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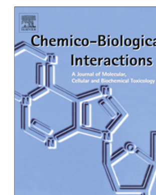

Acute and sub-chronic oral toxicity profiles of lipopeptides from *Bacillus mojavensis* A21 and evaluation of their *in vitro* anticoagulant activity

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ABSTRACT

The aim of the present study was to evaluate the acute and sub-chronic toxicity of lipopeptides mixture produced by *Bacillus mojavensis* A21 as well as their *in vitro* anticoagulant activity. A21 lipopeptides was given to mice at single dose from 75 mg to 1000 mg/kg body weight (bw). The median lethal dose (LD_{50}) of A21 lipopeptides was about 550 mg/kg bw. Sub-chronic toxicity study for 28 days was done by daily oral administration of A21 lipopeptides at doses of 40 and 400 mg/kg bw in rats. Results showed that A21 lipopeptides did not cause any change in body weights and they did not produce any marked alterations in the hematological blood parameters including hematocrit concentration, hemoglobin level, white and red cells count. However, the platelets level decreased significantly compared to control value. Moreover, no significant differences in the serum biochemical characteristics were observed for rats treated by the lowest dose. In contrast, a little enhancement of alanine-aminotransferase (ALT) activity and decrease in total cholesterol were observed with the highest dose. A21 lipopeptides were also found to cause a prolongation of the thrombin time (TT), the prothrombin time (PT) and the activated partial thromboplastin time (APTT). Overall, A21 lipopeptides may be very promising compounds for therapeutic purposes.

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1. Introduction

Thrombosis has been and continues to be a major health problem leading to mortality. Anticoagulants are pivotal for the prevention and treatment of thromboembolic disorders [1]. The most common anticoagulants used today are unfractionated heparin. Although their efficacy remains undisputed, the deleterious life-threatening side effects of these drugs have also been well documented [2,3]. In fact, these agents have hemorrhagic side effects, short half-life in the body, and are also relatively expensive. Therefore, the necessity of discovering alternative sources of anticoagulants has been arisen with interesting demand for safer anticoagulant therapy. Many organisms are important sources of thrombolytic agents. Effective anticoagulant agents have been identified and characterized from animals [4,5], plants [6] and microorganisms [7,8]. In recent years, various anticoagulant agents were successively discovered from different microorganisms, such as bacteria, actinomyces and fungi. Microbial derived anticoagulant agents have emerged as an important class of compounds which are gradually becoming economically competitive with their animal and plant-derived counterparts. Microbial derived anticoagulant agents have attracted worldwide attention as excipients due to their low toxicity, high biodegradability and ecological acceptability.

The genus *Bacillus* is a special group of microorganisms, which have been demonstrated to be excellent producers of bioactive and structurally novel metabolites [9]. Among these molecules, lipopeptides have received a great attention in different fields including phytosanitary sector, medicine, cosmetics, food and feed additives, bioremediation, etc., [10]. These amphiphilic cyclic peptides that are linked to a fatty acid hydrocarbon chain and that belong to the surfactin, iturin and fengycin families are synthesized by non-ribosomal peptide synthetases without involving messenger RNA [11]. Each family is constituted of several variants, which can differ in their fatty acid chain and their peptide moiety [12,13].

Lipopeptides have been reported to display various biological actions, including anti-fungal and anti-bacterial activities [14,15], as well as anti-viral [16] and anti-tumour activities [17,18]. In addition, several reports on whether they inhibited fibrin clot formation platelet cytosolic phospholipase A2 (PLA2) activity [19] and inflammatory mediators, such as nitric oxide (NO) and cytokines have indicated that lipopeptides may be regarded as valuable

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drugs against platelet-mediated cardiovascular diseases. Lim et al. [20] reported that surfactin was able to prevent a platelet aggregation leading to the inhibition of additional fibrin clot formation, and to enhance fibrinolysis with the facilitated diffusion of fibrinolytic agents.

However, a possible toxicity of lipopeptide molecules constitutes a drawback for their medical applications at present. In fact, there are little reports that discuss lipopeptides toxicity. So, one of the requirements for the application of these molecules as therapeutic agents would be the evaluation of their toxicity.

In our previous study we showed that *Bacillus mojavensis* A21 was able to produce lipopeptides belonging to surfactin, pumilacidin and fengycin families. The crude lipopeptides mixture has been reported to display various biological actions, including antifungal and anti-bacterial activities [15]. Regarding their interesting properties, efforts are now being developed in our laboratory to evaluate their toxicity profile. Thus, the aim of the present work was firstly to evaluate the in *vivo* potential toxicity of A21 lipopeptides and then to investigate their *in vitro* anticoagulant activity.

2. Materials and methods

2.1. Bacterial strain and lipopeptides production

The microorganism used in this study was isolated in our laboratory from marine water in Sfax, Tunisia. It was identified as *B. mojavensis* A21 based on its biochemical and physiological characteristics, and on the 16S rRNA gene sequence analysis. It was assigned the accession number EU366229 [21].

The production of lipopeptides by *B. mojavensis* A21 was performed in Landy medium. The fermentation temperature and the initial pH value were 30 °C and 8.0, respectively. The culture was conducted in a rotator shaker at 160 rpm for 72 h. A21 lipopeptides were partially purified by sequencial ultrafiltration/diafiltration. The culture broth was centrifuged 15 min at 13,000g, and the cell-free supernatant was ultrafiltered through a regenerated cellulose 10-kDa membrane (Millipore SA, Molsheim, France) then washed four times by diafiltration. During the four washings, the solution was diafiltered to obtain 10% of the initial retentate volume and fed to the initial volume with distilled water. Then methanol was added to the retentate till the concentration of methanol reached about 70% (v/v), and the solution was filtered through the 10-kDa membrane. The final filtrate was collected, concentrated and lyophilized, resulting in partially purified A21 lipopeptides mixture. The extract was then characterized by high-performance liquid chromatography (HPLC) (Ayed et al. [15]). Results showed that under the above conditions, *B. mojavensis* A21 was able to produce approximately 92 and 248 mg/L surfactin and fengycin, respectively.

2.2. Evaluation of A21 lipopeptides toxicity

2.2.1. Experimental animals

Adults male Wistar rats (weighing 150–160 g) and male Suiss albino mice (weighing 30–40 g) were purchased from SICO (Sfax, Tunisia). Animals were individually housed in clean polyethylene cages under controlled conditions of 22–25 °C, 45–50% relative humidity and 12-h light–dark cycle with free access to food and water. The experimental protocol was approved by the Local Animal Care Committee, and all the experimental procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals.

2.2.2. Median lethal dose (LD_{50}) determination

The LD_{50} of A21 lipopeptides, defined as the dose required to kill half of the test rats, was estimated in mice. Fifty animals were equally divided into five groups of ten animals per group; control group and 4 treated groups. After an overnight fast, the control group received sterile distilled water while each treated group received 75, 400, 600 or 1000 mg/kg bw, administered orally. The number of deaths that occurred within 48 h was recorded. The method of Miller and Tainter [22] was used to determine the LD_{50}.

2.2.3. Sub-chronic toxicity study

Fifteen rats were divided into three groups of 5 animals. The group I served as control and received sterile distilled water daily, while groups II and III were orally administered with A21 lipopeptides at doses of 40 and 400 mg/kg bw, respectively, for 28 days. During the experimental period, animals were weighed once a week. On the 27 day of study, animals were fasted overnight prior to blood collection.

2.2.4. Blood analysis

Heparinized blood was used for hematological study by using an automatic hematology analyzer. The parameters included: red blood cell count (RBC), white blood cell count (WBC), hemoglobin concentration (Hb), platelets (Plt) and hematocrit (Ht). For biochemical analysis, the non-haparinized blood was centrifuged to get serum. The obtained serum was assayed, by using diagnostic kits, for (AST), (ALT), Bilirubin (TBIL), total cholesterol (TC), glucose and urea. After blood collection, animals were sacrificed by decapitation and organs (liver and kidneys) were isolated and weighed.

2.3. In vitro assessment of anticoagulant activity

2.3.1. APTT assay

For the APTT assay, 45 μL of normal citrated platelet poor plasma (PPP) were mixed with 5 μL of A21 lipopeptides at various concentrations and incubated for 3 min at 37 °C. APTT reagent (CK-PREST, DIAGNOSTICA-STAGO) (50 μL) was added and the mixtures were then incubated for 3 min at 37 °C. The clotting time was immediately recorded following the addition of 50 μL of 25 mM $CaCl_2$. The clotting time is expressed in seconds and as a ratio, with the average value of a normal subject less than 1.2. The control clotting time is measured by replacing the 5 μL of lipopeptides with distilled water.

2.3.2. TT assay

For the TT assay, 10 μL of A21 lipopeptides at various concentrations were incubated with 90 μL of PPP for 3 min at 37 °C and clotting time was determined after the addition of 100 μL of thrombin (80 NIH). The TT value is expressed in seconds.

2.3.3. PT assay

For the PT assay, 5 μL of sample solution at various concentrations were incubated with 45 μL of PPP for 3 min at 37 °C and clotting time was determined after the addition of 100 μL of Néoplastine® CI (DIAGNOSTICA-STAGO).

2.4. Statistical analysis

Results were expressed as means ± SD (Standard Deviation) and all measurements were done in triplicate. Statistical analyses were performed with SPSS. V. 5.0 (Professional Edition) using ANOVA analysis. Differences were considered significant at $p < 0.05$.

3. Results and discussion

Lipopeptides represent a unique class of bioactive secondary metabolites with increasing interest in many fields. Owing their environmental acceptability and biodegradability, lipopeptides are generally considered as low or non-toxic products and

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