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β-AMYRIN AS AN ANALGESIC COMPONENT OF THE LEAVES OF *CALLISTEMON CITRINUS* (CURTIS) SKEELS: CHEMICAL, BIOLOGICAL AND *IN SILICO* STUDIES

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Abstract

The analgesic potential of the leaves of *Callistemon citrinus* was reported earlier but no active principle for this analgesia was explored. To identify the major analgesic metabolite(s), the leaves were extracted with methanol and fractionated into petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions. Based on the thin layer chromatography, the carbon tetrachloride fraction was subjected to gel permeation chromatography and a compound (**1)** was isolated, which was characterized as β-amyrin. Compound (**1)** displayed significant peripheral analgesia ($p < 0.05$) on mice model at an oral dose 200 mg/kg body weight. It also showed noticeable anti-inflammatory membrane stabilizing activity. *In silico* docking study of β-amyrin with cyclooxygenase (COX)-2 showed a good binding affinity (–9.1 Kcal/mol). Virtual pharmacokinetics and toxicity studies explore its potentials as a lead molecule having no extreme lethality.

Introduction

Medicinal plants derived metabolites are very useful as healing agents. Pain and inflammation are associated with some injuries and diseases. Many analgesic and anti-inflammatory drugs are available presently but they are not free from side-effects. Plant-derived molecules might be screened to discover desired drugs (Cazacu *et al.* 2015).

Callistemon citrinus (Curtis.) Skeels an evergreen shrub recognized as Red bottle-brush or Lemon bottle-brush belongs to Myrtaceae. Previously, some terpenoids, flavonoids, etc. have been isolated from this plant (Goyal *et al.* 2012). This plant is also known to have antimicrobial and cardioprotective properties (Oyedeji *et al.* 2009, Momin *et al.* 2011). Recently, the potential of the leaves of *C. citrinus* as analgesic has been reported on mice model but no further study has been conducted to reveal the major analgesic principle(s) (Ahmed *et al.* 2015). On the basis of this, the aim of the present study was to isolate the major analgesic compound(s) from the leaves of *C. citrinus*.

Materials and Methods

The leaves of *Callistemon citrinus* were collected from Dhaka and identified (DACB accession no. 38576) at Bangladesh National Herbarium, Mirpur, Dhaka. One kg of the powdered leaves was extracted with 5 liter of methanol for 7 days and 5 g of the concentrated extract was fractionated by modified Kupchan method to provide petroleum ether, carbon tetrachloride, chloroform and aqueous soluble materials (Van Wagenen *et al.* 1993). Based on thin layer chromatographic pattern of the major metabolites, 300 mg of carbon tetrachloride extract was subjected to gel permeation chromatography over Sephadex LH-20 (Lipophilic) and eluted with

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hexane-dichloromethane-methanol (2 : 5 : 1) mixture (300 ml) to give a total of 15 fractions (each 10 ml). Fractions 11 and 12 provided compound **1** in pure form upon re-chromatographed over Kieselgel 60 F_{254} using 1.5% methanol in chloroform as the developing solvents. ^{1}H NMR spectrum was recorded using a Bruker AMX-400 (400 MHz) instrument using deuterated chloroform.

The peripheral analgesic activity was assessed in Swiss-albino mice (either sex, 20 - 25 g, 4 - 5 weeks) using acetic acid induced writhing method (Xu *et al.* 2014). Experimental groups are explained in Table 1. Here, compound **1** (200 mg/kg body weight), diclofenac sodium (1 mg/kg body weight; positive control) and vehicle (10 ml/kg body weight of 1% tween 80 in distilled water) were administered orally to the overnight fast mice. Thirty minutes later, 0.7% (v/v) acetic acid solution (10 ml/kg body weight) was intraperitoneally administered and the writhing number was recorded.

The percentage inhibition of acetic acid induced writhing was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Mean number of writhing (control)} - \text{Mean number of writhing (drug)}}{\text{Mean number of writhing (control)}} \times 100$$

On the other hand, the tail immersion method was used to estimate central analgesic activity in Swiss-albino mice (either sex, 20 - 25 g, 4 - 5 weeks) (Olonode *et al.* 2015). Experimental groups are displayed in Table 2. The painful thermal stimulus in mice was generated by dipping the tip of the tail in the hot water. The test group was pre-treated with oral administration of 200 mg/kg body weight of isolated pure compound **1** orally. Ten ml/kg body weight of 1% tween 80 in distilled water (orally) and 2 mg/kg body weight of morphine (subcutaneously) were administered to control and standard drug-treated groups, respectively. After 30 minutes, the tail of each mouse was immersed in a water bath maintained at 55 ±1°C and the time required to remove its tail from water was recorded.

Hypotonic solution- and heat-induced erythrocyte hemolysis methods were used to evaluate the anti-inflammatory activity (Nkeh-Chungag *et al.* 2015). The prepared human erythrocyte suspension was treated with the compound **1** and the standard acetylsalicylic acid**.** In hypotonic assay, the percentage inhibition of hemolysis using optical density (OD) was calculated as $\{(OD_{control} - OD_{test\ samples})/ OD_{control}\} \times 100$. In case of heat induced assay, % inhibition of hemolysis was determined as $\{1 - (OD_{heated\ test\ sample} - OD_{unheated\ test\ sample}) / (OD_{heated\ control\ sample} - OD_{heated\ test\ sample})\} \times 100$.

Docking of β-amyrin with COX-2 was performed according to the method described by Khan *et al.* (2015). The X-ray crystal structure of mouse cyclooxygenase (COX)-2 (PDB ID: 3LN1) was prepared by PyMOL and MGLTools. Energy minimization was performed by SPDV viewer (http://www.expasy.org/spdbv/). The 3D chemical structure of β-amyrin (CID: 73145; PubChem-compound database at NCBI) was refined by the semiempirical PM6 method implemented in Gaussian 09 software. The docking was accomplished by the AutoDock vina (Table 3). The interactions were analyzed by using PyMOL and LigPlot$^+$ v.1.4 software (Laskowski and Swindells 2011).

In silico molecular pharmacokinetics, bioactivity (drug-likeness) and toxic profile evaluation of β-amyrin molinspiration server (www.molinspiration.com) and Toxtree v.2.6.0 (https:// sourceforge.net/projects/toxtree/) were used.

Results and Discussion

Compound **1** was colorless powder and identified as β-amyrin (Fig. 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.12 (1H, t, J = 3.3, H-12), 3.21 (1H, dd, J = 10.8, 4.4 Hz, H-3), 1.14 (3H, s, H_3-27), 1.07 (3H, s, H_3-28), 0.97 (3H, s, H_3-26), 0.91 (3H, s, H_3-24), 0.87 (3H, s, H_3-29), 0.87 (3H, s, H_3-30), 0.78 (3H, s, H_3-23), 0.73 (3H, s, H_3-25).

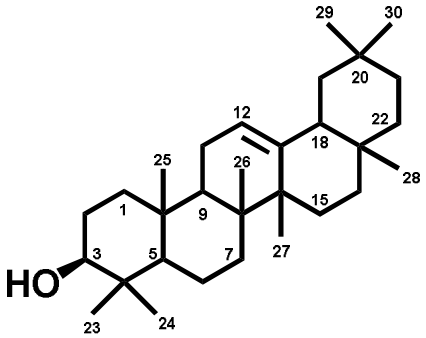

Fig. 1. Purified compound **1** was characterized as β-amyrin.

β-amyrin reduced the acetic acid-induced writhing significantly ($p < 0.05$) (Table 1) as compared to the non-treated mice indicating its peripheral analgesic potential. However, it did not show any significant central analgesic activity in the tail immersion technique (Table 2). β-amyrin (1 mg/ml) also displayed noticeable inhibition of hemolysis indicating it anti-inflammatory potential (Fig. 2). In case of docking of β-amyrin with COX-2, hydrogen bond formation and hydrophobic interactions were revealed (Table 3, Fig. 3). Scores of the *in silico* studies of pharmacokinetics and bioactivity are presented in Table 4. Fatal lethality of β-amyrin was not found.

Table 1. Effect of compound 1 on acetic acid-induced writhing in mice.

Mice (5 /group)	Initial oral treatment		Consequent 0.7% (v/v) acetic acid treatment (i.p.)	Number of writhing	% inhibition
	Material	Dose			
Control	Vehicle	10 ml/kg b.w.	10 ml/kg b.w.	28.00 ± 2.00	-
Standard	Diclofenac sodium	1 mg/kg b.w.	10 ml/kg b.w.	7.33 ± 0.57*	73.82 ± 2.28*
Test	β-amyrin	200 mg/kg b.w.	10 ml/kg b.w.	12.47 ± 0.53*	55.46 ± 1.04*

Values are presented as mean ± Sd (n = 3); Standard and test groups were compared with the vehicle-treated group; *$p < 0.05$; b.w., body weight; i.p., intraperitoneal; vehicle, 1% Tween 80 in normal saline.

C. citrinus has been studied previously to some extent and the leaves were reported to have analgesic potential (Ahmed *et al.* 2015). In the present study, attempts were made to isolate principal analgesic elements from the leaves of *C. citrinus*.

Repeated chromatographic separation of carbon tetrachloride soluble fraction and purification over silica gel led to the isolation of a compound **1**. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of this compound displayed eight three proton singlets at δ 0.73 (H_3-25), 0.78 (H_3-23), 0.87 (H_3-29), 0.87 (H_3-30), 0.91 (H_3-24), 0.97 (H_3-26), 1.07 (H_3-28) and 1.14 (H_3-27). It also showed a triplet at δ 5.12 (J = 3.3 Hz) which could be assigned to the olefinic proton at H-12 of a pentacyclic

triterpene moiety. The appeared double doublet (J = 10.8, 4.4 Hz) at δ 3.21 demonstrated the presence of an oxymethine proton at C-3. These spectral features are in close agreement to those published for β-amyrin (Vazquez *et al.* 2012). On this basis, the identity of compound **1** was confirmed as β-amyrin (Fig. 1).

Table 2. Effect of compound 1 on tail immersion test in mice.

Mice (5 /group)	Treatment	Dose (route)	After 30 min		After 60 min	
			TFLPM	%I	TFLPM	%I
Control	Vehicle	10 ml/kg b.w. (oral)	4.35 ± 0.93	-	3.22 ± 1.56	-
Standard	Morphine	2 mg/kg b.w. (s.c.)	8.90 ± 0.20*	104.59	19.12 ± 1.53*	493.79
Test	β-amyrin	200 mg/kg b.w. (oral)	4.87 ± 0.59	11.95	5.75 ± 0.58	78.57

Values are presented as mean ± Sd (n = 3). Standard and test groups were compared with the vehicle-treated group; *p < 0.05; TFLPM – Tail flick latency per minute; % I - % inhibition; b.w., body weight; s.c., subcutaneous; vehicle, 1% Tween 80 in normal saline.

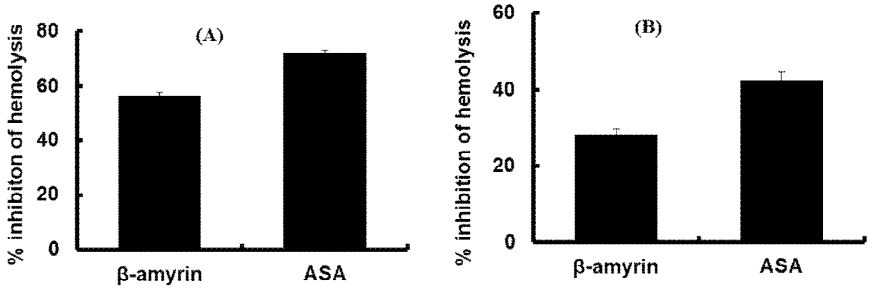

Fig. 2. Effect of compound **1** on hypotonic solution induced (A) and heat induced (B) hemolysis of erythrocyte membrane. ASA, acetylsalicylic acid (standard drug).

Table 3. Grid center, dimension and binding affinity of the docking studies.

Ligand	Protein	Grid center	Dimension	Binding affinity (Kcal/mol)
Celecoxib*	COX-2	34.858, –29.081, –9.11	40, 40, 40	–12.3
Celecoxib	COX-2	34.86, –29.055, –9.175	40, 40, 40	–12.1
β-amyrin	COX-2	34.86, –29.055, –9.175	40, 40, 40	–9.1

*Khan *et al.* 2015 was used for the validation of the present docking experimental method.

Prostaglandins produced by the cyclooxygenase (COX) pathway are responsible for the generation of pain and introducing inflammation (Ricciotti and FitzGerald 2011). In the present study, the purified β-amyrin (**1**) displayed very significant peripheral analgesic action (Table 1). However, β-amyrin mediated central analgesic action was very insignificant (Table 2). From this, it can be assumed that β-amyrin might inhibit the biosynthesis of prostaglandins. The β-amyrin oriented peripheral analgesia of the present study might be fairly supported by the result of another effective analgesic experiment conducted by the administration of an isomeric mixture of α- and β-amyrin in mice model (Aragao *et al.* 2007). More importantly, β-amyrin mediated inhibition of

COX-2 and subsequent down-regulation of inflammatory prostaglandin (e.g. PGE_2) have also been confirmed by an earlier study (Krishnan *et al.* 2014).

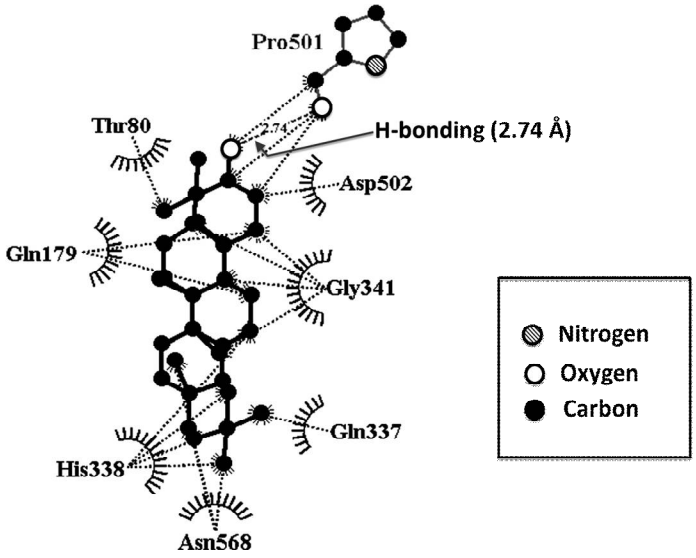

Fig. 3. Ligplot 2D map of interaction between mice COX-2- and β-amyrin. Hydrophobic contacts are represented by arcs with radiating spokes. Atom with dots is the corresponding atom involves in hydrophobic interaction.

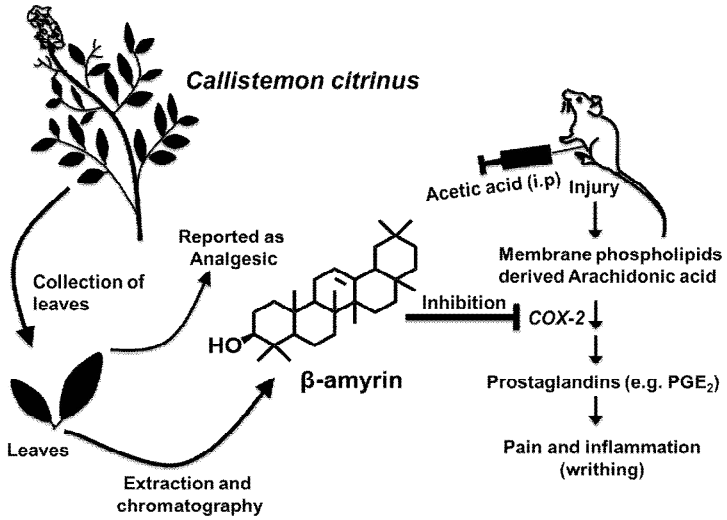

Fig. 4. Summary of the peripheral analgesic action of β-amyrin from *C. citrinus* through inhibition of COX-2. COX, cyclooxygenase; PGE_2, prostaglandin E_2; i.p., intraperitoneal.

Inflammatory stimuli (hypotonic medium, heat, etc.) can trigger the hemolysis of the erythrocyte membrane. In the present study, the β-amyrin was able to inhibit the hemolysis in both the hypotonic solution- and heat-induced assays (Fig. 2) to a noticeable extent, which indicates its potential as an anti-inflammatory agent (Shinde *et al.* 1999). Besides, docking of β-amyrin with

COX-2 showed that they can bind strongly with each other through hydrogen bond formation and hydrophobic interactions (Table 3, Fig. 3). Computational study of α-amyrin, an isomer of β-amyrin, was also performed earlier and a similar trend of inhibiting COX-2 was also noticed displaying the binding energy –8.02 Kcal/mol (Ranjbar *et al.* 2016). In the present study, the binding energy of β-amyrin with COX-2 was found –9.1 Kcal/mol. These outcomes might be related with the potential of β-amyrin to inhibit the production of prostaglandin through COX pathway. The overall β-amyrin mediated analgesic and anti-inflammatory activities demonstrated in the present study can be abridged as Fig. 4.

Table 4. *In silico* analyses of (A) molecular pharmacokinetic and (B) bioactivity (drug-likeness) properties of β-amyrin by Molinspiration server (www.molinspiration.com).

(A) Molecular pharmacokinetic			(B) Bioactivity	
Parameters		Scores	Parameters	Scores
The logarithm of octanol/water partition coefficient		8.02	G-protein coupled receptors ligand	0.22
Total polar surface area		20.23	Ion channel modulator	–0.05
Number of non-hydrogen atoms		31	Kinase inhibitor	–0.31
Hydrogen bond-	acceptors (O and N atoms) number	1	Protease inhibitor	0.11
	donors (OH and NH groups) number	1		
Molecular	weight	426.73	Enzyme inhibitor	0.56
	volume	460.70		
Number of rotatable bonds		0	Nuclear receptor ligand	0.67
Number of rule of 5 violations		1		

Virtual analyses of β–amyrin revealed various aspects of it as a drug candidate. Its pharmacokinetics parameters (Table 4A) comply well with the most of the Lipinski's rules (Lipinski *et al.* 2001) and the bioactivity scores (Table 4B) also support the biochemical potential of this secondary metabolite (Ranjbar *et al.* 2016). Toxicity analysis of β-amyrin did not expose any extreme lethal effect. Taken together, β-amyrin appears as a major analgesic metabolite of the leaves of *C. citrinus*, which might be considered as a promising lead molecule for drug development.

Acknowledgements

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