Phenolic profile and antimicrobial activity of different dietary supplements based on *Cochlospermum angolensis* Welw.

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Running title: Bioactives and antimicrobial activity of Borututu dietary supplements

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Abstract

Three different formulations (infusion, pills and syrup) of *Cochlospermum angolensis* were characterized by HPLC-DAD-ESI/MS regarding phenolic composition, and evaluated by their *in vitro* antimicrobial activity against clinical isolates of multiresistant bacteria. Infusion and pills showed the highest variety of phenolic compounds, with eleven molecules identified. Protocatechuic acid was only present in infusions, being the most abundant compound, while (epi)gallocatechin-*O*-gallate was the main molecule identified in pills and eucaglobulin/globulusin in syrup. Infusion revealed antimicrobial activity against all the studied bacteria with the exception of *Proteus mirabilis* whereas the pills revealed activity in *Escherichia coli* spectrum extended producer of β-lactamases and methicillin-resistant *Staphylococcus aureus*. In the syrup there was no antimicrobial activity detected, which is in agreement with its low concentrations of phenolic compounds. None of the formulations inhibited *P. mirabilis*. Considering the obtained results, *C. angolensis* infusion can be considered a good source of phenolic compounds as well as a good antimicrobial agent.

Keywords: *Cochlospermum angolensis*; Infusion/pills/syrup; Phenolic compounds; Antimicrobial activity; Clinical isolates.

1. Introduction

Plants of the family *Cochlospermaceae* are widely used in Angola by the native healers for the treatment and prevention of liver diseases. *Cochlospermum* is a genus of tropical trees belonging to this family that comprises eleven species occurring in pantropical regions (Poppendieck, 1981). Plants of this genus have been reported by their extensive use in the treatment of malaria, jaundice and liver injuries, including *Cochlospermum regium*, *Cochlospermum tinctorium*, *Cochlospermum planchonii*, and *Cochlospermum angolensis* (Poppendieck, 1981).

The infusion of *C. regium* roots has been used to treat intestinal, ovarian and uterine inflammation, dermatitis (Pio Corrêa, 1975), ulcers and rheumatoid arthritis (Lewis and Hanson, 1991; Ritto, 1996), being also reported the antimicrobial activity of its hydroethanolic extract and fractions against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Solon et al., 2012). The total aqueous extract of *C. tinctorium* demonstrated potent antiplasmodial activity (Traoré et al., 2006) and its rhizomes extract revealed hepatoprotective activity in aflatoxin B1 (Dalvi and Sere, 1988) and carbon tetrachloride ($CC1_4$) treated rats, and in primary rat hepatocyte cultures exposed to cytotoxic concentrations of galactosamine or $CC1_4$ (Diallo and Vanhaelen, 1987). A crude aqueous extract of *C. planchonii* showed the capacity to lower serum bilirubin levels in rats treated with $CC1_4$ and inhibited two cytochrome P-450 enzymes, aminopyrine-N-demethylase and aniline hydroxylase (Aliyu et al., 1995). *Cochlospermum angolense* roots infusion inhibited *Plasmodium falciparum* and depressed the DNA synthesis of mice erythrocytes infected by *Plasmodium berghei* (Presber et al., 1992); its bark infusion, pills, and syrup revealed strong antioxidant activity, with the infusion also demonstrating anti-hepatocellular carcinoma activity, in

previous studies performed by our research group (Pereira et al., 2013; Pereira et al., 2014).

According to some reports, the therapeutic purposes of *Cochlospermum* genus species (mainly hepatoprotective effects) can be explained by the presence of phytochemicals, specially phenolic an polyphenolic compounds, with gallic and ellagic acids and its derivatives as the major compounds (Diallo and Vanhaelen, 1987; Diallo et al., 1992; Solon et al., 2012; Ferreres et al., 2013). In particular, *C. tinctorium* revealed gallic and ellagic acids, ellegitannins and flavonoids (Diallo and Vanhaelen, 1987; Diallo et al., 1992), while *C. regium* showed ellagic and gallic acids, dihydrokaempferol, dihydrokaempferol-3-*O*-β-glucopyranoside, dihydrokaempferol-3-*O*-b-(6''-galloyl)-glucopyranoside, pinoresinol, excelsin and two triacylbenzenes (cochlospermines A and B) (Solon et al., 2012). *Cochlospermum angolensis* hydromethanolic extract revealed high levels of methyl ellagic acid and its derivatives with methyl ellagic acid pentoside isomer as the major compound, and the aqueous extract showed high amounts of ellagic acid and its derivatives (Ferreres et al., 2013).

Nevertheless, scarce studies have been carried out regarding the phenolic composition and antimicrobial activity of different formulations of this plant. In the present work, infusion, pills and syrup of *C. angolensis* were characterized by HPLC-DAD-ESI/MS regarding phenolic composition, and evaluated by their *in vitro* antimicrobial activity against clinical isolates of multiresistant bacteria (*Escherichia coli*, *Escherichia coli* spectrum extended producer of β-lactamases (ESBL), *Proteus mirabilis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*) obtained in the Hospital Center of Trás-os-Montes and Alto Douro (CHTMAD, Chaves, Portugal).

2. Material and methods

2.1. Samples

Cochlospermum angolensis Welw. (borututu) was obtained from an herbalist shop in Bragança (Portugal), as dry material for infusion preparation (bark), pills (containing 500 mg of borututu roots, diluent -microcrystalline cellulose-, anti-agglomerating -dibasic calcium phosphate anhydrous and silicium dioxide-, and lubrificant -magnesium stearate-) and a syrup (containing 10% of borututu roots, acidity regulator -citric acid-, preservatives -potassium sorbate, sodium benzoate and propyl gallate-, and sweeteners -aspartame, sodium cyclamate and saccharin).

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Phenolic standards were from Extrasynthèse (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Methanol was of analytical grade purity from Lab-Scan (Lisbon, Portugal). The culture media Mueller Hinton broth (MHB), Wilkins–Chalgren broth (WCB) and Columbia agar (CA) with 5% horse blood were obtained from Biomerieux (Marcy l'Etoile, France). The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St Louis, MO, USA) to be used as microbial growth indicator.

2.3. Characterization in phenolic compounds

2.3.1. Extraction procedures

The infusion was prepared by adding the sample (1 g) to 200 mL of boiling distilled water, left to stand at room temperature for 5 min, and then filtered under reduced

pressure; afterwards the obtained infusion was frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

The pills (1.5 g) were reduced to powder and submitted to hydromethanolic extraction by stirring with 25 mL of methanol:water (80:20 *v/v*, 25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional 25 mL of methanol:water (80:20 *v/v*) for another hour. The combined extracts were dried (Büchi R-210, Flawil, Switzerland) and purified using a C_{18} SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and the purified samples were further eluted with 5 mL of methanol. The extract was concentrated under vacuum.

The syrup (5 mL) was submitted to a purification following the procedure described above and then concentrated under vacuum.

2.3.2. Analysis of phenolic compounds

The previously described extracts and infusions were dissolved in water:methanol (80:20, *v/v*) and water, respectively and analysed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents were: (A) 0.1% formic acid in water, and (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and

370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500.

The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the

same phenolic group. The results were expressed in μg per g of lyophilized infusion or extract. For each formulation, the analyses were carried out in triplicate.

2.4. Evaluation of antimicrobial activity

2.4.1. Samples preparation

The lyophilized infusion was redissolved in distilled water (final concentration 1000 mg/mL). The pills were reduced to powder and dissolved in distilled water (final concentration 150 mg/mL). The syrup was directly used (100 mg/mL, according with the label information).

2.4.2. Microorganisms and culture media

The microorganisms used to screen the antimicrobial activity were clinical isolates from patients hospitalized in various departments of the Hospital Center of Trás-os-Montes and Alto Douro. Four Gram-negative bacteria (*Escherichia coli* isolated from urine, *Escherichia coli spectrum extended producer of β-lactamases* (ESBL) isolated from blood culture, *Proteus mirabilis* isolated from wound exudates and *Pseudomonas aeruginosa* isolated from urine) and one Gram-positive bacterium (methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from wound exudates) were used. All strains were identified using the MicroScan® panels automated methodology (Siemens, Camberley, UK).

2.4.3. Antimicrobial activity assays

Minimum inhibitory concentration (MIC) determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric

assay following the methodology previously described by the authors (Alves et al., 2012).

Initially, 50 µL of each extract (1000, 150 and 100 mg/mL for the infusion, the pills and the syrup, respectively) was diluted in 450 µL of MHB (final concentration of 100, 15 and 10 mg/mL, respectively) and then, 200 µL of this extract solution was added in each well (96-well microplate). Dilutions were carried out over the wells containing 100 µL of MHB and, afterwards, 10 µL of inoculum (1 $\times 10^8$ cfu/mL) were added to all the wells. Two negative (one with MHB and the other with the extract) and one positive (with MHB and the inoculum) controls were prepared. The plates were incubated at 37 °C, for 24 h, in an oven (Jouan, Berlin, Germany).

The MIC's of the samples were detected after adding INT (0.2 mg/mL, 40 µL) and incubating at 37 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC was defined as the lowest extract concentration that prevented this change and exhibited complete inhibition of bacterial growth. All the assays were carried out in triplicate.

2.5. Statistical analysis

The results are expressed as mean values ± standard deviation (SD). The differences between the total phenolic compounds in different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using SPSS v. 22.0 program.

3. Results and Discussion

3.1. Phenolic compounds

Table 1 presents the data obtained from HPLC-DAD-MS analysis (retention time, λ_{max} in the visible region, mass spectral data) used for the identification and quantification of phenolic compounds in borututu formulations.

Compounds 2 and 8 were positively identified as protocatechuic acid and ellagic acid, respectively, according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Compound 1 showed a fragmentation pattern characteristic of a digalloyl hexoside, with a precursor ion at *m/z* 483 and product ions at *m/z* 313 $[M-H-170]^-$, 331 $[M-H-162]^-$ and 169 $[M-H-162-152]^-$ from the losses of gallic acid and hexosyl and galloyl moieties, respectively. Compound 3 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 453 releasing MS^2 fragment ions at *m/z* 169 and 313 associated to a galloyl moiety and a galloylglucose group, respectively. A compound with similar characteristics was reported in *Rhus coriaria* by Abu-Reidah et al. (2015) and identified as hydroxymethoxyphenyl-galloyl-hexoside, so that this identity was tentatively assigned to our peak. Compound 4 ($[M-H]^-$ at *m/z* 497) also yielded fragment ions at *m/z* 169 and 313, this latter from the loss of 184 mu indicated as characteristic of oleuropeic acid (Hasegawa et al., 2008); these mass features are coherent with the structure of eucaglobulin or globulusin B (**Figure 1**), previously reported in the leaves of *Eucalyptus globulus* (Hasegawa et al., 2008; Boulekbache-Makhlouf et al., 2013).

Compound 5 was assigned as (epi)gallocatechin-*O*-gallate owing to its pseudomolecular ion ($[M-H]^-$ at *m/z* 457) and fragment ions at *m/z* 305 and 169, corresponding to the deprotonated ions of (epi)gallocatechin and gallic acid, respectively.

Compounds 6, 9, 12 and 13 presented similar UV-vis and mass spectra characteristic of ellagic acid derivatives All of them produced a major MS^2 fragment ion at *m/z* 315, from the loss of hexosyl (-162 mu, compound 6), pentosyl (-132 mu, 9 and 12) or

deoxyhexosyl moieties (-146 mu, 13), which can be interpreted as corresponding to deprotonated methyl ellagic acid; the second product ion at *m/z* 300 would derive from the further loss of a methyl group (-15 mu). Compound 7, with a molecular mass 14 mu lower than 9 and 12 and a fragment ion at *m/z* 301 (-132 mu, loss of a pentosyl moiety; ellagic acid) was assigned as ellagic acid pentoside. All these compounds, together with ellagic acid, have been previously reported as majority phenolics in hydromethanolic extracts obtained from the bark of *C. angolensis* (Ferreres et al., 2013).

Compounds 10 and 11 were assigned as flavanone glycosides based on their UV-vis and mass spectra. Compound 10 ($[M-H]^-$ at *m/z* 433 and MS^2 fragment ion $[M-H-162]^-$ at *m/z* 271) was tentatively identified as a naringenin-*O*-hexoside, whereas compound 11 ($[M-H]^-$ at *m/z* 609 and MS^2 fragment at *m/z* 301 $[M-H-308]^-$) could be associated to a hesperetin-*O*-rutinoside or hesperetin-*O*-neohesperidoside. As far as we are aware, these compounds have not been previously reported in *C. angolensis*.

A compound with a pseudomolecular ion $[M-H]^-$ at *m/z* 211 was present in the syrup and corresponded to propyl gallate added to the syrup as a preservative, as described in its label, which also explained its high levels (data not shown).

Ellagic acids, methyl ellagic acids, eucaglobulin/globulusin and (epi)gallocatechin-*O*-gallate were the common compounds found in all the different formulations. The highest concentration of phenolic compounds was found in the infusion extract (**Table 1**). Protocatechuic acid was the most abundant compound although it was only present in infusions; (epi)gallocatechin-*O*-gallate was the main phenolic in pills and eucaglobulin/globulusin in the syrup.

3.2. Antimicrobial activity

The results obtained in the screening of antimicrobial activity of the different formulations of borututu (dietary supplements) against the studied bacteria are shown in **Table 2**. The infusion extract revealed the highest antimicrobial activity, with lower MIC values and proved to be able to inhibit the growth of *E. coli*, *E. coli* ESBL, *S. aureus* and *P. aeruginosa*, with MIC values of 50, 6.2, 1.6 and 25 mg/mL, respectively. The effectiveness of this formulation against *E. coli* and *S. aureus* could be due to the presence of protocatechuic acid that revealed antimicrobial activity against *E. coli* (β-lactamases positive) in a previous study, when isolated from *Ficus ovata* (Kuete et al., 2009). Pills revealed activity against *E. coli* ESBL and *S. aureus* MRSA in concentrations of 15 and 1.9 mg/mL, respectively. Lastly, the syrup did not reveal antimicrobial activity at the studied concentration (10 mg/mL). None of the tested formulations inhibited *P. mirabilis*. As far as we know, there are no reports on the antimicrobial activity of pills or syrups containing *C. angolensis*.

Overall, among the different formulations, the infusion and pills revealed the highest variety of phenolic compounds, with eleven compounds identified, which can possibly explain their higher antimicrobial activity when compared to the syrup. In this latter, eight phenolic compounds were detected, although in low concentration, which may explain its lack of antimicrobial activity. Considering the obtained results, *C. angolensis* infusion can be considered a good source of phenolic compounds and antimicrobial agent.

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in infusion extracts, pills and syrup of *Cochlospermum angolensis* (mean ± SD).

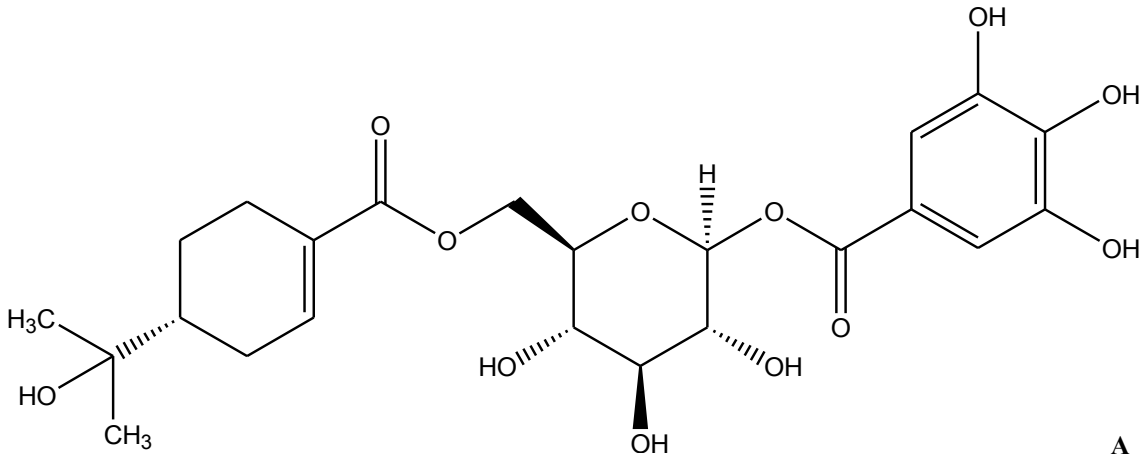
Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)		
						Infusion	Pills	Syrup
1	5.8	276	483	331(41),313(25),169(30)	Digalloyl glucoside	117±6	27±2	nd
2	6.0	259, 296sh	153	109(100)	Protocatechuic acid	1782±40	nd	nd
3	7.8	280	453	313(11),169(8)	Hydroxymethoxyphenyl-galloyl-hexoside	245±4	76±1	nd
4	15.4	282	497	313(31),169(25)	Eucaglobulin/globulusin B	492±51	430±5	381±11
5	17.6	270	457	305(5),169(10)	(Epi)gallocatechin-*O*-gallate	1040±8	777±15	155±15
6	18.7	254, 362sh	477	315(64),300(20)	Methyl ellagic acid hexoside	172±8	72±1	tr
7	19.2	248, 362sh	433	301(100)	Ellagic acid pentoside	50±8	nd	nd
8	20.7	246, 364sh	301	284(10),245(3),185(4),173(5),157(3),145(6)	Ellagic acid	216±17	tr	tr
9	21.8	250, 362sh	447	315(43),300(12)	Methyl ellagic acid pentoside	159±1	55±1	tr
10	22.3	284, 340sh	433	271(100)	Naringenin-*O*-hexoside	nd	653±32	nd
11	22.9	282, 338sh	609	301(100)	Hesperitin-*O*-rutinoside/ hesperetin-*O*-neohesperidoside	nd	111±7	nd
12	24.7	248, 366sh	447	315(54), 300(15)	Methyl ellagic acid pentoside	343±20	181±3	55±4
13	26.9	250, 360sh	461	315(95), 300(41)	Methyl ellagic acid deoxyhexoside	111±7	238±2	tr
					Total phenolic compounds	4726±7[a]	2618±58[b]	590±30[c]

tr-traces; nd-not detected. For total phenolic compounds, different letters mean significant differences ($p<0.05$).

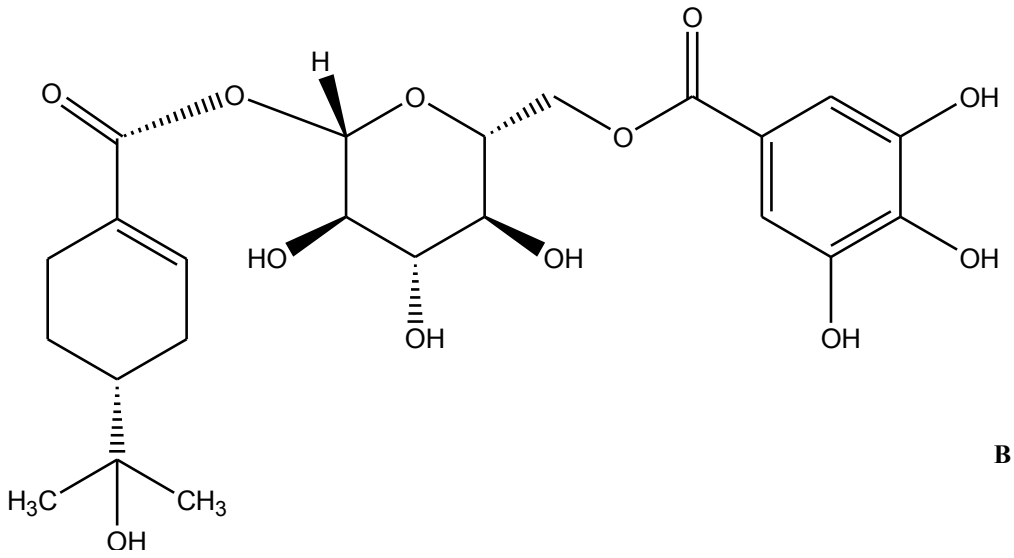
Table 2. Antimicrobial activity of infusion extract, pills and syrup of *Cochlospermum angolensis* against bacteria clinical isolates.

Bacteria	Infusion MIC (mg/mL)	Pills MIC (mg/mL)	Syrup MIC (mg/mL)
Escherichia coli	50	>15	>10
Escherichia coli ESBL	6.2	15	>10
Proteus mirabilis	>100	>15	>10
MRSA	1.6	1.9	>10
Pseudomonas aeruginosa	25	>15	>10

MIC - Minimum inhibitory concentration; ESBL - spectrum extended producer of β-lactamases; MRSA - methicillin-resistant *Staphylococcus aureus*.



A



B

Figure 1. Chemical structures of eucaglobulin (**A**) and globulusin B (**B**).