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Detection of cysteine-rich peptides in *Tragia benthamii* Baker (Euphorbiaceae) and *in vivo* antiinflammatory effect in a chick model

Abstract: *Tragia benthamii* (TBM) commonly called the climbing nettle is a tropical plant claimed to have numerous anti inflammatory effects in sub Saharan African ethnomedicine which lacks scientific evidence. Aqueous extracts of TBM were further prepurified on a RP-C18 parked solid phase system to obtain 20% aqueous fraction. This fraction was enzymatically and chemically analyzed (by MALDI TOF MS and MS/MS) to contain interesting low molecular weight cysteine-rich stable peptides within the range of 2.5–3.2 KDa. The 20% aqueous fraction was further tested *in vivo* using carrageenan-induced foot edema (acute inflammation) in seven-day old chicks with diclofenac as reference drug. The cytotoxicity of this active fraction was investigated using the brine shrimp lethality assay. The brine shrimp cytotoxicity assay produced LC_{50} above 1000 $\mu\text{g/mL}$. Pretreatment with the TBM extract (30–300 mg/kg, i.p) dose dependently ($P < 0.01$) reduced foot edema with maximal inhibition of 0.253 ± 0.180 (84.3%) at 300 mg/kg body weight, which was comparable to that of diclofenac with inhibition ($P < 0.05$) of 0.410 ± 0.271 (74.5%) at 10 mg/kg body weight. The study has therefore shown for the first time, the detection of cysteine-rich biologically active peptides in *T. benthamii* and the stable peptide extracts from this ethnomedicinal plant, which is not toxic to *Artemia salina*, exhibits anti inflammatory activity in a chick *in vivo* model. This may provide scientific evidence for its use in the treatment of inflammation and pain in traditional medicine. Further *in-depth vivo* and *in vitro* studies will be required to investigate its anti inflammatory activity including effect on HUVEC-TERT, the possible inhibition of ICAM-1 surface expression and the mechanism of the anti inflammatory effect.

Keywords: brine shrimp cytotoxicity; carrageenan; cysteine-rich peptides; inflammation; *Tragia benthamii*.

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1 Introduction

Tragia benthamii (TBM), Bak. (Euphorbiaceae) also known as climbing nettle or stinging nettle, is a medicinal plant widely distributed and used by traditional healers all over sub Saharan Africa where the plant appears to be endemic [1, 2]. The genus *Tragia* contains over 152 species including a newly reported species – *Tragia guayanensis* [1, 3] with only five that have received some scientific attention among which TBM remains the least investigated; others include *Tragia involucrata*, *Tragia cannabina*, *Tragia spathulata*, and *Tragia plukenetii*. Folkloric claims on the medicinal application of the plant include abortifacient activity, facilitation of child birth, treatment of venereal diseases, as an anti inflammatory remedy, and as an antibiotic. For instance, the Baule women in Ivory Coast have used TBM to induce abortion or to facilitate delivery [2, 4, 5]. Despite its adaptational irritating properties when the foliar organs are in contact with the human skin [6], there is a widespread ethnopharmacological applications among African populations; while this should attract scientific attention, only very limited investigation has been carried out on the plant with no available data on the bioactive constituents from the aqueous extracts and their possible effect on inflammation, a common denominator of several chronic diseases and initiator of different pathophysiological diseases [7–9].

T. benthamii is a creeping and twining tropical herbaceous plant distributed around the flood-plain thickets and flood-plain grasslands of Africa. It is commonly found in Ivory Coast, Ethiopia, Angola, Botswana, Zimbabwe, Mozambique, and Nigeria. TBM possesses stinging hairs which are covering trichome outgrowths from the surface of the leaves whose contact with the body result in local inflammation and pain lasting up to 10 min [6]. The trichome-covered leaves have leaf blade which looks more or less shallowly and cordate at the base. In Nigeria, TBM is known as *Esisi* or *Werepe* in Yoruba, *Abalagwo* in Igbo, and *Ibadidon* or *Eeghelepe* (ososo) in Edo.

A typical plant has been described as a physiological laboratory, making it a rich source of bioactive compounds with diverse structures suitable for drug design and discovery [10]. Overtime, phytomedicinal research has paid more attention to low molecular weight secondary compounds [11] while the higher molecular weight peptides have recently gained more recognition within the scientific community [11–21]. However, a major therapeutic drawback of peptides lies in their poor metabolic stability or oral bioavailability [14, 20, 22]. The cysteine-rich peptides (CRPs), particularly those containing three to six disulfide bonds have been well reported to overcome this undesirable limitation [12, 19, 23]. Of particular interest are CRPs whose molecular weights lies within the range of 2–6 kDa which are known to contain a well-defined tertiary structure arising from a uniquely coupled thiol groups resulting in a cross-linked multiple disulfide bonds (SS-bonds) that provide them with exceptional stability against thermal and enzymatic degradation [20]. Many of these plant-derived CRPs display varied interesting biological and pharmacological activities [19, 20, 24].

In the present work, we extracted the peptide-rich aqueous portion of TBM and subjected this to pre purification via solid phase extraction to yield an optimized crude peptide mixture whose biophysical properties, nature and individual masses were determined by a combination of reversed phase liquid chromatography (RP-HPLC), chemical derivatization and matrix assisted laser desorption time of flight mass spectrometry (MALDI TOF MS). Furthermore, we investigated the anti inflammatory properties of TBM using *in vivo* chick carrageenan response as a viable model for anti inflammatory response; in addition, the nauplii of *Artemia salina* were used for cellular toxicity assay using the brine shrimp cytotoxicity model. Following our new findings, we show for the first time the presence of cysteine-rich stable peptides in the aqueous extracts of TBM and also demonstrate the *in vivo* anti-inflammatory property of the peptide-rich aqueous extracts in a chick model and the safety of this extracts to cellular crustaceans.

2 Materials and method

2.1 Plant material

Shoots of *T. benthamii* Bak. (Euphorbiaceae) were collected during the raining season from the rocky areas of Egbetua land, Ososo village, and southern Nigeria in 2011. The complete plant with relevant anatomical features was identified, authenticated and a voucher specimen with the number FHI: 109853 was deposited at the Forest Herbarium Ibadan (FHI) unit of the Forest Research Institute of Nigeria (FRIN).

2.2 Extraction, SPE pre purification and preliminary MALDI TOF/TOF analysis

Dried and powdered TBM plant material was extracted with methanol: dichloromethane (1:1; v/v) for 24 h under continuous agitation at room temperature. After adding half volume of ddH₂O to the extraction system and centrifuging (for 3 min at 1000 rpm), the aqueous supernatant was separated out and diluted further with solvent A (100% ddH₂O, 0.05% trifluoroacetic acid) to less than 10% MeOH for C₁₈ flash pre purification. The aqueous phase was extracted on RP-C₁₈ solid-phase. Cartridges (Strata Gigatubes C₁₈-E; 5 g, 20 mL, Phenomenex, Germany) was first preconditioned with one volume of MeOH, activated with one volume of solvent B (10% ddH₂O in acetonitrile and 0.05% trifluoroacetic acid) and equilibrated with two volumes of solvent A, washed with 20% solvent B (to yield 80% aqueous fraction), eluted with 20 mL of 80% solvent B (yielding 20% aqueous fraction) and finally eluting with 100% solvent B (yielding the 0% aqueous fraction). Cryodesiccation of the eluates using the freeze drying equipment followed after pipetting out 200 µL each of the eluates for preliminary crude MALDI-TOF

MS analysis of peptide-like peaks. A volume of 0.5 μL each of eluted fraction was mixed well with 3 μL of matrix (alpha cyano hydroxyl cinnamic acid), spotted on the MALDI target plate and exposed to dryness away from light source. Acquired spectra were processed using the 4800 Analyzer. The freeze dried aqueous extract was kept refrigerated for RP-HPLC analysis and for bioassays [12].

2.3 RP-HPLC fractionation of C₁₈ peptide positive fraction

The dried material was reconstituted in a minimal amount of buffer A (0.05% trifluoroacetic acid prepared in distilled water). The solution was further passed through a solid phase filter (Sartorius) before purification using semi preparative RP-HPLC on Dionex Ultimate 3000 HPLC unit (Dionex, Amsterdam, the Netherlands) whose specification include 250 mm $\times$ 10 mm; 5 μm , 100 Å, Kromasil column. Linear gradient of 0.1–1% min^{-1} solvent B at a flow rate of 3 mL min^{-1} . Collected fractions were again analyzed on the MALDI TOF/TOF analyzer following the earlier described procedure. Fraction of interest was freeze dried and reconstituted in solvent A for analytical HPLC (flow rate of 1 mL min^{-1}) for hydrophobicity determination of the peptide.

2.4 Reduction/alkylation of peptides and MALDI-MS analysis

Disulphide linkages within the peptide were reduced with 2 μL of dithiothreitol after reconstituting approximately 6 nmol of the lyophilized peptides in 20 μL of 0.1 M ammonium bicarbonate (pH 8.2), and incubating at 65 °C for 10 min. Reduced sample was further alkylated with 4 μL of iodoacetamide. One microlitre of TFA was used to stop the reaction. MALDI-TOF/TOF analyzer was then used to investigate the reduced and alkylated sample. Kalata B1 was used as positive control [12].

2.5 Derivatization of peptides and MALDI TOF/TOF analysis

Reduced and alkylated peptides were enzymatically digested with endoproteinase Glu-C (Sigma-Aldrich, Austria). The previous reduction and alkylation reaction was stopped by adding 1 μL of formic acid and incubated for 10 min at 37 °C. This was followed by the addition of 2 μL of the enzyme to the reaction mixture and further incubating for 3–14 h under mild agitation. The reaction was quenched with 1 μL of formic acid. 0.5 μL of the sample was mixed with 3 μL of matrix and spotted on the MALDI target plate for MALDI TOF MS analysis. All acquired spectra were processed with the Explorer 4800 software. Digested samples were kept under minus 20 freezers for further analysis [12].

2.6 *In vivo* anti-inflammatory assay using the chick model

The carrageenan-induced edema in the footpad of chicks [25–27] was used to evaluate the anti inflammatory activities of the extract compared to the reference drug (diclofenac). Chicks were randomly divided into six groups of six each. Chicks were then randomly selected to perform one of the following study groups: control (10 mL/kg Distilled water, i.p.); *TBM* (10, 30, 100, and 300 mg kg⁻¹, i.p) and diclofenac (10 mg kg⁻¹, i.p.). Foot volumes were measured by water displacement plethysmometer [28]. Edema was induced by subplantar injection of carrageenan (10 µL of a 1% carrageenan in normal saline) into the right footpad of the chicks. The control and all the drug treated groups were pretreated with the vehicle and the drugs 30 min intraperitoneal (i.p.) before carrageenan injection. Foot volumes were measured for 5 h after carrageenan injection. The foot edema was quantified by measuring the difference in foot volume before carrageenan injection and at the various time points. All drugs were freshly prepared.

2.7 Brine shrimp cytotoxicity (lethality assay)

The brine shrimp cytotoxicity assay was used to evaluate the toxicity and potential pharmacological activity of the peptide-rich extract [29, 30]. Sea water was obtained from the Lagos Lagoon, Nigeria. The sea water was poured into the hatching tank and the cysts were added to one side of the tank that has been covered with paper foil to shade the shrimp cysts from light source and were left for two days to hatch and mature.

Solutions of the plant extract were prepared at concentrations of 0, 1, 10, 100, 500, and 1000 µg/mL and filled into vials in triplicates. Furthermore, 4 mL of sea water was added to each vial and the volume in each of the vials was made up to 5 mL each. Hatched eggs of *A. salina* were counted into each vial and left for 24 h in light; after 24 h, the brine shrimp nauplii were counted and the mortality was recorded and analyzed using the Finney computer program for probit analysis to determine the LC₅₀ values and 95% confidence intervals [30].

3 Results

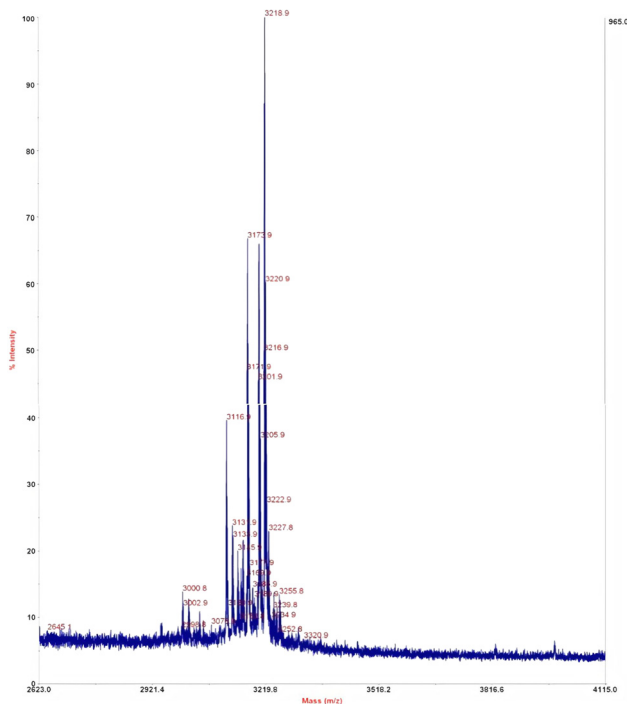
3.1 Extraction of cysteine-rich peptides and mass spectrometry experiment

Powdered leaves obtained from the shoot system of *T. benthamii* (TBM) was extracted using 50% (v/v) methanol in dichloromethane and the aqueous portion further extracted with half volume of double distilled water and freeze dried to yield the crude aqueous

extract. The CRP peptides in the aqueous extract were obtained by pre purification using the solid phase extraction and the peptides profiled by the matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS). Distinct MS profiles within a short mass window were observed (Figure 1), which ranged between 3000 and 3400 Da. To further characterize the peptides in the mixture as potential CRPs, the crude peptide extract was chemically derivatized via reduction and alkylation to obtain a mass shift of +348 Da (Figures 2 and 3) and enzymatically digested with trypsin and endo-proteinaseGluC to result in a complete fragmentation (Figure 4) instead of a +18 Da observed with cysteine-rich circular peptides. The characteristic hydrophobicity of CRPs was evident in their late elution profile (25–50% acetonitrile) in RP-HLC (Figure 5).

3.2 Effect of extract on carrageenan-induced foot edema in chicks and brine shrimp cytotoxicity

Intradermal injection of 10 μ L of 1% carrageenan-induced a time-dependent edema response in the seven-day old chicks that peaked between 2 and 3 h post carrageenan injection (Figure 6a) (Roach and Sufka, 2003).



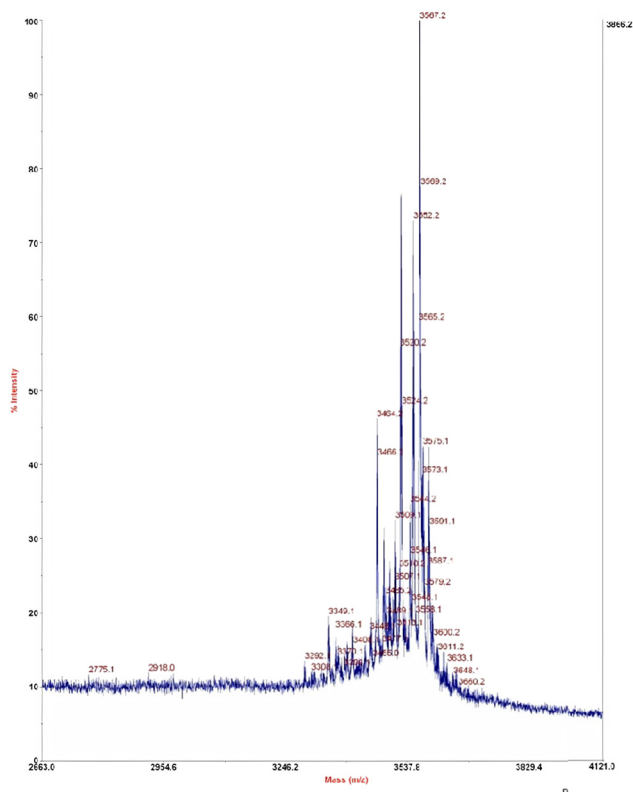


Figure 2: MS profile for the reduced and alkylated peptide enriched prepurified extracts of TBM showing a typical mass shift of +348 Da following chemical modification achieved by the incubation of peptides with dithiothreitol and iodoacetamide.

Two-way ANOVA (treatment x time) from the time-course curves and one-way ANOVA from the area under the time course curves (AUCs) (Figures 6a and b) revealed significant anti inflammatory effects for the reference drugs and the extract. *TBM* [$F_{5, 180} = 9.25$, $P < 0.0001$] significantly reduced total edema with maximal inhibitory effect of 0.253 ± 0.180 at 300 mg/kg as shown in (Table 1). Diclofenac (10 mg/kg, i.p.), an NSAID, also showed significant effect on the time course [$F_{1, 60} = 11.73$, $P < 0.001$] (Figure 6a); and the total edema (AUC), with maximal inhibitory effect of 0.410 ± 0.271 (Figure 6b). Result obtained from the brine shrimp lethality assay indicates that the aqueous peptides extract of TBM is nontoxic to the nauplii of *A. salina* (Table 2).

4 Discussion

T. benthamii is an ethnomedicinal plant endemic to tropical Africa. Traditional medicinal practitioners (TMPs) and spiritual healers in Ghana were known to mix TBM

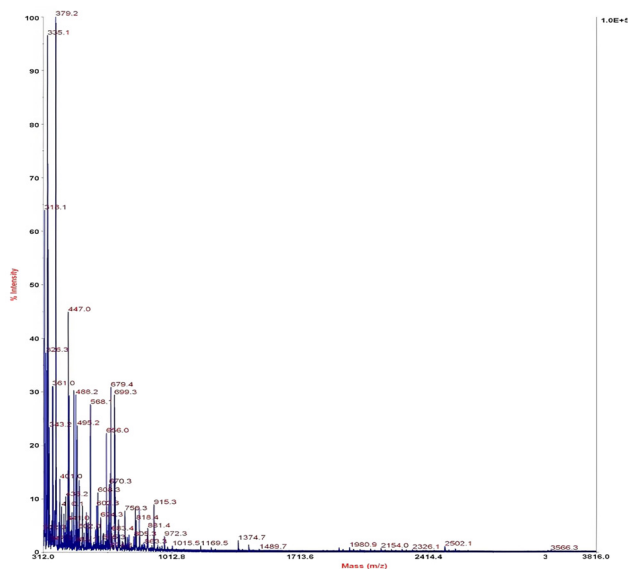


Figure 3: MS profile of digested CRPs showing heavy fragmentation of the earlier reduced and alkylated peptide masses; an addition of +18 Da was absent to confirm the linearity of the peptide or if circular, the unlikely event of the presence of more than one glutamic acid in all the digested peptides.

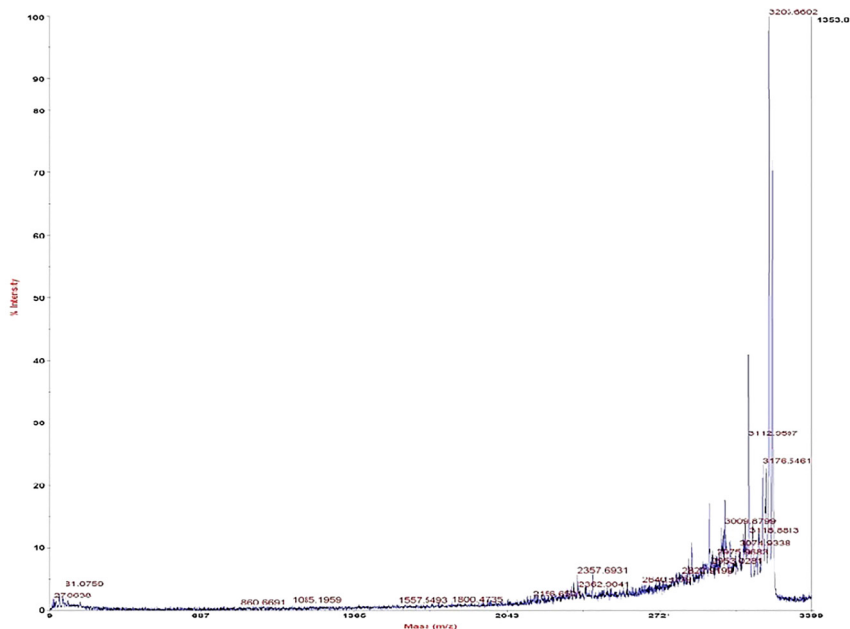


Figure 4: Very poor fragmentation pattern of the selected native mass (3218.9 Da) earlier detected in TBM following aqueous extraction and pre purification and further subjected to a tandem mass spectrometry experiment; this is indicative of an intact and stable peptide typical of cysteine-rich peptides particularly the knottin peptides.

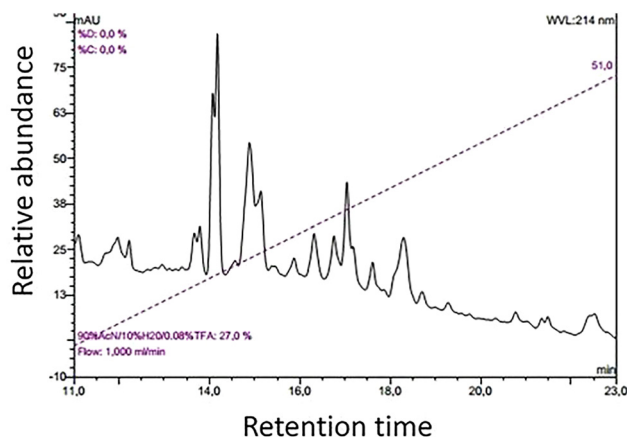


Figure 5: Analytical RP-HPLC showing the hydrophobicity behavior of the peptides and late elution profile for the 20% peptide optimized aqueous fraction obtained by solid phase extraction of C_{18} -bound peptides using a combination of 80% buffer B (10% ddH₂O (v/v) in acetonitrile and 0.05% (v/v) trifluoroacetic acid) and 20% buffer A (100% ddH₂O, 0.05% trifluoroacetic acid).

leaves with eggs for washing themselves while the aerial part of TBM is spread on the floor on which their sacred objects are laid. In Yoruba folklore, TBM is believed to possess some mystical powers and is thus used in proverbs and adages while in other parts of tropical Africa, TBM has commonly been used to treat pain and inflammation [2, 4]. The complete phytochemical profile of TBM has not been reported not to mention bioactive metabolites from the aqueous part of the plant. Recently, Balogun et al. [2] isolated 2, 5-dithia-3, 6-diazabicycloheptane and carried out the GC-MS analysis of the silyl group (R_3Si) derivatized extract. Following very limited number of published articles, the genus *Tragia* is known to accumulate alkaloids, phenolic compounds, sterols, and essential oils [4, 5, 31]. For instance, the genus is well known for its stinging defense capability due to the presence of the chemotaxonomically important shellsol and calcium oxalates which are released following skin contact with the foliar organs normally surrounded by these covering trichomes and this leads to itching, inflammation, and pain [4]. However, no class of bioactive cysteine rich peptides (CRPs) has been documented for any member the genus.

Plant CRPs are emerging therapeutics whose molecular weights are lower than small-molecule metabolites. They come with additional advantages of an optimized on-target specificity and decreased off-target toxicity [11]. An interesting property of plant CRPs that has attracted further scientific attention is their high stability and their phylogenetically conserved structural configuration, which have been described as potential scaffolds that are useful for grafting bioactive peptide epitopes to increase their metabolic stability and retain efficacy [10, 20].

The MALDI TOF MS analysis of peptide optimized aqueous fraction of TBM detected peptide masses whose reduction and alkylation experiment resulted in a mass

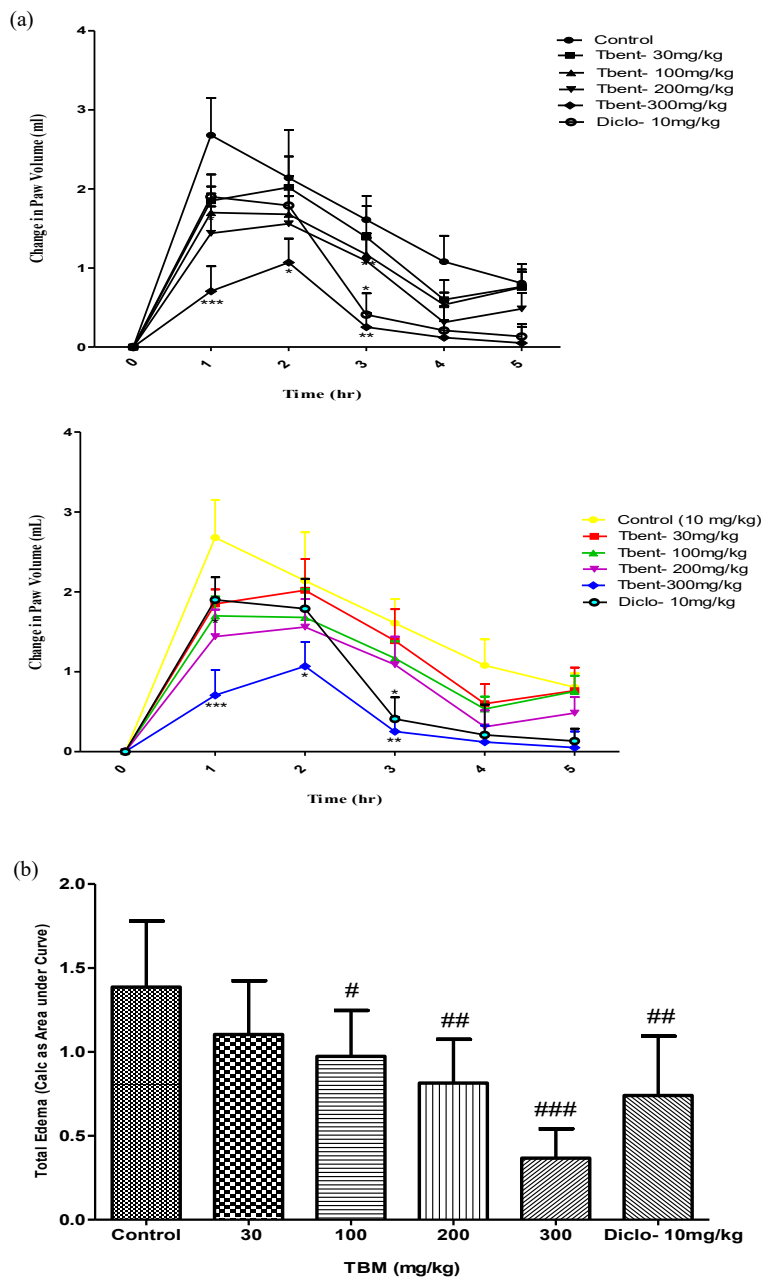


Figure 6: Effect of TBM (10–300 mg kg⁻¹, *i.p.*), diclofenac (10 mg kg⁻¹, *i.p.*) on time-course curves (a) and the total edema response (b) in the carrageenan-induced foot edema in chicks. Values are Mean $\pm$ S.E.M. ($n = 6$). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ compared to vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). ### $P < 0.001$; ## $P < 0.01$; # $P < 0.05$ compared to vehicle-treated group (one-way ANOVA followed by Newman–Keuls *post hoc* test).

Table 1: Change in the foot volume of chicks following the administration of graded doses of the cysteine-rich aqueous extracts of *Tragia benthamii*.

Treatment	1 h	2 h	3 h	4 h	5 h
Control (10 mg/kg)	2.680 ± 0.472	2.140 ± 0.606	1.610 ± 0.301	1.080 ± 0.327	0.807 ± 0.174
TBM (30 mg/kg)	1.850 ± 0.181	2.020 ± 0.391	1.390 ± 0.393	0.600 ± 0.247	0.763 ± 0.289
TBM (100 mg/kg)	1.700 ± 0.242	1.680 ± 0.352	1.170 ± 0.211	0.535 ± 0.155	0.755 ± 0.196
TBM (200 mg/kg)	1.440 ± 0.338*	1.560 ± 0.349	1.090 ± 0.350	0.312 ± 0.206	0.483 ± 0.200
TBM (300 mg/kg)	0.707 ± 0.316***	1.070 ± 0.303*	0.253 ± 0.180**	0.120 ± 0.207	0.052 ± 0.202
Diclofenac (10 mg/kg)	1.900 ± 0.283	1.790 ± 0.374	0.410 ± 0.271*	0.210 ± 0.380	0.133 ± 0.156

Values are Mean ± S.E.M. (n = 6). ***P < 0.001; **P < 0.01; *P < 0.05 compared to vehicle-treated group (two-way ANOVA followed by Bonferroni's post hoc test).

Table 2: Brine shrimp lethality of the peptide-rich aqueous extracts of *Tragia benthamii* Bak. (Euphorbiaceae).

Concentration of extract	Mortality/10		Total mortality/30	Percentage mortality (%)	Expected probit	Calculated probit
	Replicates ^a					
1000	4	3	4	11	36.67	4.68
500	4	2	1	7	23.33	4.55
100	3	3	4	10	33.33	4.23
10	0	2	1	3	10.00	3.79
1	0	0	1	1	3.33	3.34
Control	0	0	0	1	1.00	—

^aAn average of 10 nauplii were used for each experimental replicate. Chi squared degree of freedom = 3.4979, ED₅₀ = 6127.0890, G value = 0.3187, upper confidence limit = 1.337, 236.00%, lower confidence limit = 1073.

shift of 348 Da indicative of the presence of six cysteine residues involved in three disulphide bonds. The mass shift is a result of an addition of ~58 Da following reduction with dithiothreitol and iodoacetamide alkylation of six reduced sulfhydryl radicals [12]. Considering the detection of six cysteines involved in cystine bonds, the observed hydrophobic behavior of the peptide demonstrated by their late elution profiles and the resistance of the peptides to MALDI-TOF MS/MS fragmentation, these peptides appear to be members of the cystine knot peptides, commonly represented by knottins and hevein-like peptides which are the most reported six-cysteine CRPs having a knotted disulfide connectivity that bridges the cysteine residues I–IV, II–V, and III–VI [10, 12, 19]. However, the configuration and structure of these peptides are important for their biological function. This gives cysteine-rich short molecular weight peptides a stability advantage over other larger bioactive peptides of lower stability. The intercystine connectivity common to cysteine-rich peptides impacts additional stability to extreme conditions of heat, enzymatic and chemical attack. In this work, we applied a reversed phase system to obtain non-peptide fraction (80% ddH₂O, 0.05% TFA in acetonitrile) and aqueous peptide fraction (20% ddH₂O, 0.05%TFA in acetonitrile) using solid phase extraction. The MALDI TOF MS assisted characterization of the peptides showed that the mini proteins are cysteine-rich peptides. Cysteine-rich peptides identified in TBM are low molecular weight peptides rich in cysteine residues. The thiol bonds resulting from the disulphide linkages induce a folding pattern in the peptides which confers additional stability on these peptides to survive many hostile environments and several unfriendly solvent extraction steps and purification [32]. Digestion with endoproteinase Glu-C did not result in ring opening associated with +18 Da on MALDI TOF MS after reduction and alkylation. Instead, there was fragmentation of the peptide. This suggests that TBM peptides may belong to the cystine knot inhibitor (ICK) family. The ICK family of peptides is linear [33]. Gressent et al. [32], described similar class of peptides purified from *Pisum sativum*. *P. sativum* peptides were described to be bioactive, linear, containing six cysteine residues and showing high stability profiles after boiling and rigorous solvent extraction steps [32, 34].

Inflammation is a complex sequence of events initiated by the presence of foreign stimuli, infection or trauma [35, 36]. The local site of inflammation is characterized by redness, swelling, pain, warmth and often loss of functionality. Inflammation is followed by the movement of cells into the site triggered by the upregulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and E-selectin on the surface of endothelial cells, which allows leukocyte binding and subsequent migration [36]. There appear to be a big shift of interest from the less complex synthetic molecules to more complex phytochemicals mainly as a result of unavoidable side effects of synthetic drugs [37]. For instance, the commonly used drugs for the management of inflammatory diseases are the nonsteroidal anti inflammatory drugs (NSAIDs) [38]. These drugs come with undesirable side effects, especially after prolonged use such as gastric lesions, gastrointestinal damage, and kidney dysfunction [39]. Several other

diseases such as heart attacks, Alzheimer's diseases, and cancer whose underlying cause is tied to chronic inflammation have not found an efficient scientific solution. Natural compounds have been used in folklore to treat inflammatory problems. Bioactive peptides have shown great potentials as anti inflammatory molecules of pharmaceutical relevance [40, 41]. The effect of TBM extract on carrageenan-induced foot edema in chicks has shown that the cysteine-rich peptide extracts from the ethnomedicinal plant *T. benthamii* exhibit anti inflammatory activity in a chick model. This represents the first report on the anti inflammatory activity of the peptide optimized aqueous fraction which has been shown in this study to be nontoxic following the brine shrimp lethality assay. The safety data appear to be in agreement with previous studies on two members (*T. plukenetii* and *T. involucrata*) of the genus which were reported to free from acute, oral, and dermal toxicities [4, 42]. While documented data on the anti inflammatory activity of members of the genus is still very scanty, the anti inflammatory potentials of *T. involucrata*, a close member of the genus *Tragia* has been reported [1, 4, 43]. These findings may give some scientific credence to its use for the treatment of inflammation and pain in traditional medicine.

5 Conclusions

Indigenous knowledge on the medicinal application of plants will continue to be relevant in the study of crude drugs and the bioactive extracts or fractions derived from them. Findings from this work expand the current knowledge and library on the occurrence and distribution of cysteine-rich peptides in flowering plants and represents the first report on the identification of nature-derived CRPs with anti inflammatory activity in a chick model from the genus *Tragia* and family Euphorbiaceae. Therefore, a further *in-depth vivo* and *in vitro* studies will be required to investigate its anti inflammatory activity including effect on human umbilical vein endothelial cell-immortalized by telomerase reversed transcriptase (HUVEC-TERT), the possible inhibition of ICAM-1 surface expression and the mechanism of the anti inflammatory effect.

Acknowledgement: Authors wish to thank Associate Professor Christian W. Gruber of the center for physiology and pharmacology, mass spectrometry facility, Medical University of Vienna, Austria for access to the mass spectrometry laboratory during the experiment.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: Authors declare no funding was received for this work.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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