accumulation and biosynthesis. Phytochemistry 49, 1029–1036.

Mabry, T., Markham, K.R., Thomas, M.B. 1970. The Systematic Identification of Flavonoids. Springer Verlag, New York.

Harborne, J.B., 1999. Plant chemical ecology. In: Barton, D., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 8. Pergamon, Oxford, pp. 137–196.

Preparation of Illustrations

All illustrations should be suitable for reproduction (which may include reduction) without retouching. Illustrations (figures, tables, etc.) should be prepared for either single or double column format. For submission, illustrations should be included in the manuscript and also submitted separately as high-resolution files. Figures must be prepared at a minimum resolution of 300 dpi (for further information please see http://www.elsevier.com/artworkinstructions). Refer to all photographs, charts and diagrams as "Figure(s)" and number them consecutively in the order in which they are cited. They should accompany the manuscript, but should not be included within the text. All illustrations should be clearly marked with the figure number and the author's name (a clear file name). All figures are to have a caption, which should be supplied on a separate page.

Note: Illustrations of the following type generally will not be accepted for publication within the article: (1) diagrams or photographs of chromatograms (PC and TLC), electrophoretic separations, or recorder traces of GC and HPLC data which are given merely to prove identification; (2) straight-line graphs; (3) generalized pH and temperature–denaturation curves of enzymes; (4) illustrations of IR, UV, NMR or MS (values can be quoted in the text or Experimental); (5) flow sheets illustrating isolation of compounds; (6) expectable MS fragmentation patterns; (7) formulae of well-known compounds or reaction schemes; (8) tables giving either single values for each parameter which could be easily quoted in the text, or repeating data shown elsewhere. Such illustrations may be published as supplementary data (see below).

Lettering in tables, figures, etc.: Lettering in formulae, figure axes, etc. must be large enough to be legible after reduction. Lettering should be drawn in 6–7pt Helvetica (Arial) font to ensure optimum visibility.

Chemical formulae must be made absolutely clear: printers are not chemists and much delay is caused by poor drawing. Aromatic rings must be drawn with alternate double bonds and conformation of single bonds shown by thickened (–) or dashed (|||) lines according to convention. Formulae should be numbered consecutively in Arabic numerals. If graphics are created using ChemDraw or ISISDraw, the preferred settings are: font 10 pt Helvetica (Arial), chain angle 120°, bond spacing 18% of length, fixed (bond) length 14.4 pt (0.508 cm), bold width (bond thickness) 2.0 pt (0.071 cm), line width 0.6 pt (0.021 cm), margin width 1.6 pt (0.056 cm), and hash spacing 2.5 pt (0.088 cm). The overall size should be not more than 95 mm (single column) or 194 mm (double column) by 283 mm.

Tables must be typed on separate pages, numbered consecutively, given a suitable caption and arranged to be viewed vertically. They must be so constructed as to be intelligible without reference to the text.

Every table must have an Arabic number and a title, and each column must be provided with an explanatory heading. No vertical rules should be used. Tables should not duplicate

results presented elsewhere in the manuscript (e.g. in graphs). Footnotes may be used to expand column headings, etc. and should be referenced by superscript lowercase letters a,b,c rather than symbols. Results should be cited only to the degree of accuracy justified on the basis of the errors of the method and usually only to three significant figures. Units must always be clearly indicated and chosen so as to avoid excessively high (>100) or low (<0.01) values. The figure zero should precede the decimal point for all numbers below one (e.g. 0.1).

Half-tone photographs must have good contrast and not be more than 25 cm wide and not more than 30 cm high. Original photographs (or high-resolution graphic files of at least 500 dpi) must be supplied as they are to be reproduced (e.g. black-and-white or colour). If necessary, a scale should be marked on the photograph. Please note that photocopies of photographs are not acceptable.

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In order to ensure that your submitted material is directly usable, please ensure that data are provided in one of our recommended file formats. Supplementary data must be saved in files separate from those for the manuscript and figures, and all file names must be supplied. Supplementary files should either be referred to from within the text of your manuscript in the same way as for figures and tables, or their presence be indicated by adding a paragraph entitled “Supplementary Data” at the end of the manuscript, detailing which data is supplied. In addition, authors should also provide a concise and descriptive caption for each file.

When supplying supplementary material, authors must state whether data files are either (i) for online publication or (ii) to be used as an aid for the refereeing of the paper only. All supplementary data will be subject to peer-review. For more detailed instructions please visit http://www.elsevier.com/artworkinstructions .

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Abbreviations

About, approximately: ca.

Anhydrous: dry (not anhyd.)
Aqueous: aq.
Circular dichroism: CD
Concentrated (or mineral acids): conc.
Concentrations: ppm (never ppb!), μM, mM, M, %, mol
Dry weight: dry wt; fresh weight: fr. wt
Electricity: V, mA, eV
Force due to gravity (centrifugation): g; rpm (revolutions min-1)
Gas chromatography: GC
Gas chromatography–mass spectrometry: GC–MS trimethylsilyl derivative: TMSi (TMS cannot be used as this refers to the internal standard tetramethylsilane used in 1H NMR)
High performance liquid chromatography: HPLC
Infrared spectrophotometry: IR
Length: nm, μm, mm, cm, m
Literature: lit.
Mass spectrometry: m/z [M]+ (molecular ion, parent ion)
Melting points: uncorr. (uncorrected)
Molecular mass: Da (daltons), kDa
Molecular weight: Mr
Nuclear magnetic resonance: 1H NMR, 13C NMR, Hz, δ
Numbers: e.g. 1, 10, 100, 1000, 10,000: per or -1
Optical rotatory dispersion: ORD
Paper chromatography: PC
Precipitate: ppt.
Preparative thin-layer chromatography: prep. TLC
Radioactivity: dpm (disintegrations per min), Ci (curie), sp. act (specific activity), Bq (1 becquerel = 1 nuclear transformation sec-1)
Repetitive manipulations: once, twice, x3, x4, etc.
RRt (relative retention time), Rt (Kovat's retention index), ECL (equivalent chain length—term frequently used in fatty acid work)
Saturated: satd.
Solution: soln.
Solvent mixtures including chromatographic solvents: abbreviate as follows n-BuOH–HOAc–H2O (4:1:5)
Statistics: LSD (least significant difference), s.d. (standard deviation), s.e. (standard error)
Temperature: (with centigrade), mp, mps, mmp, bp
Temperature: temp.
Thin-layer chromatography: TLC, Rf
Time: s, min, h, day, week, month, year
Ultraviolet spectrophotometry: UV, A (absorbance, not OD—optical density)
Volume: l (litre), μl, ml
Weight: wt, pg, ng, μg, mg, g, kg
Inorganics, e.g.
AlCl3 (aluminum chloride), BF3 (boron trifluoride), Cr-, CO2, H2, HCl, HClO4 (perchloric acid), HNO3, H2O, H2O2, H2SO4, H3BO3 (boric acid), He, KHCO3 (potassium bicarbonate), KMnO4 (potassium permanganate), KOH, K-Pi buffer (potassium phosphate buffer), LiAlH4 (lithium aluminium hydride), Mg2+, MgCl2, N2, NH3,

(NH4)2SO4, Na+, NaBH4 (sodium borohydride), NaCl, NaIO4 (sodium periodate), NaOH, Na2SO3 (sodium sulphite), Na2SO4 (sodium sulphate), Na2S2O3 (sodium thiosulphate), O2, PPi (inorganic phosphate), SO, Tris (buffer).

Organics, e.g.

Ac2O (acetic anhydride), n-BuOH (butanol), C6H6 (benzene), CCl4 (carbon tetrachloride), CH2Cl2 (methylene chloride), CHCl3 (chloroform), CH2N2 (diazo-methane), CM (carboxymethyl), DEAE (diethylaminoethyl), DMF (dimethylformamide), DMSO (dimethyl sulphoxide), EDTA (ethylene-diaminetetra-acetic acid), Et2O (diethyl ether), EtOAc (ethyl acetate), EtOH (ethanol), HCO2H (formic acid), HOAc (acetic acid), iso-PrOH (iso-propanol), Me2CO (acetone), MeCOEt (methyl ethyl ketone), MeOH (methanol), NaOAc (sodium acetate), NaOMe (sodium methoxide), petrol (not light-petroleum or petroleum ether), PhOH (phenol), PrOH (propanol), PVP (polyvinylpyrrolidone), TCA (trichloroacetic acid), TFA (trifluoroacetic acid), THF (tetrahydrofuran).

1H NMR solvents and standards: CDCl3 (deuterochloroform), D2O, DMSO-d6 [deuterodimethylsulphoxide, not (CD3)2SO], pyridine-d5 (deuteropyridine), TMS (tetramethylsilane).

For further terms used in biochemistry and molecular biology the authors should see the websites of the nomenclature committees (www.chem.qmul.ac.uk/iubmb/).

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6.2. Indicadores de produção 2006-2008

6.2.1 Resumos em congressos

Resumos apresentados na II Reunião Regional da FeSBE

04.102 - Produtos Naturais
ISOLAMENTO DA PROTEÍNA TIPO VICILINA DE *Cratylia mollis* E AVALIAÇÃO DA ATIVIDADE ANTITUMORAL Ramos, A. L. G.; Silva, C. B. da; Coelho, L. C. B. B.; Lima, V. L. M.; Souza, I. A.; Paiva, P. M. G.;

04.105 - Produtos Naturais
AVALIAÇÃO DA ATIVIDADE ANTITUMORAL DO EXTRATO AQUOSO DE FOLHAS DE *Indigofera suffruticosa* Mill EM CARCINOMA DE EHRLICH. Silva, C. B.da; Pereira, D. R.; Ramos. A. L. G.; Souza, I. A.; Lima, V. L. M.;