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Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

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Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

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Tristan, 1993,1995), (Kumasi et al., 2001)
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Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

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ARTICLES

Full Length Research Paper

Polymeric microparticles containing protein prepared using a controllable combination of diffusion and emulsification steps as part of the salting out procedure

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The key to success of proteins as biopharmaceuticals is to have in place an efficient drug delivery system that allows in gaining access to their target sites. A novel procedure of preparing ovoalbumin-loaded Eudragit® S100 microparticles, based on combining salting out and double emulsion-evaporation steps was developed. The ratio of a water miscible solvent (acetone and isopropanol) to a non-water-miscible solvent (chloroform) and salt addition to aqueous phase external were shown to be the primary determinants of size, polydispersity index (PI) and encapsulation efficiency (EE). Once optimized, using an organic phase of 3:0.5:1.5 (acetone:isopropanol:chloroform, v/v/v), further control was exerted using modification of acetone diffusion by alterations in $MgCl_2$ concentration. Diffusion control, using 75% w/w $MgCl_2$ solution, produced microparticles with a mean size of 26.3 µm, a good PI of 0.36 and 56.5 ± 0.5% EE. Electron microscopy showed the particles to be smooth and spherical. Ovoalbumin release studies using different buffers demonstrated immediate release in the buffer at alkaline pH. Calorimetry studies suggested that ovoalbumin existed in the microparticle as a molecular dispersion. Thus, Eudragit® S100 microparticles have great potential as oral carriers for delivery of proteins to the intestines.

Key words: Double emulsion/solvent evaporation, emulsification-diffusion, microparticles, protein encapsulation.

INTRODUCTION

Proteins and peptides exhibit the widest structural and functional variation and are integral to the regulation and maintenance of all biological processes. The greater biochemical and structural complexity of proteins compared compared with conventional drug-based pharmaceuticals makes formulation design for delivery of therapeutic proteins a very challenging and difficult task.

During the last two decades, parenteral drug delivery

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systems based on polymeric microparticles (MP) have extensively been investigated (Liechty et al., 2010). Although the microencapsulation of peptides has led to several commercially available products (Hougaard et al., 2013), the encapsulation of proteins still presents considerably difficulties (Aamir and Ahmad, 2010; Wieber et al., 2010).

Polymeric drug-loaded MP are colloidal systems, typically 1 to 1000 μm in diameter, with a therapeutic payload entrapped, adsorbed or chemically coupled to an orbicular polymer matrix (Licciardi et al., 2012). Since the microencapsulation procedure offers a large number of possibilities for modifications, the success of this technology strongly depends on the adaptation of process variables, including polymer composition and concentration, rates of solvent diffusion, drug loading, phase ratio of emulsion system, emulsion stability, homogenization techniques and temperature (Chaisri et al., 2009; Sartori et al., 2013; Ogawa et al., 1988).

The multiple emulsion evaporation method differs fundamentally from diffusion in that the main phases stay immiscible at all times, being removed later by evaporation. This, biphasic emulsion systems ($W_1/O/W_2$) have been described and used to incorporate water-soluble drugs, such as proteins (Joshi et al., 2013; Xia et al., 2013) and propranolol HCl (Ubrich et al., 2004). However, several difficulties are still encountered when using this technique, such as poor encapsulation efficiency of the drug and high polydispersity index (PI) (Win and Fend, 2005). PI is a unit-less expression for the tightness of the particle size distribution. Theoretically, PI is zero for a monodisperse colloidal suspension, however standard latex particles with a polydispersity index of about 0.05 are practically monodisperse. The values greater than 0.5 indicate a very broad size distribution (Jain et al., 2004). In many respects, the salting out-diffusion procedure, as first introduced by Allemann et al. (1992) and modified by Konan et al. (2002), draws on aspects of the method described earlier. The addition of sufficient electrolyte is used for separating an aqueous phase from an organic phase (usually acetone), the two being fully miscible before any intervention or for at least to minimize such drug partitioning between the two miscible phases. Controlled dilution of the electrolyte content thereafter gives rise to particle formation, as acetone diffuses from the organic droplets.

The aims of the present study were to evaluate the influence of certain physicochemical properties of the aqueous and organic phases used during protein loaded-MP preparation, and their effect on the characteristics of polymeric microparticles produced by the combination of double emulsion solvent diffusion-evaporation and salting out methods, to obtain particles with a size > 20 μm to allow the encapsulation of biological interest proteins. In line with our laboratory internal focus, the release profiles were performed at different conditions, slightly acidic,

neutral and alkaline pH as these are characteristic media for the release of insecticidal proteins.

MATERIALS AND METHODS

Chemicals

Microparticles were prepared from Eudragit® S100 (Helm) and stabilized using poly (vinyl alcohol) (PVA, Mowiol 4-88, Clariant®). Ovoalbumin (OA, lyophilized powder, ≥ 98% agarose gel electrophoresis, Sigma-Aldrich, St. Louis, MO). Magnesium chloride (Merck, Naucalpan de Juarez, Estado de México) was used as the salting out agent. All others reagents, such as acetone, isopropanol and chloroform were of appropriate laboratory grade (analytical grade, M TEDIA high purity solvents) and used without further purification.

Microparticles preparation and purification

Ovoalbumin (OA) was identified through the determination of ultra violet (UV) absorption maximum (Genesys 10UV scanning, *Thermo scientific,* Madison Wisconsin). Bradford based method was used for determination of OA, using protein estimation. The ovoalbumin-Eudragit® S100 microparticles (OA-MP) were prepared using controllable combination of diffusion and emulsification steps as part of the salting out procedure. Briefly, the inner aqueous phase (IAP) with 0.3 ml aqueous solution of OA (0.33%, w/v) was added to a 100 mg of Eudragit® S100 previously dissolved in 5 ml of an organic phase (OP), comprising different volume ratios of acetone (AC), chloroform (CH), and isopropanol (ISP) (Deepti et al., 2004: Liu et al., 2010). The IAP was then emulsified with the OP by mechanical stirring (EUROSTAR power-b, IKA® WERKE), for an optimized time period. The primary emulsion (W/O) was then immediately emulsified with 13 ml external aqueous phase (EAP) of PVA (optimized percentage (w/w) in MgCl2 aqueous solution) by mechanical stirring (EUROSTAR power-b, IKA® WERKE) to an optimized speed, on an ice bath. Later, the organic solvent was evaporated under reduced pressure, in order to obtain an aqueous suspension of OA-MP. OA-MP were then separated from the bulk aqueous phase by centrifugation at 1,630 g (rotor F1202, Beckman Coulter Allegra 64R Centrifuge, Fullerton, California) for 10 min at 4°C. After subsequent washing and centrifuged with cold distilled water, the pellet was lyophilized (LYPH-LOCK 6, Labconco, Kansas city, Missouri). Optimization encapsulation efficiency was determined along with the measurement of particle size, PI and particle shape of different formulations.

First, EAP volume and $MgCl_2$ $6H_2O$ concentration was modified in order to determine the effect on size. Further, variations in the organic phase composition where the ratio volume of AC:ISP:CH was 1:0.5:3.5; 2:0.5:2.5 and 3:0.5:1.5, in one-part incremental steps, were used. Further, stirring speed was needed to be optimized in order to obtain a sufficiently high yield of microparticles, with a desired size distribution and acceptable encapsulation efficiency. The stirring speeds used were 100, 300 and 500 rpm. The microparticles were produced at different PVA concentrations of 1, 2 and 3% (w/w) in order to determine the effect of PVA concentration on size and encapsulation efficiency.

Particle size measurement

The size and shape of OA-MP were estimated using optical microscopy (Micromaster® Fisher Scientific, with the software:

Table 1. Mathematical equations for the models used to describe release characteristics of OA from MP

Model	Equation
Zero order kinetics	$Q_t = Q_0 + K_0 t$
First order kinetics	$Q_t = \log Q_0 + K_0 t$
Higuchi	$Q_t = Q_0 + K_H t^{1/2}$
Korsmeyer-Peppas	$Q_t = K_{MB} t^n e^{(-et)}$

Table 2. Initial conditions of polymeric microparticles formulation

Parameter	Conditions
Volume ratio of ACT:ISP:CH(mL)	2.0: 0.5: 3.5
Concentration of PVA in EAP (% w/w)	3.0
Concentration of $MgCl_2$ in EAP(% w/w)	15-75
Volume of EAP(mL)	10

Westover Digital MCD Model 2300). Formulation and dried microparticles re-dispersed in water were spread into a glass slide. Measurements of 100 consecutive particles were observed to microscope, were carried out at 25°C, each sizing determination done in triplicate and an average particle size (intensity average) expressed as the mean diameter ± standard deviation (SD). The PI was calculated by dividing standard deviation with average particle size (Jain et al., 2004). For external morphology, the OA-MP was coated with gold, using sputter coater, and the sample was randomly analyzed using a scanning electron microscope (SEM) (LEO-435VP, Cambridge, UK). The effect on particle size before and after drying was evaluated only by the optimized formulation.

Estimation of encapsulation efficiency

OA- MP were dissolved in AC and centrifuged to remove the supernatant because the acetone led to the OA precipitation. Then, the pellet was suspended in carbonate buffer 50 mM at pH 10.3 and analyzed using UV absorption spectroscopy at 583 nm, using appropriate calibration and blanking procedures with the Bradford method. Measurements were done in triplicate; the encapsulation efficiency was calculated using the ratio of the mass of protein determined analytically to the theoretical protein loading. Encapsulation efficiency was calculated as:

$$\text{Encapsulation efficiency } (\%) = \frac{\text{Actual protein loaded (mg)}}{\text{Theoretical protein loading (mg)}}$$

"In-vitro" release

The in vitro release profile of OA-MP was determined. Aliquots of 0.5 ml of the formulations were suspended separately in volumetric flasks containing dissolution media (pH 5.0, 7.4, or 10.3; acetate, phosphate or carbonate buffer 50 mM, respectively). Volumes were made up to 2 ml with each buffer and the flasks were incubated at 25°C under 100 cycles min^{-1} shaking. At various time intervals, flasks were removed, one at a time, and the contents were centrifuged at 33,250 g (rotor F1202, Allegra® 64R) for 25 min at

4°C. The supernatants were then used to determine the released OA by the Bradford method (n = 3; mean ± standard deviation). To analyze the in vitro release data, various kinetic models were used to describe the release kinetics. The zero order rate describes the systems where the drug release rate is independent of its concentration (Hadjiioanou et al., 1993). The first order describes the release rate is concentration dependent (Bourne, 2002). Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

Table 1 shows the equations, where Qt is the amount of drug released in time, Q0 is the initial amount of the drug in table. The following equations were made: cumulative (%) protein release versus time (Zero order kinetic model); log cumulative of protein (%) release versus square root of time (Higuchi model) and log cumulative protein (%) release versus log time (Korsmeyer model) (Korsmeyer et al, 2010). Korsmeyer peppas et al. derived a simple relationship which described drug release from polymeric system (Peppas, 1983). To find out the mechanism of drug release, first 60% drug release data was fitted in Korsmeyer model (Table 1).

Then, the data of the dissolution profiles were summarized numerically and 95% confidence intervals for the differences between pHs in the mean dissolution profiles at each dissolution time point were evaluated using statistical package for social sciences (SPSS) Statistics 17.0 program.

Differential scanning calorimetry

The thermal characteristics of OA powder, Eudragit® S100, physical mixture of OA and Eudragit® S100 and OA-MP were determined using a differential scanning calorimeter (DSC-40, NETZSCH). Samples were crimped in a standard aluminum pan and heated from 40 to 330°C, at a heating rate of 10°C per minute, under constant purging of nitrogen at 30 ml min^{-1}.

RESULTS

Particle size measurement and determination of encapsulation efficiency

The effect of formulation variables on size, IP and encapsulation efficiency of OA-MP prepared by controllable combination of diffusion and emulsification steps as part of the salting out procedure was determined. Table 2 shows the initial conditions of OA-MP. EAP used was normal MgCl2 aqueous solution, to prevent the diffusion of any solvent to the aqueous phase. Different amounts of $MgCl_2$ $6H_2O$ (15, 35 and 75, w/w) increments were studied (Figure 1), while keeping the organic phase composition constant (AC: ISP: CH, 2.0:0.5:2.5, v/v/v). Therefore, the formulation with 75% w/w $MgCl_2$ $6H_2O$ was chosen to evaluate tensio-active concentration effect because it showed a PI of 0.38, indicating a less dispersion in particle size. Figure 2 shows the effect of PVA concentration on the particle size. It was observed that when PVA concentration was reduced, the particle size also decreased. Once determined that the $MgCl_2$ $6H_2O$ at 75% (w/w) and 3% (w/w) PVA are required to allow the formation of the MP, the influence of the composition of OP was assessed in the incorporation of the model protein through increases in the proportion of

Table 3. Effect of organic phase composition on mean particle size, polydispersity index and OA encapsulation. OA-MP were prepared using 3 % w/w PVA and 75% w/w MgCl$_2$ 6H$_2$O, using 300 rpm of mechanical stirring at and 10 mL of EAP.

Volume ratio of (AC:ISP:CH mL)	Particle size (µm ± SD)	Polydispersity Index	Aggregation	% EE
1.0:0.5:3.5	18.24 ± 11.3	0.62	+	14.1 ± 0.6
2.0:0.5:2.5	16.2 ± 6.1	0.38	--	32 ± 2.7
3.0:0.5:1.5	9.7 ± 3.4	0.35	--	19.3 ± 1.6

+ presence, -- without

Table 4. Effect of volume EAP on mean particles size, polydispersity index and OA encapsulation. OA-MP were prepared using 3% (w/w) PVA and 75% (w/w) MgCl$_2$ 6H$_2$O, using mechanical stirring at 300 rpm and the organic volume ratio of 3:0.5:1.5. (AC:ISP:CH).

Volume EAP (mL)	Particle size (µm ± ds)	Polydispersity Index	Aggregation	% E E
7	3.0 ± 1.2	0.38	---	15.45 ± 1.2
10	9.7 ± 3.4	0.35	---	19.3 ± 1.6
13	10.7 ± 3.8	0.35	---	57.5 ± 8.9

+ presence, -- without.

Table 5. Effect of mechanical stirring speed on mean particles size, polydispersity index and OA entrapment. MP were prepared using 13 mL of 1% (w/w) of PVA to 75 % (w/w) MgCl$_2$ 6H$_2$O in EAP and an organic volume ratio of AC:ISP:CH (3.0:0.5:1.5).

Stirring (rpm)	Particle size (µm)			Polydispersity Index	% EE
	Mean	Moda	Median		
100	8.60 ± 5.2	7.8	7.9	0.61*	9.3 ± 1.0
300	26.3 ± 9.7	22.8	25.5	0.36	56.5 ± 0.5
500	7.9 ± 3.9	4.6	7.1	0.49*	4.9 ± 0.3

* Polymer aggregation.

acetone. The results in Figure 3 show that MP properties, such as PI, can be improved by alterations in this diffusion rate once the solvent ratio in the organic phase has been optimized, as done in Table 3.

Variations in the ratio of solvents in OP were shown to have effect on the observed OA encapsulation efficiency, which was greater than 13 and less than 35%. The results from this part of the work indicated that a ratio of 3:0.5:1.5 (AC:ISP:CH) emulsified in 10 ml 3% (w/w) PVA and 75% (w/w) MgCl$_2$ 6H$_2$O of EAP with 300 rpm of mechanical stirring produced MP with a mean particle size of 9.7 ± 3.4 µm, PI of 0.35 and the encapsulation efficiency was 19.3 ± 1.6%. Although this formulation presents low encapsulation efficiency percentage, no presence of aggregates was shown (Table 3 and Figure 3). The volume of EAP was increased from 7 to 13 ml, the encapsulation efficiency and particle size also increased (Table 4). With the decreasing on PVA concentration from 3 to 1% (w/w), the surface tension increased and it was found that the particle size also increased (from 10 to 26 microns), whereas the encapsulation efficiency remained around 56% (Figure 4 and Table 5) with a speed of 300 rpm.

Clearly, some measure of control is needed to improve the particle size distribution, probably due to affording more speed for the mechanical stirring process to produce a narrower size distribution of emulsified droplets, as observed in Table 5.

Morphology of polymeric microparticles

The external morphology and shape of the optimized formulation were studied by scanning electron microscopy (SEM) (Figure 5), which revealed fairly spherical OA-MP with a smooth surface. A student t-test was made to analyze the influence of drying on particle size of an optimized formulation. The drying process has no effect on the morphology and size of OA-MP (n = 3; p < 0.05). It is clear that the shape of OA-MP is obtained and can be assumed to be spherical with smooth surfaces (Figure 5).

In vitro release profile

The *in vitro* release profile of OA-MP was performed to

Table 6. Mathematical Model used to describe the drug release

pH buffer	Zero order kinetics	First order kinetics	Higuchi model	Korsmeyer-Peppas model		Type of transport
		Regression co-efficient (R^2)			n	
5.0	0.807	0.715	0.886	0.897	0.703	Anomalous (non-Fickian)
7.4	0.720	0.639	0.742	0.987	0.682	Anomalous (non-Fickian)
10.3	0.835	0.833	0.841	0.814	0.038	----
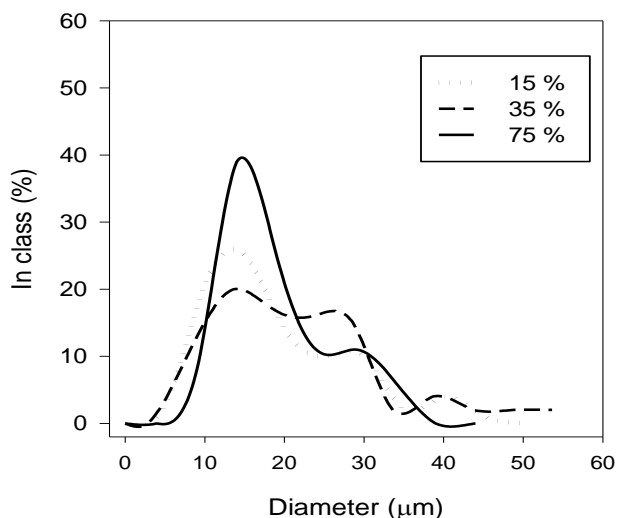
Figure 1. Effect on microparticle size distribution profiles by MgCl$_2$ 6H$_2$O in EPA.
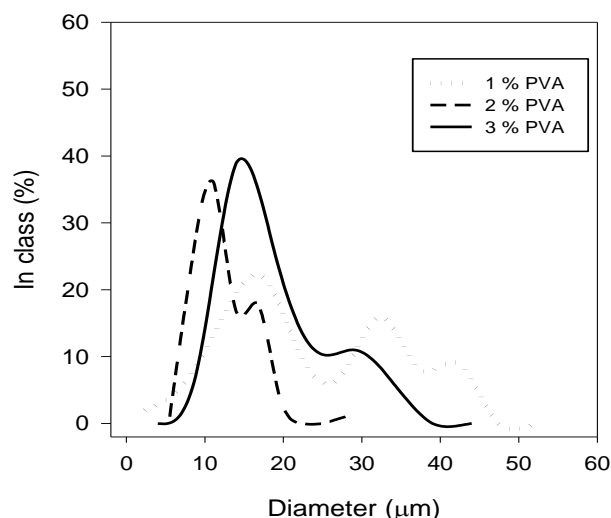
Figure 2. Effect on microparticle size distribution profiles by changes in PVA concentration of EPA.

determine the effect of pH on the release of the protein. Figure 6 shows that the formulation released about 95% of the protein model after 10 min in the dissolution medium having pH of 10.3. In contrast, at pH 5, it showed a gradual release, exhibiting 21% of release at 10 min. The kinetic analysis of the *in vitro* release data of OA from the prepared Eudragit® S100 MP are presented in Table 6. According to the determination coefficients (R2), the *in vitro* release data were in favor of Korsmeyer Peppas model (pH 7). Fast kinetic release of OA from MP at alkaline pH was followed by optical microscopy which showed the dissolution of the particles (Figure 7). Finally, we performed a statistical analysis to compare the releases at different values between pH 5.0 and 10.3.

Analysis of variance (ANOVA) showed that there was a significant difference (p-value < 0.05), thus confirming that the release depends on the environment in which the MP are exposed.

Differential scanning calorimeter (DSC) analysis

The DSC technique is one of the most convenient methods for investigating the compatibility of polymer
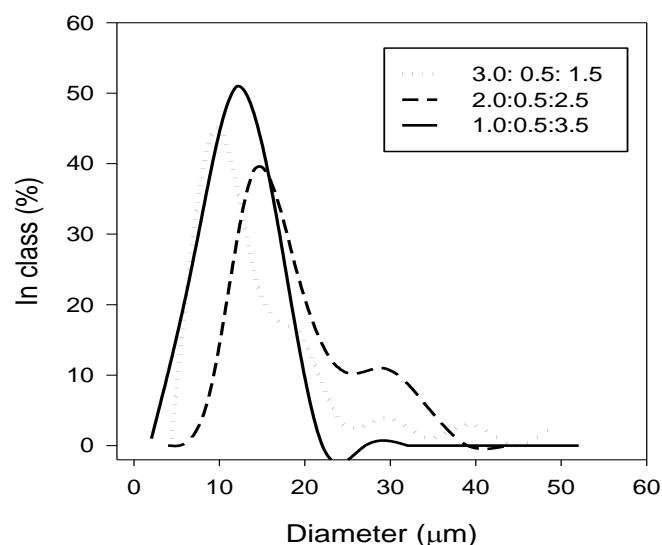
Figure 3. Effect on microparticle size distribution profiles by changes in organic phase composition (AC:ISP:CH).

blends; therefore it was used to evaluate thermodynamic compatibility between ovoalbumin and Eudragit® S100 in
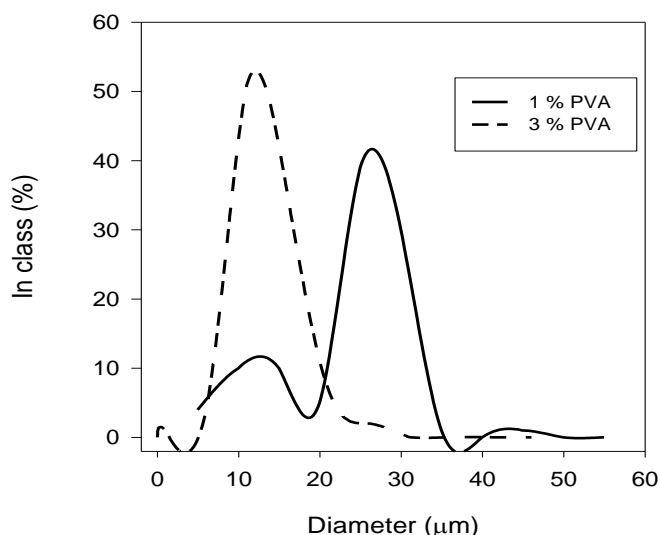
Figure 4. Effect on particle size by PVA. MP were prepared using 13 mL to 75 % w/w MgCl$_2$ 6H$_2$O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.
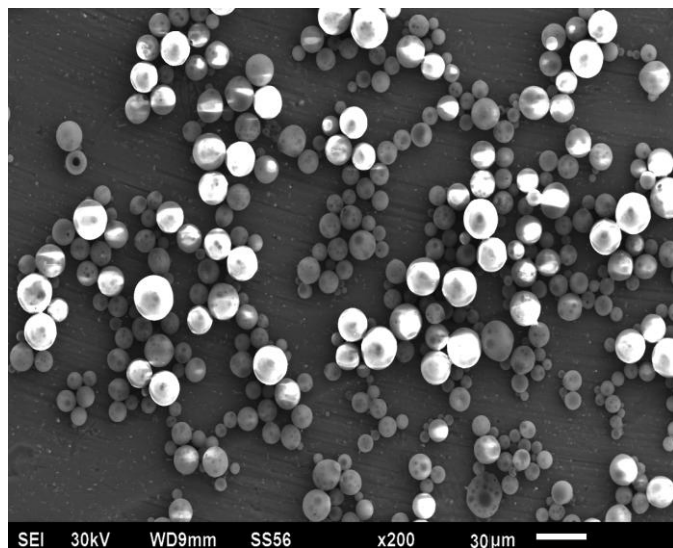
Figure 5. SEM of ovoalbumin-loaded MP. MP were prepared using 13 mL of 1 % w/w PVA and 75 % w/w MgCl2 6H2O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

the OA-MP, based on crystalline melting temperature and the glass transition temperature. Figure 8 depicts the obtained DSC thermograms. In the Eudragit® S100; an endothermic peak at 217.9°C was observed as a melting point. The raw material OA showed a sharp endothermic peak at 92.5°C that corresponded to its melting point, indicating its crystalline nature. A physical mixture of the protein with polymer resulted in the decrease in such a
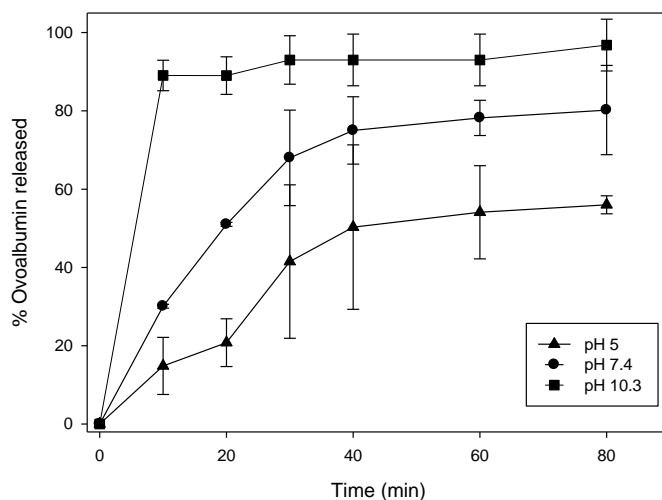
Figure 6. Ovoalbumin release profiles. Error bars show mean + SD; n=3. MP were prepared using 13 mL of 1 % w/w PVA to 75 % w/ MgCl$_2$ 6H$_2$O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

fusion peak.

DISCUSSION

Particle size measurement and determination of encapsulation efficiency

The effect of formulation variables on size, IP and encapsulation efficiency of OA-MP prepared by controllable combination of diffusion and emulsification steps as part of the salting out procedure was determined. Maintaining some diffusion control is advantageous as at all concentrations of MgCl$_2$ 6H$_2$O, low polydispersity is evident with 75% (w/w) MgCl$_2$ 6H$_2$O (Figure 1). With no modulating control over acetone diffusion (15 and 35% w/w MgCl$_2$ 6H$_2$O) the polydispersity was found to be excessive (> 0.5) because there were observed particles with sizes > 200 microns (data not shown). Thus, the rate of acetone diffusion is controlled primarily by the concentration of MgCl$_2$ 6H$_2$O On the other hand, the size of the droplets was dependent mainly on the amount of PVA, mechanical energy used to form the emulsion, and the volume of EAP. PVA is a popular stabilizer for the production of polymeric MP (Vandervoort and Ludwig, 2002), whereby its hydrocarbon regions adsorb strongly on the surface of the MP via hydrophobic bonding with Eudragit® S100. AC diffusion from nascent particles has been shown to precipitate PVA and in turn, acts as an effective stabilizing agent around the droplet and restricting aggregation (Murakami et al., 1997). At a concentration of 1% (w/w), particles < 1 micron were obtained (data not
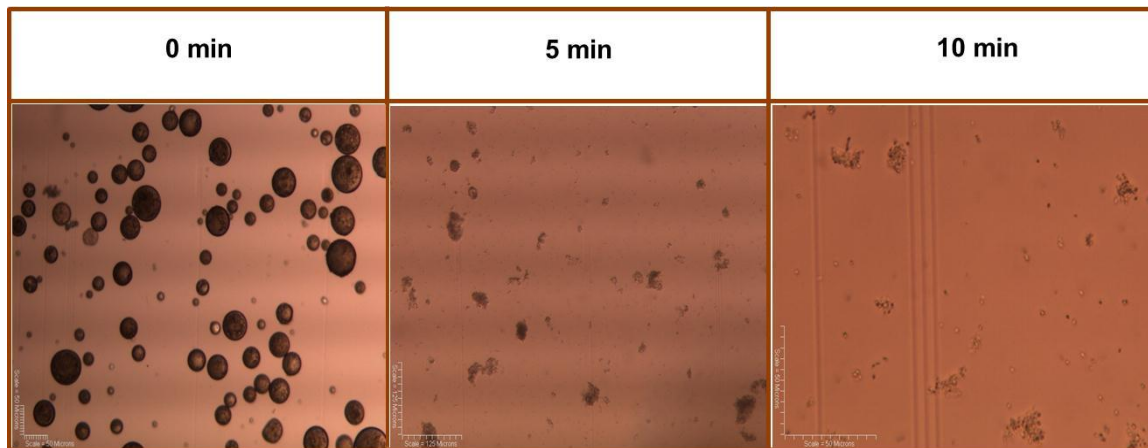
Figure 7. Optical microscopy of ovoalbumin-loaded MP in pH 10.3, at 0.0, 5.0 and 10.0 minutes of exposition.
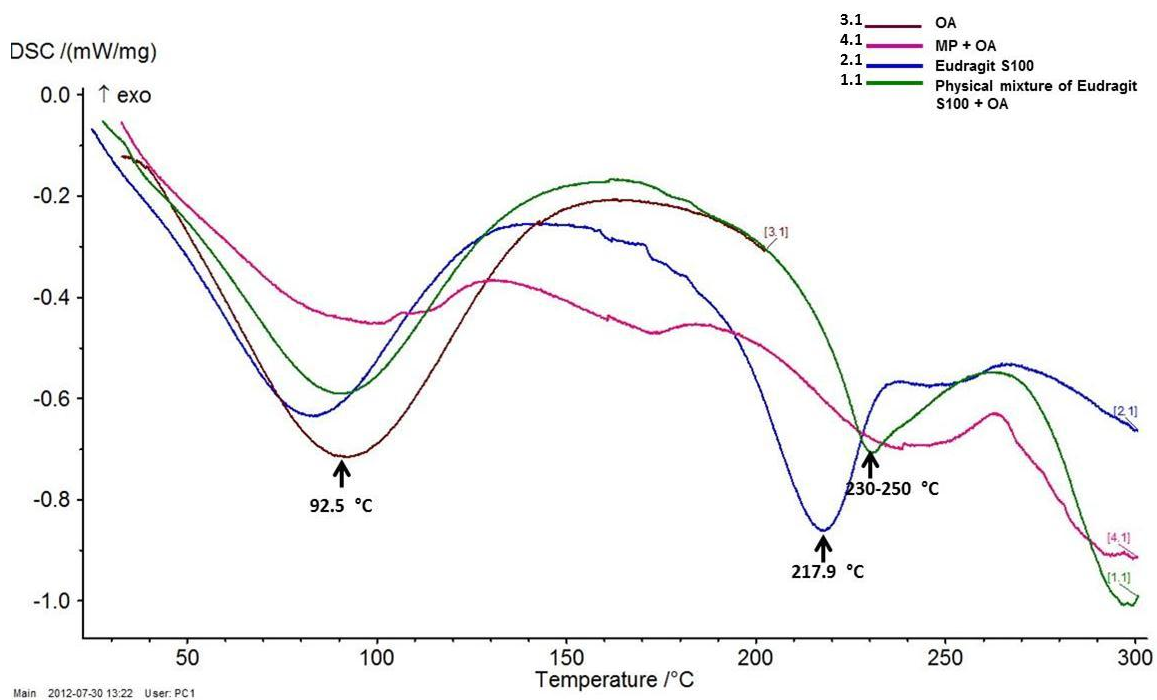
Figure 8. Differential scanning calorimetry for OA-loaded Eudragit® S100 microoparticles, OA pure, Eudragit® S100 pure and physical mixture of OA and Eudragit® S100. MP were prepared using 13 mL of 1 % w/w PVA to 75 % w/w MgCl2 6H2O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

shown), and a polymer aggregates possibly because the interfacial tension resulted in low globules formation, because the PVA concentration was insufficient to stabilize the globules. The formulation of PVA 3% (w/w) presented a size around 16.2 ± 6.1 microns with no aggregates (with a PI of 0.38), allowing globules stability. This result is based on the fact that the acetone fraction was able to diffuse to EAP which is instrumental in forming a shell around the quasi-MP during the former part of the particle assembly.

In Table 3 and Figure 3, it can be observed that as the AC ratio increases in the organic phase, the particle size decreased probably due to the solvent mixture having a low interface tension with the aqueous phase, allowing more stable globules. As Eudragit® S100 is solubilized by the solvent mixture, the ratio of the three solvents was

critical. Therefore, removing a relatively small volume of AC precipitates the polymer (Eudragit® S100) and a coacervate phase may not actually be formed. This fast precipitation impacts the morphology and size of the capsules produced (Atkin et al., 2004; Murakami et al., 1997). It could be mentioned that the organic phase composition influenced the encapsulation efficiency, but this effect was minimal on the PI. Despite low percentage encapsulation efficiency, formulation with the best PI (OP composed by AC: ISP: CH (3.0:0.5:1.5, v/v/v)) from Table 3 was chosen to evaluate the influence of EAP volume on particle size and encapsulation efficiency.

With the decrease on volume of EAP, the EE and particle size also increased. Less effective stirring (300 rpm) and accumulation of internal phase globules generated an emulsion with larger globules and increased the particle size (Zolfaghanan and Mohammadpour, 2009). Furthermore, it was observed that the effect on particle size by EAP volume would not yield to obtain particles > 20 microns (Table 4). However, a formulation was obtained with a PI and acceptable encapsulation efficiency percent at a volume of 13 mL of EAP (0.35 and 57.5 ± 8.9, respectively). Due system behaved as an emulsion, in order to increase particle size it was decided to decrease the concentration of PVA.

Table 5 shows that preparation at a high speed (500 rpm) had submicron-sized particles and polymer aggregates (data not shown), possibly because tensoactive concentration was not enough to prevent coalescence among yielded globules, giving origin to a polymer film. On the otherhand, low speed stirring (100 rpm) yielded higher polymer aggregates and less particle production because the energy provided during emulsification was not enough to promote the formation of a disperse system (Makame et al., 2005). This can be sustained by the polydispersity values, which showed t- value of ≥ 0.4, indicating heterogeneity in particle size or the presence of polymer aggregates. The PI and encapsulation efficiency were acceptable for the formulation produced at a speed of 300 rpm. A lack of diffusion control gives rise to a bimodal distribution with particle sizes around 15 µm (Boury et al, 1995; Shakesheff et al., 1997).

Manu Sharma et al. (2011) encapsulated papain in Eudragit® S100 (and other polymers) microparticles using a W_1/O/W_2 emulsion solvent evaporation and observed an encapsulation efficiency of 82.35%. In the present experiment, the best encapsulation efficiency of OA was 56.5%. The higher drug loading observed in the study of Manu Sharma et al. (2011) appears to be due to the use of a higher polymer: protein ratio compared with the present study (100: 3.5 against 100:10, respectively).

In vitro release profile and mechanism of drug release

The release may be due to dissolution and diffusion,

because the polymer (Eudragit® S100) is pH-dependent (dissolves at pH > 7.0). It could be demonstrated that the alkaline medium promotes the almost immediate release of OA. Furthermore the isoelectric point for OA (4.7) contributes too, because as the polymer, OA exists in anionic form at the alkaline pH (West et al., 1974; Palmieri et al., 2000; Bykov et al., 2000). To investigate the kinetics and mechanism of drug release, the release data were fitted to zero order, first order, Higuchi and Korsmeyer-Peppas models. The 'n' value that can be obtained from the slope of a plot of log Mt/M∞ versus log time (Korsmeyer-Peppas model) is indicative of drug release mechanism. If n is 0.45 or less, the release mechanism follows Fickian diffusion, higher values (0.43 < n < 0.85) for mass transfer follow a non-Fickian model (anomalous transport), where release is controlled by a combination of diffusion and polymer relaxation. When n reaches a value of 0.85 or above, the mechanism of drug release is regarded as case-II transport or super case-II transport which means the drug release rate does not change over time and involves polymer relaxation and chain disentanglement (Cox et al., 1999; Harland et al., 1988).

The values of n were > 0.43 and < 0.85, indicating non Fickian (anomalous) transport for the investigated formulation in these pH. Thus, it was proposed that these formulations delivered their active ingredient by coupled diffusion and relaxation. Whereas the kinetic release of OA from MP at alkaline pH was not fitted to mathematical models used and fast release was supported by optical microscopy showing the dissolution of the particles at less than 10 min of exposure (Figure 7).

DSC analysis

OA crystals still exist in physical mixture (Bharate et al., 2010; Pignatello et al., 2001). This type of interaction was previously observed in the physical mixture of piroxicam with Eudragit® S100 and diflunisal with Eudragit RL 100 (Pignatello et al., 2001; Maghsoodi et al., 2010). However, for microparticles, the intensity of melting peak of OA decreases considerably. The reduction of the protein endothermic peak in the microparticles suggested that OA might be imbedded into Eudragit® S100 and existed in an amorphous state in the microparticles, indicating a thermodynamic compatibility between OA and Eudragit® S100 (Hu et al., 2011). In this way, the intermolecular interaction between protein molecules is favored.

Conclusion

Ovoalbumin was successfully encapsulated in enteric microparticles by controllable combination of diffusion and emulsification steps as part of the salting out procedure, optimizing the various formulation parameters

in order to attain maximum encapsulation efficiency and a spherical shape, with an almost monodispersed particle size distribution and an optimum *in vitro* release profile. The release profiles for the formulations allowed pH-dependent release of ovoalbumin in alkaline pH.

Eudragit® S100 particles disintegrated and showed complete release of ovoalbumin in the pH environment of the ileum intestine within few minutes. Thus, Eudragit® S100 particles have great potential as oral carriers for delivery of proteins to the small and large intestines to facilitate their digestion.

Abbreviations

MP: Polymeric microparticles, **PI:** polydispersity index, **PVA:** poly(vinyl-alcohol), **OA:** ovoalbumin, **IAP:** inner aqueous phase, **AC:** acetone, **OP:** organic phase, **CH:** chloroform, **EPA:** external aqueous phase, **ISP:** isopropanol, **SEM:** scanning electronic microscopy, **EE:** entrapment efficiency.

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Full Length Research Paper

Screening of antibacterial activity of 20 Chinese herbal medicines in Yunnan

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The 80% ethanol extracts from 20 Chinese herbal medicines collected in Yunnan Province, China, were screened for antibacterial activity against *Staphylococcus aureus* (SA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA), including antifugal activity against *Candida albicans* (CA) by the agar hole diffusion test. The extracts were prepared through macerating at room temperature and hot refluxing procedures. 19 of the 20 extracts showed effects with different potency and their inhibition zone diameters (IZD) ranged from 9 to 27 mm. The extracts from *Psychotria henryi* Levl and *Marsdenia tinctoria* R. Br. (maceration and reflux extraction) were the most active against both SA and MRSA, with mean IZDs = 19.7 to 22.0 mm. The anti-SA and MRSA active extracts were further determined for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by serial dilution with a standard broth microdilution method. 12 extracts showed effects on both SA and MRSA strains with MICs/MBCs ranges among 32 to ≥ 1024 / 64 to ≥ 1024 mg/L, respectively. *P. henryi*, *M. tinctoria* and *Sapium baccatum* Roxb also showed inhibition against EC, PA and CA strains, and displayed a broad spectrum of antimicrobial activities.

Key words: Chinese herbal, methicillin-resistant *Staphylococcus aureus* (MRSA), minimum inhibitory concentrations (MIC).

INTRODUCTION

Since methicillin-resistant *Staphylococcus aureus* (MRSA) occurred in early 1961 (Jevons, 1961), it has become a troublesome pathogen responsible for serious infections owing to its resistance to a large group of antibacterial agents, resulting in high morbidity and mortality. It is resistant not only to β-lactam antibiotics, but also to other antibacterial groups, such as fluoroquinolones, tetracyclines, macrolides, lincosamides and aminoglycosides. Some strains that are partially or fully resistant to vancomycin have been found (Pantosti and Venditti, 2009). The search for novel drugs to combat MRSA infection is urgently needed.

Traditional Chinese medicine (TCM) has accounted for a wealth of health needs over thousands of years in China (The Compiler Group, 2009). A great number of plant species have been used as healing herbs in TCM for the treatment of infectious diseases, such as skin ulcers, cold, diarrhea, pneumonitis and tuberculosis, etc (Jiangsu New Medical College, 1977; Sima et al., 2012; Jinous and Fereshteh, 2012; Muhammad et al., 2012). There is increasin interest in researching Chinese herbal drugs for application as antibacterial agents (Zuo et al.,

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Table 1. The antibacterial agent-susceptibility testing results of MRSA strains.

MRSA Strain	Resistant (R)	Intermediate (I)	Susceptible (S)
MRSA092	PEN,OXS,AMP,AZM,CFZ,CAT,FUR,LEV	None	VAN,GAT,PIP/T,FOS,LNZ
MRSA098	PEN,OXS,CFP/SU,LEV,CLI,AZM,	GM	VAN,FOS,LNZ
MRSA111	PEN,OXS,FUR, LEV,ERY,PIP/T, CTX V	None	VAN,LNZ

AMP: ampicillin; AZM: azithromycin; CAT: cefathiamidine; CFP/SU: cefoperazone /sulbactam; CFZ: cefazolin; CLI: clindamycin; CTX: Cefotaxime; ERY: erythromycin; FOS: fosfomycin; FUR: cefuroxime; GAT: gatifloxacin; GM: gentamicin; LEV: levofloxacin; LNZ: linezolid; OXS: oxacillin; PEN: Penicillin; PIP/T: piperacillin/tazobactam; CFP/SU: cefoperazone /sulbactam; VAN: Vancomycin.

2008). For the sake of finding more anti-MRSA plants, we herein report the screening of 20 southern Yunnan medicinal plants for *in vitro* inhibition against clinical MRSA isolates, including their effects on pathogens of *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

MATERIALS AND METHODS

Bacterial strains

Strains of *S. aureus* (SA, ATCC25923), *E. coli* (EC, ATCC25922), *P. aeruginosa* (PA, ATCC27853), *C. albicans* (CA, ATCCY0109) and antibiotic susceptibility disks were provided by the National Institute of Control of Pharmaceutical and Biological products (NICPBP, Beijing, China). MRSA strains were clinical isolates from infectious samples of critically ill patients in Kunming General Hospital (KGH) (Table 1). Pathogen purification and identification (including colonial morphology, gram staining and coagulase testing) were conducted in our clinical microbiology laboratory and further confirmed by standard cefoxitin disk diffusion test following Clinical and Laboratory Standards Institute (CLSI, 2007) standard procedures. The tested MRSA strains were multi-drug resistant to β-lactams, aminoglycosides, fluoroquinolones and macrolides, and they were susceptible to vancomycin (Table 2).

Plants

The selected plants were collected in Xishuangbanna of Yunnan Province in August, 2011. They were identified at the Botany Department, Kunming Institute of Botany (KIB), the Chinese Academy of Sciences. The voucher specimens were preserved at the herbarium of KIB (Table 2).

Media used

Standard Mueller-Hinton agar and broth (MH-A and MH-B), and Sabouraud agar and broth (S-A and S-B) (Tianhe Microbial Agents Co., Hangzhou, China) were used as the bacterial and fungal culture media, respectively.

Extract preparation

Two types of 80% ethanol extracts were prepared from the 20

Southern Yunnan plants by the macerating extraction at room temperature (25°C) and the hot reflux extraction (85°C), respectively as the follows: An amount (50 g, each) of powdered air-dried aerial parts of the plant material was macerated with 80% ethanol (150 ml) for one week, filtered and the residue was further macerated twice with the same amount of solvent overnight and filtered after sonication for 30 min. The filtrates were combined and the solvent was evaporated at 40°C under reduced pressure to afford each of the plant extracts. Another amount (50 g, each) of the same plant material was refluxed with 80% ethanol for two hours, filtered and the residue was further refluxed twice with the same amount of solvent and filtered. The filtrates were combined and the solvent was evaporated at 40°C in vacuum to afford each of the plant extracts (Table 3).

Susceptibility test

The ethanol extracts of the 20 plants were initially subjected to susceptibility test according to the agar diffusion method on MH-A (for the bacteria) or S-A (for *C. albicans*) plates (Zuo et al., 2008). Briefly, crude extracts were prepared at concentrations of 50 mg/ml in dimethylsulfoxide (DMSO). The microbial suspension was firstly plated onto the agar plates with inoculums of 1.5×10^8 CFU/ml for the bacteria and 5×10^5 CFU/ml for *C. albicans*. Then, holes of 6.0 mm diameter each in the agar plates were punched with a sterilized hole-puncher. The samples were pipetted into the holes with no overflowing. The plates were incubated at 35°C for 24 h and measured and the inhibition zone diameters (IZDs) recorded. For every experiment, a sterility check (DMSO and medium), negative control (DMSO, medium and inoculums) and positive antibacterial control (vancomycin) were included (Table 3). All experiments were performed in triplicate.

The samples of IZDs > 12 mm against standard *S. aureus* (SA) were subjected to assay of their IZDs against MRSA (Table 4). The subsequent samples with IZDs > 12 mm against one of the MRSA strain (MRSA111) were further subjected to assay of their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for MRSA by standardized broth microdilution techniques (Zuo et al., 2008). Briefly, the starting inoculums were 5×10^5 CFU/ml. The 96-well plates were incubated at 35°C for 24 h according to CLSI guidelines (CLSI, 2006, 2007). All experiments were determined in duplicate, with concentrations ranging up to 2048 mg/L. For the MBC assays, 0.1 ml aliquots from drug dilution wells with no visual growth inhibition were plated onto MH-A. The lowest drug concentration that yielded three or fewer microorganism colonies was recorded as the MBC NCCLS, 1999). Vancomycin (Eli Lilly Japan K.K., Seishin Laboratories) was also used as a positive control anti-MRSA agent (Table 5).

Table 2. Species of the 20 medicinal plants.

No	Species	Family	Specimen number	Folk use
1	*Aphanamixis grandifolia* Bl.	Meliaceae	HITBC7568	Rheumatism and analgesia
2	*Blumea densiflora* DC.	Compositae	KUN3413	Rheumatism and analgesia
3	*Cinnamomum bejolghota* (Buch.-Ham.) Sweet	Lauraceae	KUN48	Ulcer, dysmenorrheal, rheumatism and ostalgia, injuries from falls
4	*Citrus medica* L.	Rutaceae	HITBC5184	Dyspepsia, cough
5	*Croton tiglium* L.	Euphorbiaceae	KUN51	Expelling phlegm, anthelminthic
6	*Duranta repens* L.	Verbenaceae	KUN662	Malaria, subduing swelling and detoxicating
7	*Fructus piperis* Nigri	Piperaceae	KUN5478	Dissolving phlegm and detoxicating, dyspepsia, diarrhea, epigastralgia
8	*Hedyotis capitellata* Wall. ex G. Don	Rubiaceae	KUN1217	Cold and rheumatism, malaria, fractures
9	*Heracleum candicans* Wall. ex DC	Umbelliferae	KUN12451	Asthma, cough and cold, chronic bronchitis, stomach ache, rheumatalgia, injury from fall
10	*Illicium simonsii* Maxim.	Magnoliaceae	IBSC125	Ulcer,anthelminthic
11	*Lignum santali* Albi	Santalaceae	HITBC5636	pneumonitis, cough and asthma, nausea and vomiting, ecchymoma, paralysis
12	*Marsdenia tinctoria* R. Br.	Asclepiadaceae	KUN722	Epigastralgia, hepatomegaly, rheumatism and osteodynia
13	*Millettia pachycarpa* Benth.	Leguminosae	YFS1005	Injury from fall, fracture, acute gastroenteritis
14	*Nerium indicum* Mill.	Apocynaceae	HITBC383	Cardiotonic and dieresis, expelling phlegm, analgesia, removing blood stasis, epilepsy
15	*Psychotria henryi* Levl	Rubiaceae	KUN6642	Strengthening spleen and dehumidify, regulating vital energy and analgesia
16	*Radix curcumae* Wenyujin	Zingiberaceae	KUN753	Menoxenia, jaundice, hemuresis, epilepsy
17	*Rumulus uncariae* Macrophyllae cum Uncis	Rubiaceae	KUN14506	Headache, serious dysentery, convulsion and hypertension
18	*Sapium baccatum* Roxb	Euphorbiaceae	KUN270	Menoxenia, dyspepsia, pa-leng
19	*Scutellaria discolor* Wall. *ex* Benth.	*Lamiaceae*	KUN688	Tympanitis,diminish inflammation, rheum, fever, tuberculosis, injury from fall
20	*Toona ciliata* Roem.var.pubescens (Franch.) Hand.-Mazz.	Meliaceae	PE53414	Tinea, chronic diarrhea, chronic dysentery, uterine bleeding

RESULTS

The antibacterial agent susceptibility spectrum of the tested three MRSA strains is listed in Table 1. They were used for anti-MRSA evaluation. The 20 Yunnan medicinal plants are shown in Table 2, together with their plant families, specimen numbers and folk uses. The initial antimicrobial screening results (IZDs) of 50 mg/ml ethanol extracts of the 20 plant extracts against standard SA, that is, methicillin-susceptible *Staphylococcus aureus* (MSSA, ATCC25923) and other standard strains of EC, PA and CA are shown in Table 3. 19 of the 20 extracts showed effects with different potency and their IZDs ranged from 9 to 27 mm.

Table 3. Antimicrobial activities of 20 plant ethanol extracts (IZD unit: mm).

Species	Extraction yield (%)	SA	EC	PA	CA
A. grandifolia (macerating extraction)	6.68	0	0	0	0
A. grandifolia (reflux extraction)	6.53	9	0	0	0
B. densiflora (macerating extraction)	5.51	0	0	0	0
B. densiflora DC. (reflux extraction)	3.02	0	0	0	0
C. bejolghota (macerating extraction)	6.20	0	0	0	20
C. bejolghota (reflux extraction)	7.68	16	0	0	10
C. medica (macerating extraction)	9.95	0	0	0	0
C. medica (reflux extraction)	41.42	0	0	0	0
C. tiglium (macerating extraction)	4.31	12	0	0	0
C. tiglium (reflux extraction)	9.18	13	0	0	14
D. repens (macerating extraction)	7.00	11	0	0	0
D. repens (reflux extraction)	15.51	13	9	0	11
DMSO	0	0	0	0	0
F. piperis (eflux extraction)	21.90	0	0	0	0
F. piperis (*macerating extraction*)	7.39	0	0	0	0
H. candicans (macerating extraction)	5.94	0	0	0	0
H. candicans (reflux extraction)	21.45	9	0	0	12
H. capitellata (macerating extraction)	3.02	0	0	0	0
H. capitellata (reflux extraction)	8.75	0	0	0	0
I. simonsii (macerating extraction)	5.76	15	0	0	0
I. simonsii (reflux extraction)	10.14	10	0	0	16
L. santali (macerating extraction)	9.53	0	9	0	0
L. santali (reflux extraction)	16.91	0	0	0	13
M. pachycarpa (macerating extraction)	22.17	0	0	0	0
M. pachycarpa (reflux extraction)	40.97	0	0	0	0
M. tinctoria (macerating extraction)	7.94	22	9	0	20
M. tinctoria (reflux extraction)	14.47	22	0	0	11
N. indicum (macerating extraction)	8.75	0	0	0	12
N. indicum (reflux extraction)	29.29	0	0	0	0
P. henryi (macerating extraction)	7.23	23	0	0	15
P. henryi (reflux extraction)	4.88	27	12	0	0
R. curcumae (macerating extraction)	1.75	0	0	0	0
R. curcumae (reflux extraction)	9.25	0	0	0	16
R. uncariae (macerating extraction)	7.82	13	0	0	0
R. uncariae (reflux extraction)	23.33	16	0	0	0
S. baccatum (macerating extraction)	4.62	20	0	0	13
S. baccatum (reflux extraction)	16.95	16	10	0	11
S. discolor (macerating extraction*)	16.05	0	0	0	0
S. discolor (reflux extraction)	9.03	15	0	0	0
T. ciliata Roem. var. pubes (reflux extraction)	18.92	0	0	0	0
T. ciliata Roem.var.pubescens (Franch.) Hand.-Mazz. (macerating extraction)	3.99	12	0	0	0

SA: *Staphylococcus aureus* (ATCC25923); EC: *Escherichia coli* (ATCC25922); PA: *Pseudomonas aeruginosa* (ATCC27853); CA: *Candida albicans* (ATCCY0109).

The anti-MRSA activities of the selected 17 extracts which were active with IZDs ≥ 10 mm against MSSA were shown in Table 4. The extracts from *Psychotria henryi* Levl and *Marsdenia tinctoria* R. Br. (maceration and reflux extraction) were the most active against both SA and MRSA, with mean IZDs = 19.7 to 22.0 mm (Table 4).

Table 4. The testing results of the 17 extracts with IZD ≥ 10mm against SA and MRSA strains (unit: mm).

Species	SA	MRSA92	MRSA98	MRSA111	Mean ± SEM (MRSA, n=3)
P. henryi (reflux extraction)	27	25	24	17	22.0±2.5
M. tinctoria (macerating extraction)	22	20	23	21	21.3±0.9
M. tinctoria (reflux extraction)	22	21	19	22	20.7±0.9
P. henryi (macerating extraction)	23	21	17	21	19.7±1.3
S. baccatum (macerating extraction)	20	19	16	18	17.7±0.9
S. baccatum (reflux extraction)	16	16	24	14	18.0±3.1
I. simonsii (macerating extraction)	15	17	22	14	17.7±2.3
R. uncariae (reflux extraction)	16	18	19	14	17.0±1.5
C. bejolghota (reflux extraction)	16	19	15	15	16.3±1.3
I. simonsii (reflux extraction)	10	16	16	15	15.7±0.3
S. discolor (reflux extraction)	15	15	17	12	14.7±1.5
C. tiglium (reflux extraction)	13	16	17	0	11.0±5.5
D. repens (macerating extraction)	11	10	10	13	11.0±1.0
D. repens (reflux extraction)	13	14	15	0	9.7±4.8
C. tiglium (macerating extraction)	12	0	0	12	4.0±4.0
R. Uncariae (macerating extraction)	13	0	0	10	3.3±3.3
T. ciliata (macerating extraction)	12	0	0	0	0.0±0.0
DMSO	0	0	0	0	0.0±0.0

SA: *Staphylococcus aureus* (ATCC25923); MRSA: methicillin-resistant *Staphylococcus aureus*.

The anti-SA and MRSA active extracts were further determined for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by serial dilution with a standard broth microdilution method. 12 extracts showed effects on both SA and MRSA strains with MICs/MBCs ranges between among 32 to ≥ 1024 / 64 to ≥ 1024 mg/L, respectively. *P. henryi*, *M. tinctoria* and *Sapium baccatum* Roxb also showed inhibition against EC, PA and CA strains, and displayed a broad spectrum of antimicrobial activities.

DISCUSSION

The screening results revealed that most of the plant extracts were much more active against Gram positive pathogen (SA) than those of Gram negative pathogens (EC and PA), which is a common phenomenon of plant antimicrobial property (Tegos et al., 2002). None of the plants showed strong inhibition against PA (Table 3). The 4 plant extracts from *D. repens* (reflux extraction), *M. tinctoria* (macerating extraction), *S. baccatum* (reflux extraction) and *P. henryi* (reflux extraction) showed IZDs of 9 to 12 mm against standard EC. There were 15 plant extracts active against standard CA and the IZDs were in the ranges of 10 to 20 mm. Some plants showed a wide spectrum of antimicrobial activity and were in agreement with their folk uses (Table 3). However, several extracts showed no antimicrobial activity at 50 mg/ml which were not consistent with their folk uses for infectious diseases. It might be due to the antimicrobial potency which was much lower in this study and the different criterion for the antimicrobial judgment. We also noted the inconsistent values between IZD and MIC of an extract; this might be due to the different diffusion capacity of the active constituents in the culture media of agar and broth.

It was found that different extraction methods produced different yields of active extracts. Meanwhile, the higher temperature of extraction may cause the active ingredient change, hence the antimicrobial activity decreases (Tables 3 to 5).

In this study, the 80% ethanol extracts of 20 plant materials were screened for their antimicrobial activities for the first time. We found that *P. henryi* was active against not only Gram-positive bacteria but also Gram-negative bacteria. The chemical composition and pharmacological effects of *P. henryi* has not been reported yet, but there were reports (Muhammad et al., 2003; Kerber et al., 2001) about the same genus, that is, *P. sychotria brachyceras* and *P. sychotria klugii* which contained indole alkaloids. So, it is worthy to further investigate the anti-MRSA components. Our study suggested that *M. tinctoria* showed promising activity against MSSA and MRSA. The plant contained steroidal components such as

Table 5. MICs and MBCs of the 12 extracts with IZD ≥ 10mm against SA, MRSA and PA strains (unit: mg /L).

Strain	Parameter	PA	ATCC 25923	MRSA 092	MRSA 098	MRSA 111
P. henryi (reflux extraction)	MIC	1024	32	64	128	32
	MBC	n.a.	64	128	512	64
S. baccatum (macerating extraction)	MIC	n.a.	32	512	128	32
	MBC	n.a.	128	1024	512	128
R. Uncariae (reflux extraction)	MIC	n.a.	64	64	512	1024
	MBC	n.a.	256	256	1024	2048
S. baccatum (reflux extraction)	MIC	2048	64	512	256	64
	MBC	n.a.	128	1024	512	256
P. henryi (macerating extraction)	MIC	2048	128	128	512	512
	MBC	n.a.	1024	1024	2048	2048
M. tinctoria (macerating extraction)	MIC	n.a	128	128	1024	64
	MBC	1024	512	512	2048	512
M. tinctoria (reflux extraction)	MIC	n.a.	256	256	1024	64
	MBC	1024	1024	1024	2048	512
I. simonsii (macerating extraction)	MIC	n.a.	512	256	256	512
	MBC	1024	1024	512	512	1024
I. simonsii (reflux extraction)	MIC	n.a.	1024	256	512	1024
	MBC	n.a	2048	512	1024	2048
D. repens L. (macerating extraction)	MIC	n.a.	1024	1024	1024	512
	MBC	n.a	2048	2048	2048	2048
C. bejolghota (reflux extraction)	MIC	n.a.	1024	1024	512	1024
	MBC	n.a	2048	2048	2048	2048
S. discolor (reflux extraction)	MIC	n.a	1024	512	256	512
	MBC	n.a.	2048	2048	2048	2048
Vancomycin	MIC	0.244	0.244	0.488	0.244	0.488
	MBC	0.488	0.488	0.976	0.976	0.976

SA: *Staphylococcus aureus* (ATCC25923); MRSA: methicillin-resistant *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa* (ATCC27853); n.a.: not active at the concentration up to 2048 mg/L.

tinctoralactone and marsdenone (Chowdhury et al., 1993) and showed antifertility activity (Chowdhury et al., 1994). But its anti-MRSA effect has not been noted previously. Further research for the corresponding active components from these plants and their systematic anti-MRSA properties are been carried out.

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Full Length Research Paper

Activity exerted by a benzamide derivative on injury by ischemia/reperfusion in an isolated heart model

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Several studies indicate that some benzamide-derivatives have activity at cardiovascular level; nevertheless, there is scarce information about the effects exerted by the benzamide derivatives on cardiac injury caused by ischemia/reperfusion (I/R). In this experimental study, a new benzamide-derivative was synthetized with the objective of evaluating its activity on I/R in an I/R model of rat heart using the Langendorff technique. In addition, molecular mechanism involved in the effect induced by the benzamide derivative on perfusion pressure and coronary resistance was evaluated by measuring left ventricular pressure in the absence or presence of following compounds; nifedipine, indomethacin, propranolol and metoprolol. The results showed that the benzamide-derivative reduces infarct size compared with control. Other results showed that the benzamide derivative significantly increase the perfusion pressure and coronary resistance in isolated heart. Other data indicate that the benzamide-derivative increase left ventricular pressure in a dose-dependent manner (0.001 to 100 nM); however, this phenomenon was significantly inhibited by propranolol and metoprolol at a dose of 1 nM (p=0.05). In conclusion, these data suggest that cardioprotective activity of the benzamide-derivative is by stimulating catecholamine production and consequently induce changes in the left ventricular pressure levels. This phenomenon results in decrease of myocardial necrosis after ischemia and reperfusion.

Key words: Heart, benzamide derivative, ischemia, propranolol, metoprolol.

INTRODUCTION

Myocardial infarction is a major cause of death and disability worldwide (Yusuf et al., 2005; Thygesen et al., 2007); this cardiovascular disease is due to cell death of cardiac myocytes caused by ischaemia, which is the result of a perfusion imbalance between supply and demand. In addition, acute myocardial infarction can produce alterations in the topography of both the infarcted and noninfarcted regions of the ventricle (Pfeffer, 1995). There are some reports which indicate that the most

effective method of limiting necrosis is the restoration of blood flow; however, the effects of reperfusion itself may also be associated with tissue injury (Klone et al., 1989). In this sense, there are studies which show that some drugs reduce myocardial necrosis in rabbits after ischemia and reperfusion (Hale et al., 1996). For example, astudy showed that rosiglitazone reduced myocardial infarction and improved contractile dysfunction caused by ischemia/reperfusion injury; nevertheless, the

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cardioprotective effect of rosiglitazone was most likely due to inhibition of the inflammatory response (Yue et al., 2001). Other data indicate that methylene blue can decrease injury by ischemia/reperfusion by reduction of molecular oxygen (Salaris et al., 1991). Also, other report showed that injury by ischemia/reperfusion is reduced with Levosidan via K_{ATP} Channels (Toit et al., 1999).

On the other hand, a report indicate that some benzamide derivatives also can exert effect on injury by ischemia/reperfusion; for example, a study showed that the compound N-(3,5-Bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamidecan decreasethe ischemia-reperfusion injury by inhibition of nuclear translocation of factor-kappa B (Onai et al., 2004). In addition, there are data which indicate that 3-aminobenzamide exert significant protective effects in myocardial reperfusion injury via activation of poly (ADP-ribose) synthetase which plays a role in the pathophysiology of acute myocardial infarction (Zingarelli et al., 1997). All these data show that several benzamide derivatives exert effects on the cardiovascular system; nevertheless, they do not show clearly the cellular site and actual molecular mechanisms of these compounds; therefore, data are needed for characterizing the activity induced by benzamide derivatives on ischemia-reperfusion injury. To test this aspect, the present experimental study was designed to investigate the effects induced by a benzamide derivative in a myocardial ischaemia/reperfusion model using Langendorff technique. In addition, it was thought desirable to evaluate the molecular mechanism involved in the activity of the benzamide derivative on left ventricular pressure, using some pharmacological tools for blocking various biological systems such as nifedipine [calcium channel antagonist] (Henry, 1980), indomethacin [prostanglandin synthesis blocker] (Owen et al., 1975), propranolol [$β_1$ receptor blocker] (Sklar et al., 1982), metoprolol [selective $β_1$ receptor antagonist] (Bengtsson et al., 1975).

MATERIALS AND METHODS

Chemical synthesis

Compound 1 (1-[(2-Amino-ethylamino)-phenyl-methyl]-naphthalen-2-ol) was prepared according to a previously reported method (Figueroa-Valverde et al., 2013). The other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting point for the danazol derivative was determined on an Electrothermal (900 model). Infrared spectra (IR) were recorded using KBr pellets on a Perkin Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in $CDCl_3$ (deuterated chloform) using tetramethylsilane (TMS) as internal standard. Electron impact mass spectroscopy (EIMS) spectra were obtained with a Finnigan Trace Gas Chromatography Polaris Q Spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of 2,4-dinitro-N-(2-{[(1E)-phenylmethylene]amino}ethyl)benzamide (compound 3)

A solution of compound 1 (100 mg, 0.29 mmol), benzaldehyde (50 mg, 0.29 mmol) and boric acid in 10 ml of methanol was stirred for 24 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, the residue was washed 3 times with water. Then, the precipitate was separated and dried at room temperature.

Biological method

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of University Autonomous of Campeche (No. PI-420/12) and were in accordance with the guide for the care and use of laboratory animals (Bayne, 1996). Male Wistar rats weighing 200 to 250 g were obtained from University Autonomous of Campeche.

Reagents

All drugs were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v).

Experimental design

Briefly, male rat (200 to 250 g) was anesthetized by injecting them with pentobarbitalat a dose rate of 50 mg/kg body weight. Then, the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice cold physiologic saline solution. The heart was trimmed of non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. The perfusion medium was the Krebs-Henseleit solution (pH = 7.4, 37°C) composed of (mmol); 117.8 NaCl, 6 KCl, 1.75 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 24.2 $NaHCO_3$, 5 glucose and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5/5%). The coronary flow was adjusted with a variable speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

Perfusion pressure

Evaluation of measurements of perfusion pressure changes induced by drugs administration in this study were assessed using a pressure transducer connected to the chamber where the hearts were mounted and the results entered into a computerized data capture system (Biopac).

Inotropic activity

Contractile function was assessed by measuring left ventricular developed pressure (LV/dP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium (Figueroa-Valverde et al., 2011a). The latex balloon was bound to cannula which was linked to pressure transducer that was connected with the MP100 data acquisition system.

First stage

Ischemia/Reperfusion model: After of 15 min equilibration time, the hearts were subjected to ischemia for 30 min by turning off the perfusion system (Booth et al., 2005). After this period, the system was restarted and the hearts were reperfused 30 min with Krebs-Henseleit solution. The hearts were randomly divided into 2 major

treatment groups with n = 9: Group I, hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution); Group II, hearts were subjected to ischemia/reperfusion and treated with benzamide derivative (0.001 nM) before ischemia period (for 10 min) and during the entire period of reperfusion. At the end of each experiment, the perfusion pump was stopped, and 0.5 ml of fluorescein solution (0.10%) was injected slowly through a side armport connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded.The areas of the normal left ventricle non risk region, area at risk, and infarct region were determined using the technique reported by Boot et al. (2005). Total area at risk was expressed as the percentage of the left ventricle.

Second stage

Effect induced by the benzamide derivative on perfusion pressure: Changes in perfusion pressure as a consequence of increases in time (3 to 18 min) in absence (control) or presence of the benzamide derivative at a concentration of 0.001 nM were determined. The effects were obtained in isolated hearts perfused at a constant-flow rate of 10 ml/min.

Evaluation of effects exerted by the benzamide derivative on coronaryresistance: The coronary resistance in absence (control) or presence of the benzamide derivative at a concentration of 0.001 nM was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. Since a constant flow was used, changes in coronary pressure reflected the changes in coronary resistance.

Third stage

Effects of the benzamide derivative on left ventricular pressure through the calcium channel: Intra coronary boluses (50 µl) of the benzamide derivative [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine at a concentration of 1 nM (duration of the pre-incubation with nifedipine was for aperiod of 10 min).

Effect exerted by the benzamide derivative on leftventricular pressure in the presence of indomethacin: The boluses (50 µl) of the danazol derivative [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The bolus injection administered was done in the point of cannulation. The dose response curve (control) was repeated in the presence of indomethacin at a concentration of 1 nM (duration of the pre-incubation with indomethacin was for a period of 10 min).

Effects induced by the benzamide derivative on left ventricular pressure through β₁- adrenergic receptor: Intracoronary boluses (50 µl) of the benzamide derivative (0.001 to 100 nM) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of propranolol or metoprolol at a concentration of 1 nM (duration of preincubation with propanolol or metoprolol was by a 10 min equilibration period).

Statistical analysis

The obtained values are expressed as average ± standard error (SE), using each heart (n = 9) as its own control. The data obtained were put under analysis of variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 program (Hocht et al., 1999). The differences were considered significant when p was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The yield of the reaction product (compound 3, Figure 1) was 70% with melting point of 306 to 308°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) at 3320, 1638 and 1350. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR and^{13}C NMR for the benzamide derivative are shown in Tables 1. Finally, the results of mass spectroscopy (MS) (70 eV) was shown as m/z 342.02. Additionally, the elementary analysis data for the benzamide derivative ($C_{16}H_{14}N_4O_5$) were calculated (C, 56.14; H, 4.12; N, 16.37; O, 23.37) and found (C, 56.10; H, 4.09).

### Biological activity

First stage

Effect of benzamide derivative on ischemia/reperfusion injury: The results (Figures 2 and 3) showed that the benzamide derivative reduces infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (control).

Second stage

In this study, the activity induced by the benzamide derivative on perfusion pressure and coronary resistance in the isolated rat hearts was evaluated. The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to18 min) in absence (control) or in presence of benzamide derivative (Figure 4), showed that benzamide derivative [1 nM] significantly increase the perfusion pressure (p=0.05) in comparison with the control conditions [1 nM]. In addition, another result (Figure 5) showed that coronary resistance, calculated as the ratio of perfusion pressure at coronary flow assayed (10 ml/min) was higher in the presence of the benzamide derivative in comparison with the control conditions (p=0.05) at a concentration of 1 nM.

Third stage

Other results showed that activity exerted by the benzamide derivative [0.001 to 100 nM] increased the left
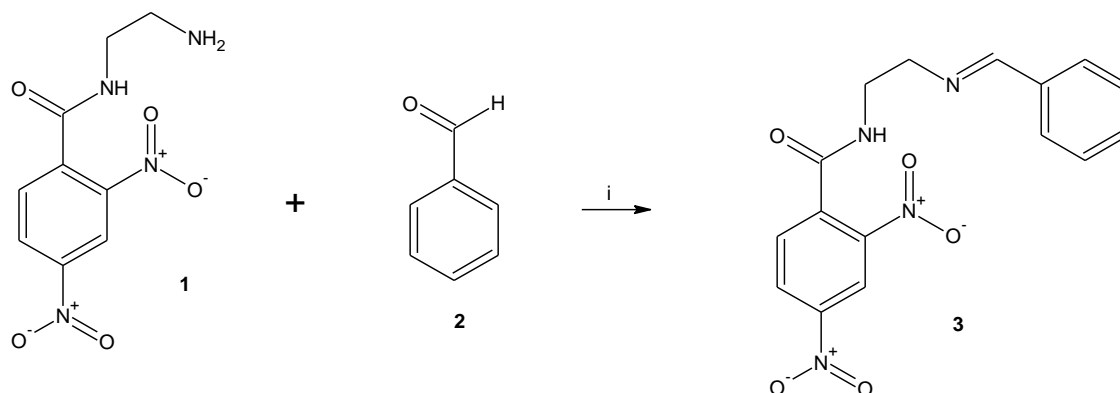
Figure 1. Synthesis of 2,4-dinitro-*N*-(2-{[(1*E*)-phenylmethylene]amino}ethyl)benzamide (**3**). Reaction of *N*-(2-aminoethyl)-2,4-dinitrobenzamide (**1**) with benzaldehyde (**2**) to form compound **3**. i = boric acid.
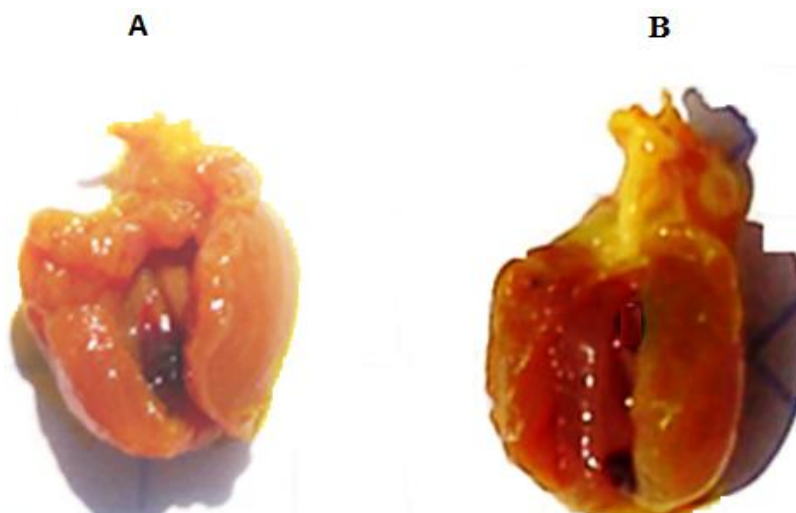
Figure 2. Comparison of cardioprotective effect of the benzamide derivative (**B**) at a dose of 1 nM with the control (**A**) on the functional recovery of rat hearts subjected to ischemia and reperfusion.

ventricular pressure and this effect was not inhibited in presence of nifedipine or indomethacin drugs (Figure 6 and 7) at a concentration of 1 nM. Finally, other data obtained (Figure 8) indicate that the benzamide derivative induces an increase in left ventricular pressure in a dose dependent manner [0.001 to 100 nM] and this effect was significantly inhibited by propranolol (p = 0.05) and metoprolol (p = 0.05) at a dose of 1 nM.

DISCUSSION

Chemical synthesis

In this study, a straight forward route for the preparation of (2,4-dinitro-*N*-(2-{[(1*E*)-phenylmethylene]amino}ethyl)benzamide (compound 3) was reported. The synthesis was achieved by the formation of an imine group (Schiff base) involved in compound 3 (Figure 1). There are several procedures for the synthesis of imines which are described in the literature (Shirayev et al., 2005; Uppiah et al., 2009; Figueroa-Valverde et al., 2012). In this study, the synthesis of the compound 3 was developed by the reaction of *N*-(2-aminoethyl)-2,4-dinitrobenzamide with benzaldehyde using boric acid as catalyst to form the compound 3. The structure of the benzamide derivative was confirmed using IR and NMR spectroscopy (Tables 1 and 2). The IR spectra contained characteristic vibrations at 33200 for imino group; at 1638 for amide group and 1350 for nitro groups. The ^{1}H NMR spectrum of the benzamide derivative shows signals at 3.72 to 3.75 ppm for arm bound to both imino and amino groups; at 7.38 to 7.68 ppm for protons involved in the phenyl group which is bound to imino group; at 7.83 ppm for amide group; at

Figure 3. Effect exerted by BD on cardiac ischemia/reperfusion with the control. The results showed that BD significantly reduced infarct size expressed as a percentage of the area at risk compared with the vehicle-treated hearts (p = 0.05). Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.
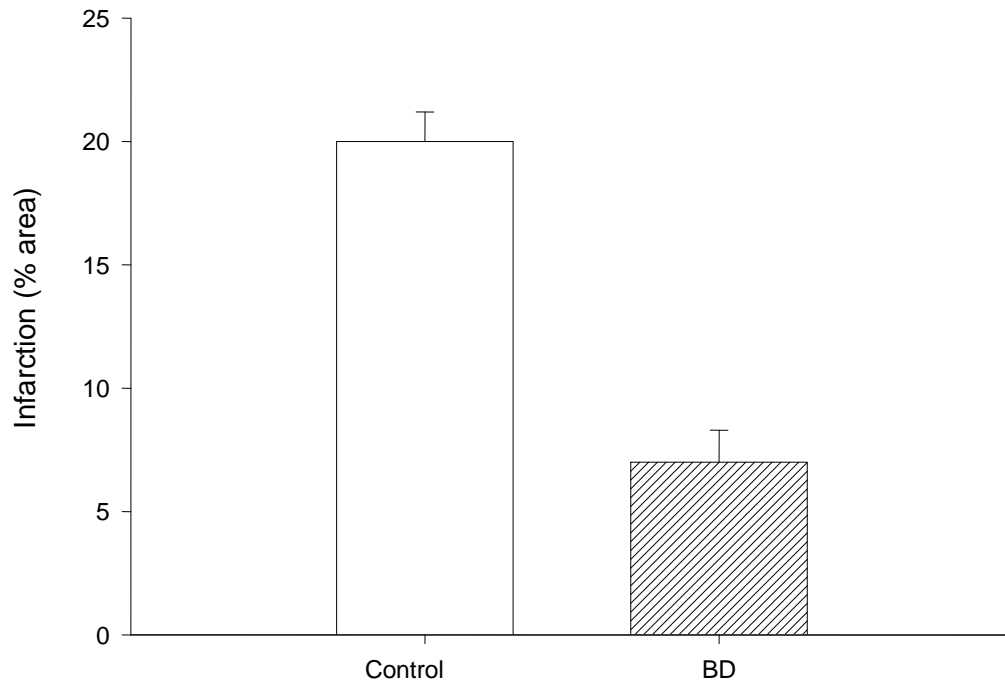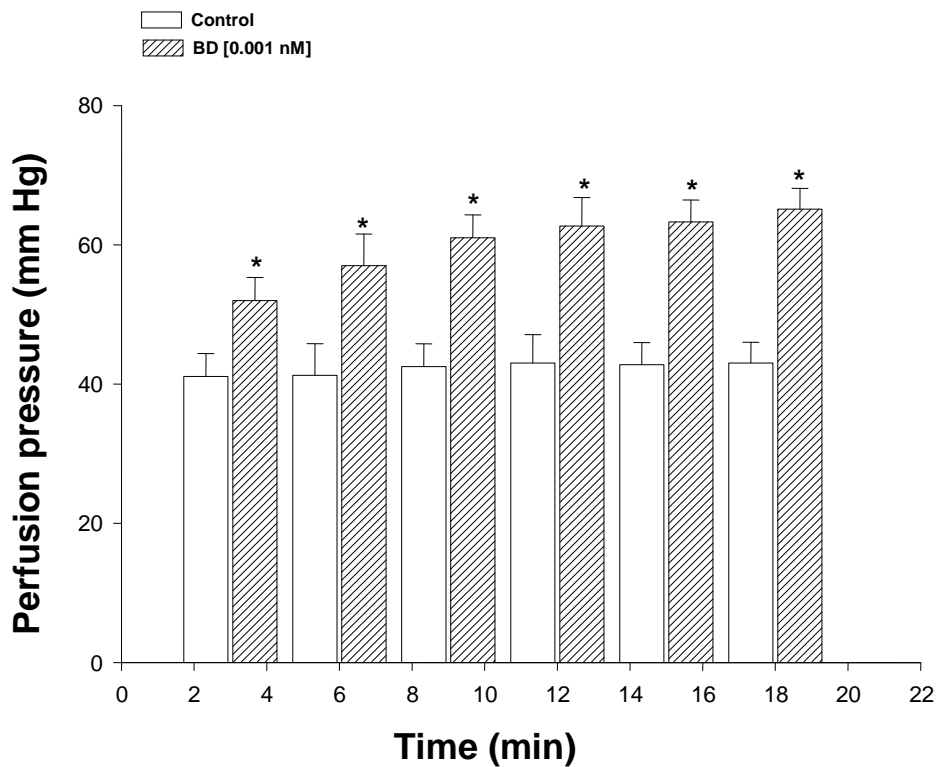

Figure 4. Effect induced by BD on perfusion pressure. The results show that BD significantly increase perfusion pressure (**p** = 0.05) through time in comparison with the control conditions. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.
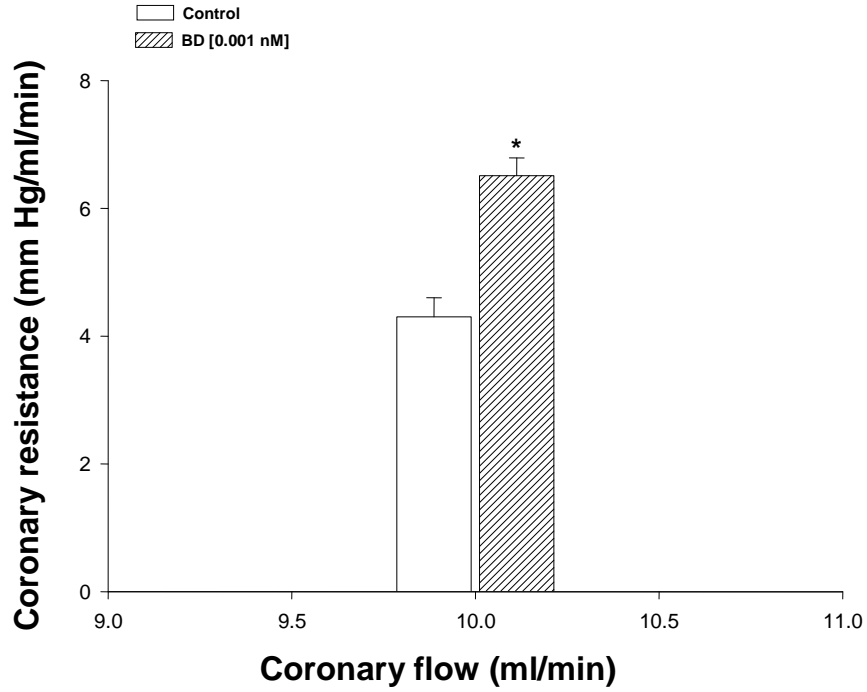
Figure 5. Activity exerted by BD on coronary resistance. The results show that coronary resistance was higher (**p** = 0.06) in the presence of BD in comparison with the control conditions. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.
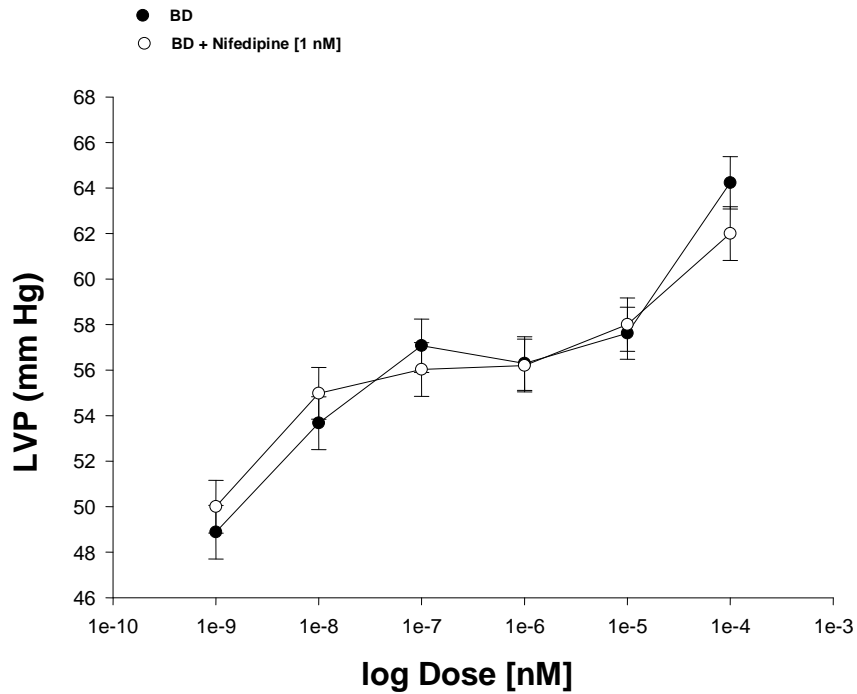
Figure 6. Effects induced by BD on LVP through calcium channel activation. Intracoronary boluses (50 µl) of BD [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined. The results showed that BD increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of nifedipine. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.
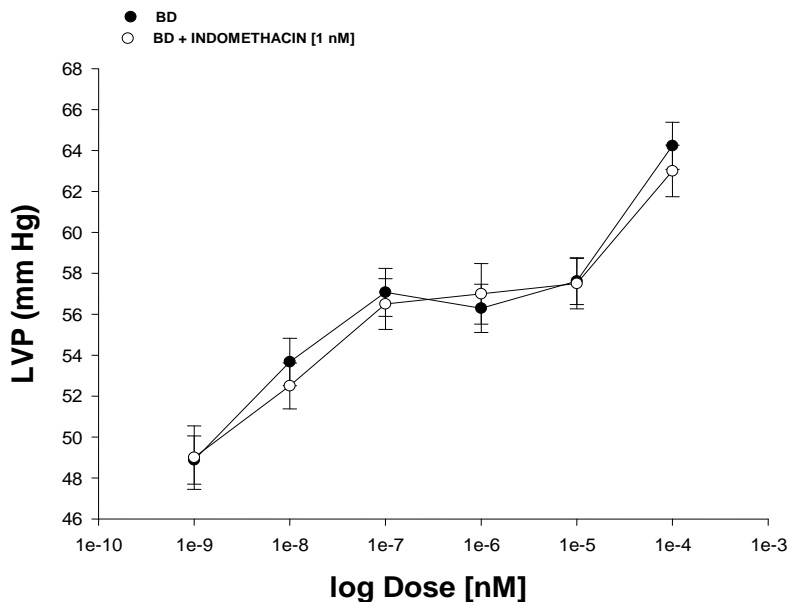
Figure 7. Effects induced by BD on LVP through prostaglandins synthesis. Intracoronary boluses (50 µl) of BD [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined. The results showed that BD increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of indomethacin. Each bar represents the mean ± S.E. of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.
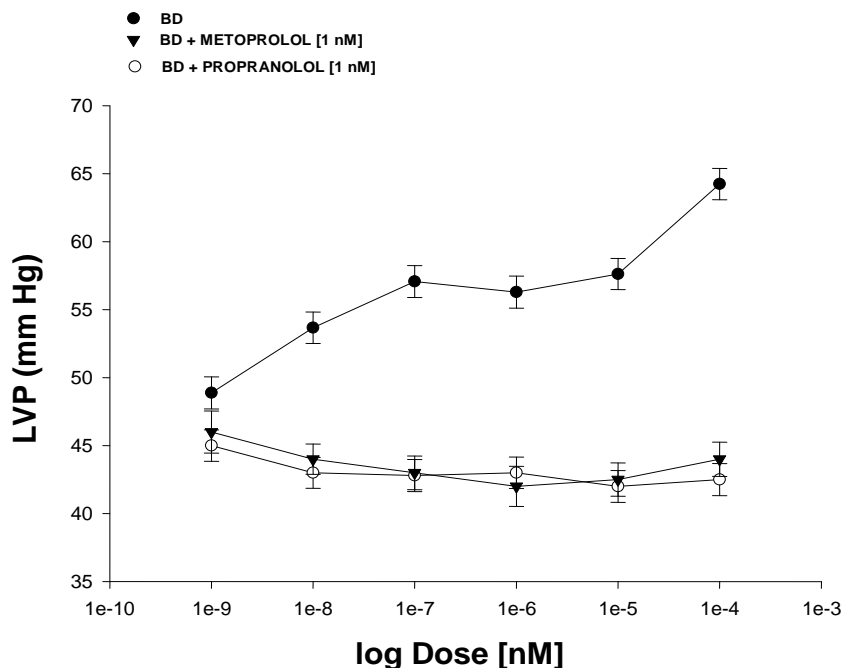
Figure 8. Activity exerted by BD on LVP through of β-adrenergic receptors.BD [0.001 to 100 nM] was administered (intracoronary boluses, 50 µl) and the corresponding effect on the LVP was evaluated in the absence and presence of propranolol or metoprolol. The results showed that activity induced by BD on LVP was significantly inhibited in the presence of propranolol ($p = 0.05$) or metoprolol ($p = 0.05$). Each bar represents the mean ± S.E. of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.BD = benzamide derivative; LVO = left ventricular pressure.

Table 1. Analysis of spectroscopicdata (300 MHz, CDCl[3]).

Parameter	^{1}H NMR(ppm)	[3]C NMR (ppm)
	3.72 (m, 2H)	35.78 (**C**-16)
	3.75 (m, 2H)	62.59 (**C**-17)
	7.38-7.68 (m, 5H)	120.91 (**C**-2)
	7.83 (broad, 1H)	128.04 (**C**-4)
	8.24 (d, 1H, J = 1.20)	128.18 (**C**-5)
	8.33-8.81 (d, 3H, J = 8.24)	128.20 (**C**-21,**C**-25)
	-	129.28 (**C**-22, **C**-24)
Benzamide derivative	-	130.62 (**C**-23)
	-	136.66 (**C**-20)
	-	141.57 (**C**-6)
	-	143.43 (**C**-1)
	-	147.50 (**C**-3)
	-	156.96 (**C**-19)
	-	163.77 (**C**-11)

8.24 ppm for methylene group bound to both amino and phenyl groups; at 8.33 to 8.81 ppm for protons involved in phenyl group bound to both nitro groups. The ^{13}C NMR spectra displays chemical shifts at 35.78 to 62.59 ppm for arm bound to both imino and amino groups; at 120.91 to 128.18 ppm for protons involved in phenyl group which is bound to both nitro groups; at 128.20 to 136.66 ppm for carbons of phenyl group bound to imino group; at 141.57 to 147.50 ppm for carbons involved in phenyl group bound to nitro groups. Finally, other signals at 156.96 ppm for methylene group bound to both imino and phenyl groups; at 163.77 ppm for amide group were found. In addition, the presence of benzamide derivative was further confirmed from mass spectrum which showed a molecular ion at m/z 342.02.

Biological evaluation

In this study, the activity of benzamide derivative was evaluated in an ischemia-reperfusion model. The results showed that benzamide derivative reduced infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (control). This phenomenon can be conditioned by activation of some structure biological (that is, ionic channels or specific receptors) involved in the endothelium of coronary artery (Bouis et al., 2009) or by the influence exerted by benzamide derivative on blood pressure which consequently bring reduction in the infarct size, and decrease the myocardial injury after ischemia-reperfusion similar to other reports for other compounds such as estrogens (Beer et al., 2002). In order to evaluate this hypothesis, the effect exerted by the benzamide derivative on blood vessel capacity and coronary resistance, translated as changes in perfusion pressure was evaluated in an isolated rat heart model. The results show that the benzamide derivative

significantly increases the perfusion pressure over time (3 to 18 min) compared to the control conditions. These data suggest that the benzamide derivative exerts effects on perfusion pressure which could subsequently modify vascular tone and coronary resistance of heart. Therefore, in this study, the activity exerted by the benzamide derivative on coronary resistance was evaluated. The results indicate that coronary resistance was increased in the presence of this compound. These data suggest that the benzamide derivative exerts effect on vascular tone through of generation or activation of vasoactive substances such as intracellular calcium happening with other type of compounds as the carbamazepine-alkyne derivative (Figueroa-Valverde et al., 2011a).

In order to characterize the molecular mechanism of this phenomenon and analyzed the reports of some investigations which indicate that some steroid derivatives induces its effect on blood pressure via the calcium channels activation (Figueroa-Valverde et al., 2011b). In addition, a report showed that some positive cardiotonic agents act by an increase in intracellular Ca^{2+} and consequently induce an increase in the sensitivity of contractile proteins to Ca^{2+} ions or by combinations of the two mechanisms (Bowman et al., 1999). Therefore, in this study, the activity induced by the benzamide deri-vative on left ventricular pressure was evaluated in the absence or presence of nifedipine. The results showed that effect exerted by the benzamide derivative was not inhibited in the presence of nifedipine. Furthermore, these data indicate that activity exerted by the benzamide derivative was not via activation calcium channel.

Analyzing experimental data obtained, validating the effect induced by some steroid derivatives on perfusion pressure via prostanglandins synthesis was also considered (Sheillan et al., 1983) and to evaluate the possibility that the activities exerted by the benzamide derivative involve stimulation and secretion of prostaglandins.

In this sense, in this experimental study,the activity exerted by the benzamide derivative on left ventricular pressure in the absence or presence of indomethacin was evaluated. The results showed that effect induced by the benzamide derivative on left ventricular pressure was not blocked by indomethacin. These results indicate that the molecular mechanism involved in the effect exerted by the benzamide derivative was not via prostaglandins.

Moreover, in the search of the molecular mechanism involved in activity induced by the benzamide derivative on left ventricular pressure and analyzing previous reports, which indicate that some substances such as progesterone can stimulate catecholamine (Tollan et al., 1993) which has an important role in the development or maintenance of elevated blood pressure (Lilley et al., 1976); in this study, the effect exerted by the benzamide derivative on left ventricular pressure was evaluated in the absence or presence of propranolol or metoprolol. The results showed that the effect induced by the benzamide derivative was significantly inhibited in the presence of this compound. All these data suggest that the molecular mechanism involved in the activity of benzamide derivative is via adrenergic system. This phenomenon is similar to activity exerted by other drugs on left ventricular pressure (Thiemermann et al., 1997) which may contribute to decrease cell death caused by ischemia/reperfusion in men.

Conclusion

The benzamide derivative is a particularly interesting drug, because the activity induced on injury by ischemia/reperfusion involves a molecular mechanism different in comparison with other drugs. This phenomenon may constitute a novel therapy for ischemia/reperfusion injury.

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Full Length Research Paper

A pilot study on the novel effect of Neu-P11 on reducing the intraocular pressure in normotensive rabbits

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Melatonin is involved in the regulation of intraocular pressure. To evaluate the effect of a melatonin receptor agonist (Neu-P11) on intraocular pressure, a multiple-dose study was performed in 30 ocular normotensive rabbits by regularly measuring the intraocular pressure within 7 h. The results indicated that topical applications of Neu-P11 and melatonin could both produce dose-dependent reductions in intraocular pressure with maximal reduction being observed at 1 h for Neu-P11 and melatonin in 10^{-5} mol/L. Moreover, there were significant differences in the declinations of intraocular pressure between Neu-P11 and melatonin at the doses of over 10^{-9} mol/L ($p < 0.05$) in the treatment group. This study suggests that Neu-P11 is a potential substance for the reducing and treatment of ocular hypertension.

Key words: Intraocular pressure, melatonin, Neu-P11, rabbit.

INTRODUCTION

Variations in intraocular pressure (IOP) are the diurnal rhythmic activities with a low level in the light and a high level in the dark in rabbits (Liu and Dacus, 1991) and reflect a balance between the inflow and outflow of aqueous humors (Civan and Macknight, 2004). Evidence has indicated that the variations of IOP are influenced by melatonin, that can regulate osmotic exchanges between the blood and intraocular fluid (Bucolo et al., 2013; Musumeci et al., 2013). Therefore, substances such as melatonin and its receptor agonists are potential drugs for the treatment of abnormal IOPs (Bucolo et al., 2013).

Melatonin is a neurohormone expressed in the tissues, including pineal gland, retina (Wiechmann 1986; Tosini 2000; Zawilska et al., 2009), ciliary body (Martin et al., 1992), and lens (Abe et al., 1999; Itoh et al., 2007). This

neurohormone is involved in the regulations on rhythmic activities such as circadian seasonal rhythms (Alarma-Estrany and Pintor 2007), sleep and cyto-/neuroprotection (Hardeland 2010). So far, clinical studies have shown that melatonin could protect ocular tissues by effectively scavenging free radicals and excessive amounts of nitric oxide (NO) generated in the glaucomatous eyes (Rosenstein et al., 2010; Agorastos and Huber 2011). As the analogues of melatonin, in recent years, much evidence has indicated that melatonin agonists can activate the melatonin receptors and have therapeutic applications in the treatment on sleep disorders (Hardeland et al., 2008) and depression (Guardiola-Lemaitre, 2007; Spadoni et al., 2011). Based on the mechanics of melatonin and melatonin agonists,

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we predicted that some melatonin agonists might be more available for regulating the IOPs.melatonin agonists which might be more available for regulating the IOPs.

Neu-P11 is one of the novel melatonin agonists with high affinity to melatonin receptors. A recent study has demonstrated that the agonist can improve the energy metabolism and insulin sensitivity of eyes (She et al., 2009). Moreover, the results from animal studies have revealed that this substance has obvious effects on the treatments of antidepressant and anxiolytic activities in rodent models (Tian et al., 2010). However, its effects on IOP remained unclear although this melatonin agonist could improve the sleep of mice (Oertel et al., 2013) and energy metabolism of animals by increasing superoxide dismutase, catalase and glutathione-reductase activities (Chuffa et al., 2011). In the present study, to evaluate the effect of Neu-P11 on IOP, a multiple-dose study was performed by regularly measuring the IOPs within 7 h in 30 ocular normotensive rabbits.

MATERIALS AND METHODS

Animals

Thirty male New Zealand white rabbits (2.5 to 3.0 kg) were kept in cages individually, with free access to food and water. They were submitted to control in light/12 h and dark/12 h cycles. All the protocols and the statement for the use of animals in ophthalmology and vision research were described complying with the Association for Research in Vision and Ophthalmology (ARVO). This study was in accordance with the European Communities Council Directives (2010/63/EU).

Compounds

The Neu-P11 ($C_{13}H_{16}N_2O_4$) and melatonin ($C_{13}H_{16}N_2O_2$) used in the study were provided by Neurim Pharmaceuticals Ltd. (Tel-Aviv, Israel), and the Oxibuprocaine/tetracaine anaesthetic was purchased from Cusi laboratories (Spain) (Figure 1).

Preparation for formulations

The stock solutions of melatonin and Neu-P11 were prepared by dissolving in dimethylsulfoxide (DMSO) in 1 mM. Then, the stock solution was diluted with isotonic saline solution (sodium chloride) to different final concentrations (10^{-14} to 10^{-5} mol/L). All different final concentration solutions contained less than 1% DMSO and were kept at room temperature.

Intraocular pressure measurement

The IOPs, including right eye (Oculus Dexter, OD) and left eye (Oculus Sinister, OS) were measured by using a Tonopen XL contact tonometer supplied with Mentor (USA). Each cornea was anaesthetized by applying 10 µl anesthetic containing 4 mg oxibuprocaine and 1 mg tetracaine. Bilateral IOPs were measured as resting IOP before melatonin or Neu-P11 treatment was cornea

unilaterally to the at a fixed volume of 10 µl, while the contralateral eye was received 10 µl physiological saline.

Pharmacological protocols

10 µl melatonin or Neu-P11 was assayed in a wide range of doses from 10^{-5} to 10^{-14} mol/L to generate the dose-effect curves. In the experiments, IOPs were measured during the maximal effect of the agent. Moreover, to study the time-course effect of single dose (10^{-5} mol/L) of melatonin or Neu-P11, IOPs were regularly measured crossing 7 h. In the experiments, one single dose was tested for a single animal, and there was a 2 days interval between the dose tests for the same animal.

Statistical analysis

Statistical differences between treatments were calculated with two-way repeated-measures analysis of variance (ANOVA) by using SPSS15.0. Significant statistical difference was refered to as $p < 0.05$ in the study. Plotting and fitting were carried out with MICROCAL ORIGIN 7.0 (Micro Software, Inc., Northampton, MA, USA). Data was displayed as means ± standard deviations (M ± SD).

RESULTS

Resting intraocular pressure measurement

The resting IOPs of 30 male New Zealand white rabbits were measured as value at 20.65 ± 3.80 mmHg before melatonin and Neu-P11 treatments were performed.

### Treatments of melatonin and Neu-P11 on intraocular pressures

Fifteen white rabbits were treated with melatonin and the other 15 were treated with Neu-P11. The fixed volumes (10 µl) of melatonin and Neu-P11 were assayed with across concentrations ranging from 10^{-14} to 10^{-5} mol/L. The results indicated that the two substances could both induce obvious reductions in the levels of IOPs with the increasing doses. Furthermore, the Neu-P11 treatment group, in comparison with the melatonin group, showed a significant decrease in IOP when the concentration exceeded 10^{-9} mol/L ($p < 0.05$) (Figure 2).

Time-course effect of Neu-P11 on intraocular pressures

We selected an optical dose of melatonin or Neu-P11 at 10 µl (10^{-5} mol/L) for unilateral eye. In the treatment, 10 µl working solution of Neu-P11 (10^{-5} mol/L) solution in saline DMSO was instilled in saccus conjunctivae of the contralateral eye. IOPs were measured at 0, 0.5, 1, 2, 3,
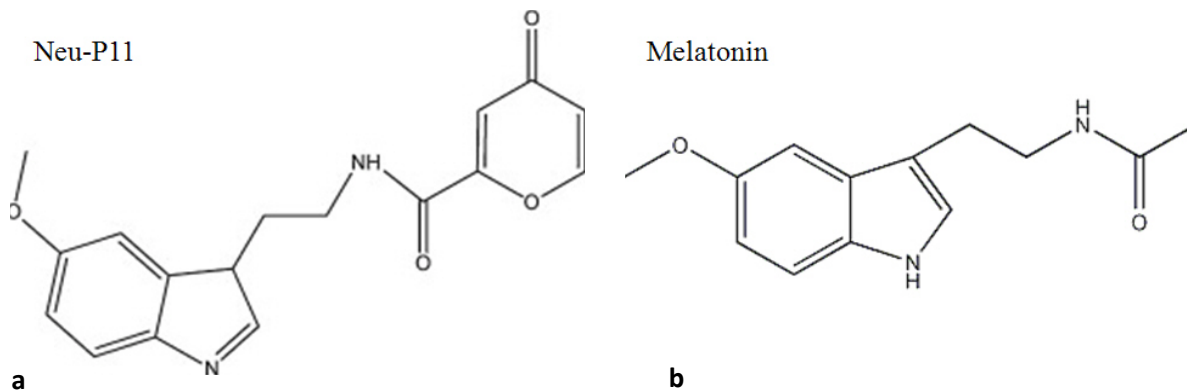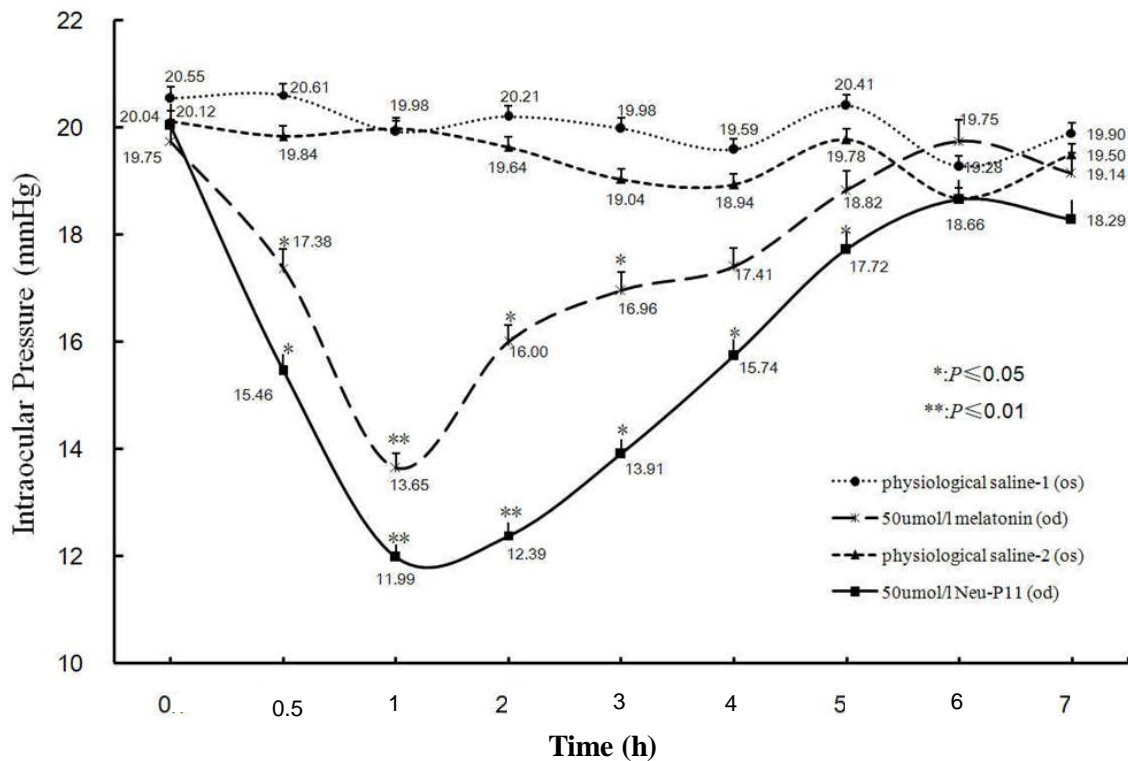
Figure 1. Formula of Neu-P11 and Melatonin
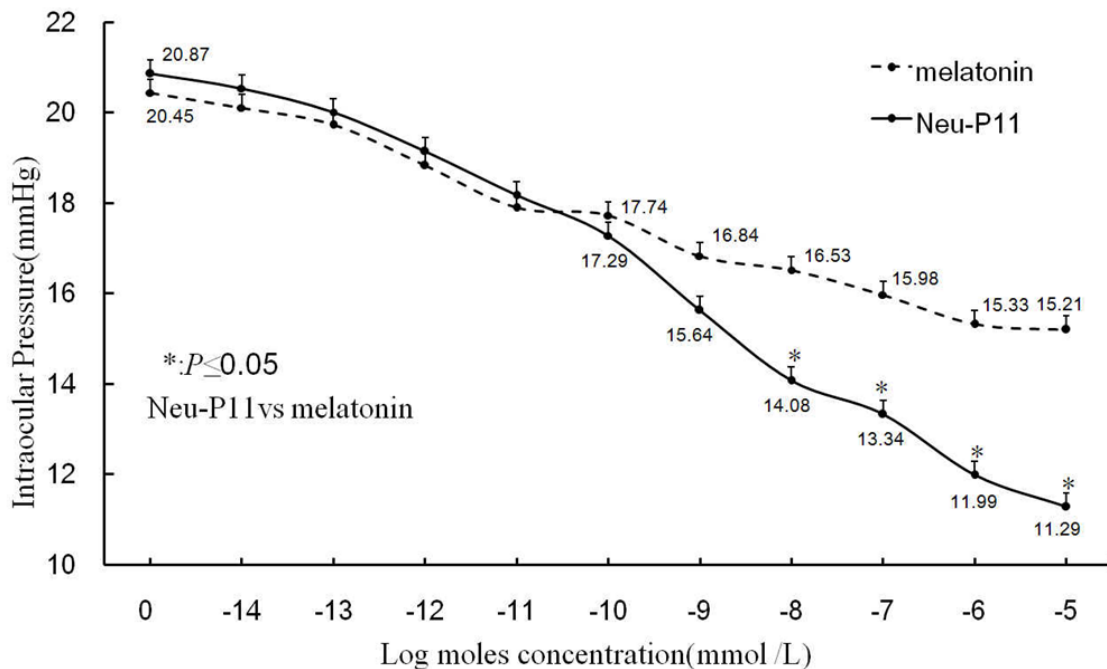
Figure 2. Dose-response curves for Neu-P11 and melatonin on intraocular pressure in New Zealand white rabbits. 10 μl of Neu-P11 and melatonin were tested at concentration range varied from 10^{-14} to 10^{-5} mol/L. Values represent mean ± SD of ten independent experiments

4, 5, 6 and 7 h after the instillation. There was no a significant difference in the variation of IOP between melatonin and Neu-P11 in the vehicle group ($p > 0.05$). Moreover, both melatonin and Neu-P11 treatments reduced IOP rapidly at 0.5 h and maximally at 1 h with 15.46 ± 2.28 and 11.99 ± 2.45 mmHg. On the contrast, the effect of Neu-P11 was more sustained than that of melatonin. The reductions in IOPs were lasting for 3 h in melatonin group and 5 h in Neu-P11 group (Figure 3). Then, the pressures returned back to baseline

DISCUSSION

The role of melatonin in IOP and the relationship of the variations in IOPs and the presence of melatonin receptors have been reported in previous studies (Rohde et al., 1985; Samples et al., 1988; Pintor et al., 2003; Alarma-Estrany et al., 2008; Musumeci et al., 2013). However, there was no evidence about the regulation of Neu-P11 on IOP. We found that Neu-P11 could reduce IOP in a dose-dependent manner over 5 h and showed a

Figure 3. Time-course effect of Neu-P11 and melatonin on rabbit intraocular pressure. Values represent the mean ± SD of ten independent experiments.

maximum reduction with single dose at 1 h.

Some previous studies indicated that the topical applications of N-butanoyl-2-(2-methoxy-6H-isoindolo [2,1-a]indol-11-yl) ethanamine (IIK7) and 5-Methoxycarbonylamino-N-acetyltryptamine 5-MCA-NAT) could produce lower reductions in IOP in New Zealand rabbit normotensive eyes (Pintor et al., 2001; Pintor et al., 2003; Alarma-Estrany et al., 2008). The effects were similar to the Neu-P11 in our study. These findings suggested that Neu-P11 had a hypotensive effect similar to the well-known MT-melatonin receptor agonists. So far, studies have evidenced that melatonin and its analogues can cause concentration- dependent reductions of SNP-released NO and cGMP production via activation of MT2 receptors in human NPCE cells, which play a role in melatonin agonist- induced regulation of aqueous humor secretion and IOP (Dortch-Carnes and Tosini, 2013). Therefore, we believed that Neu-P11 has similar mechanism in the regulation of IOPs as MT-melatonin receptor agonists.

In the present study, we observed that the Neu-P11 had a better hypotensive effect than melatonin. These findings provided further supports on the previous findings that melatonin analogues had an advantage over melatonin in reducing IOP (Pintor et al., 2003;

Alarma-Estrany et al., 2008; Musumeci et al., 2013). In the present study, Neu-P11 produced a lasting reduction in IOP in the cornea as compared to melatonin. The result implies that Neu-P11 possibly produced a longer inhibition in the adenylyl cyclase, an increasing formation of cyclic adenosine monophosphate (cAMP) and a larger protein kinase A (PKA) activity (Dubocovich et al., 2010). Thus, we deduced that Neu-P11 was a better hypotensive substance than melatonin in the treatment of ocular hypertension. Furthermore, considering the roles of Neu-P11 in energy metabolism and insulin sensitivity, depression and anxiety (She et al., 2009), this substance would have some advantages in treatment on ocular hypertension cases with abnormal energy metabolism, insulin sensitivity and mental condition. However, further evaluator studies are required to assess the veracity of this claim.

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Full Length Research Paper

Inhibitory effect of betulinic acid and 3β-acetoxybetulinic acid on rat platelet aggregation

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Platelet aggregation is one of the major causes of cardiovascular diseases. Our search for bioactive molecules from nature, led to the isolation of betulinic acid (BA) and structural modification of BA to 3β-acetoxybetulinic acid (BAA). Both inhibited blood platelet aggregation induced by thrombin, adenosine diphosphate (ADP) and epinephrine. BAA showed an enhance inhibition of platelet aggregation, in the thrombin-induced platelet aggregation (54.5 ± 0.01 at 1 mg/ml, 63.5 ± 0.17 at 3 mg/ml and 73.5 ± 0.15 at 10 mg/ml; IC_{50} 0.81 mg/ml) which was observed to be significantly ($p<0.05$) similar to that of the standard aspirin (65.4 ± 0.07 at 1 mg/ml, 72.1 ± 0.03 at 3 mg/ml and 76.5 ± 1.22 at 10 mg/ml; IC_{50} 0.33 mg/ml). The results clearly shows that functional group modification of BA to give BAA led to enhanced activity, hence BAA provides a better option as lead in the search for anti-platelet aggregation agents from nature.

Key words: Anti-platelet aggregation, aggregation inducer, Betulinic acid, acetoxybetulinic acid, *Melalueca bracteata*.

INTRODUCTION

Platelets are cells in the blood that help in the formation of clot. They play an important role in the hemostasis and in pathophysiological processess such as thrombosis (Shattil et al., 1998; Stouffer and Smyth, 2003). Intravascular thrombosis is central to the development of a wide variety of cardiovascular diseases (Grenache et al., 2003; Huo and Ley, 2004). Platelet over aggregation is usually the main cause of internal blood clot formation and if not checked, can be fatal leading to artherothrombotic diseases such as strokes and heart attack (Valko et al., 2005). Thrombin, adenosine diphosphate (ADP), epinephrine, arachidonic acid, collagen and other risk factors such as free radicals, inflammation, stress and hypercholesterolemia significantly contribute to platelet dysfunction (Ambrosio et al., 1997; Davi and Patrono, 2007; Bakdash and Williams, 2008; Verhamme and

Hoylaaert, 2009). The activation of platelet by thrombin is mediated through two protease activating receptors PAR-1 and PAR-4, in the activation of platelets; these receptors work cooperatively (Fabre and Gurney, 2010). ADP acts through G-protein coupled receptors P2Y1 and P2Y12, they activates phospholipase C and thus resulting in the elevation of intracellular calcium concentration (Davi and Patrono, 2007). Epinephrine is a weak platelet agonist and exerts its effect on human platelets through α_2-adrenergic receptors (A2AR) and potentiates the aggregation potency of other inducers (Choi, 2002).

Antiplatelet drugs help stop blood clot formation and this significantly contributes to the management of pathogennesis of cardiovascular diseases. Among the many drugs used in the management of the condition is aspirin,
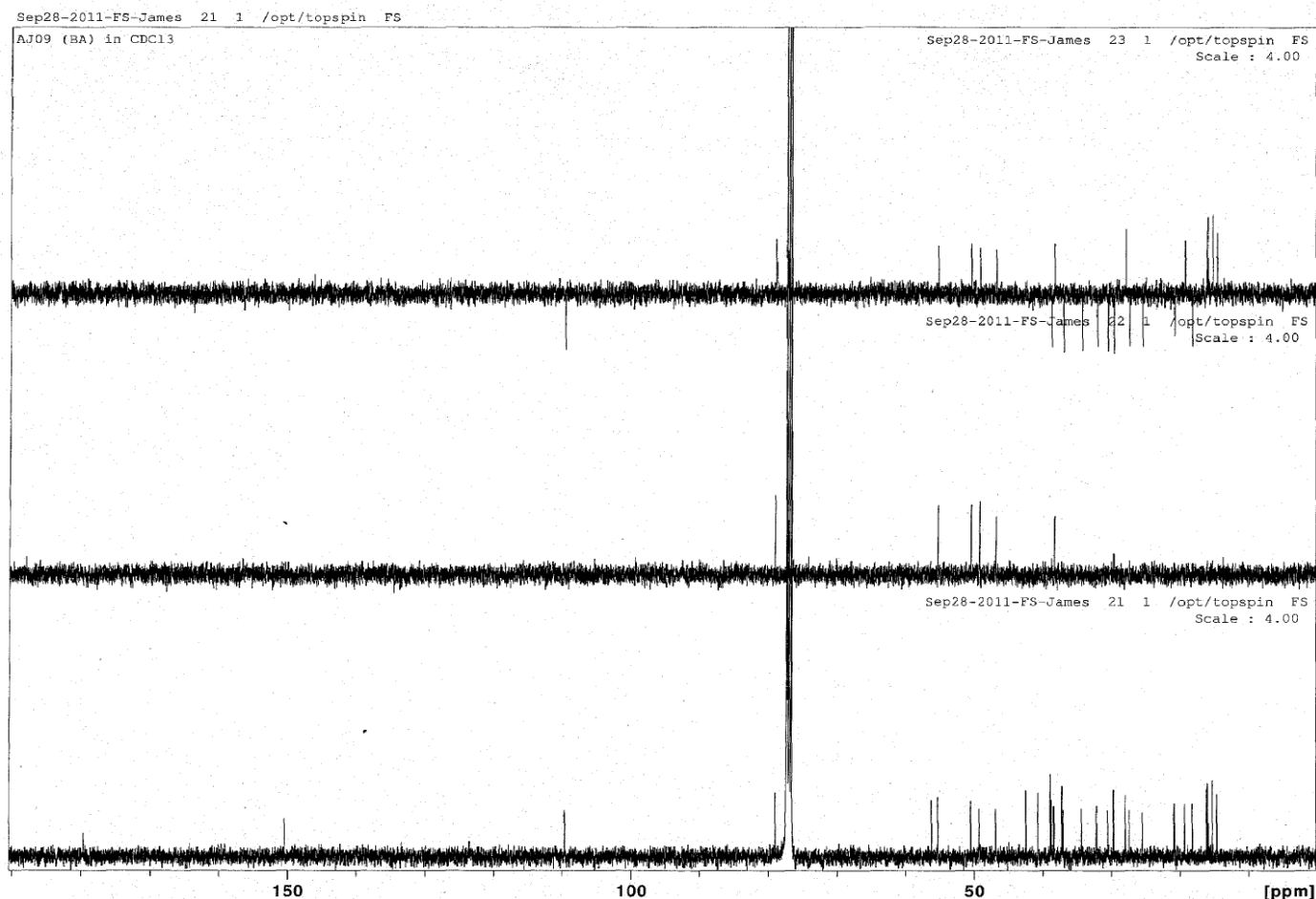
Figure 1. C-NMR, DEPT 90 and DEPT 135 spectrum of BA.

but these drugs are not without side effect, fuelling the search for more effective drugs from natural origin that will help in overcoming the challenges of toxicity and other undesirable side-effects.

The triterpene betulinic acid (BA) is one of the major components of *Malaleuca bracteata*, a plant native to Australia. The essential oil from the leaves of the plant has antiseptic, germicidal and insecticidal properties (Cribb and Cribb, 1981; Yatagai, 1997). The leaves and their volatile oils are locally used in West Africa in the treatment of skin infection (Oliver, 1960; Irvine, 1961; Howes, 1974; Goldstein et al., 1990; Belousova and Denisova 1992). BA possesses diverse biological func-tions, which includes, but not limited to anticancer (Amico et al., 2006; Huang et al., 2007), HIV-1 maturation inhibition (Fukoja et al., 1994) and antibacterial (Chandramu et al., 2003). The present study reports our findings on blood platelet aggregation (BPA) inhibition of BA and the effect of change in the functionality of BA on BPA.

MATERIALS AND METHODS

Plant

Melaleuca bracteata was supplied by Prof. F. O. Shode of the School of Chemistry, University of KwaZulu-Natal, Durban, South Africa. The plant was identified and a voucher specimen (Glow 001) was prepared and deposited in the Herbarium, School of Biological Sciences, University of KwaZulu-Natal, Durban, South Africa.

Extraction and isolation of BA from *M. bracteata*

The pulverized plant material (2.5 kg) was extracted by cold maceration in dichloromethane (5 L×2) for 48 h, filtered and concentrated under reduced pressure at 40°C using rotary evaporator and allowed to dry under room temperature. The dried residue was defatted with n-hexane to yield a solid mass (9.8 g). The solid mass (5 g) was subjected to column chromatography using ethyl acetate:hexane (8:2) as solvent of elution to give a white amorphous powder which was identified as betulinic acid (BA). The structure was confirmed by nuclear magnetic resonance (NMR) spectra (Figure 1) and comparison with literature values (Mahato and Kundu, 1994).
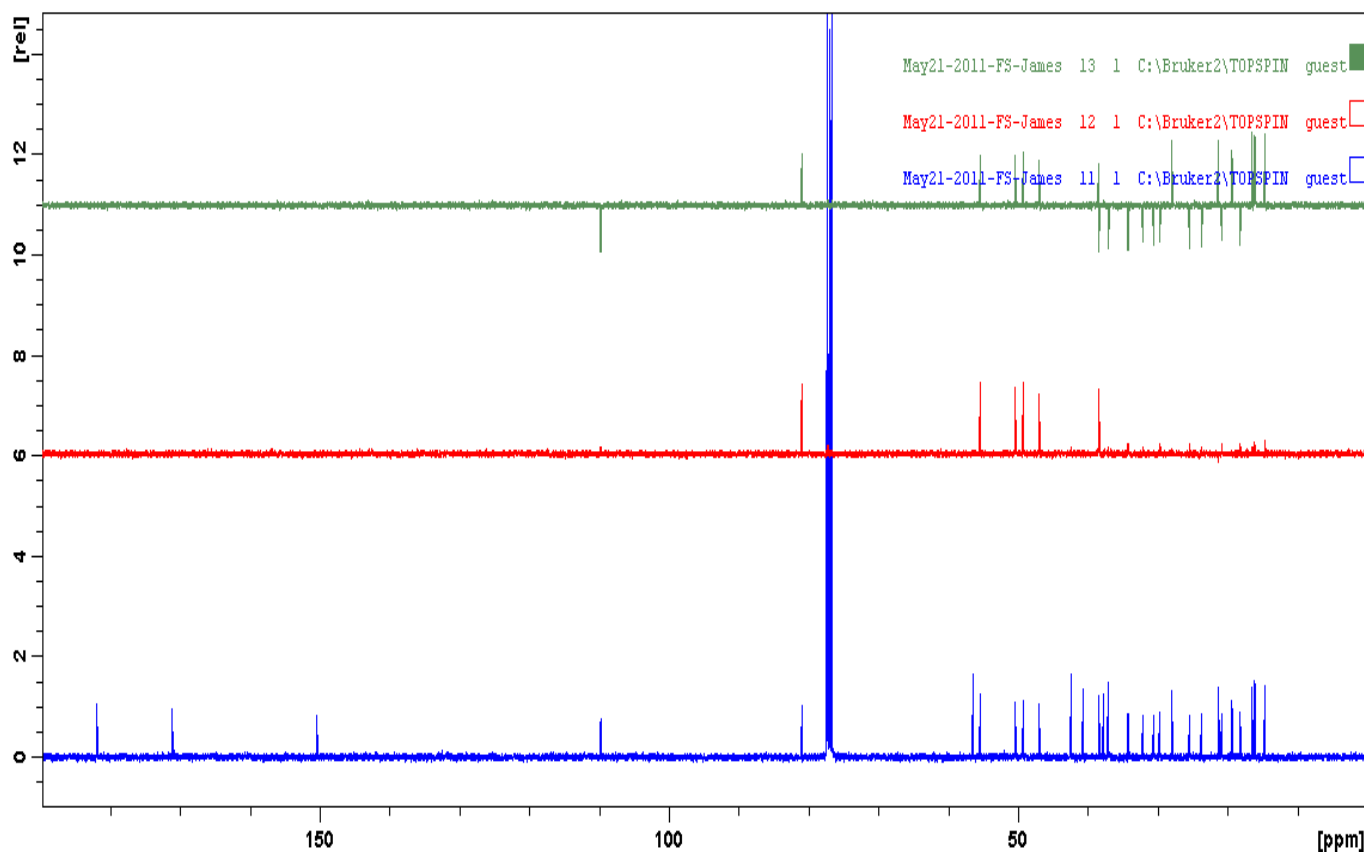
Figure 2. C-NMR, DEPT 90 and DEPT 135 spectrum of BAA.

Preparation of 3β-acetoxy betulinic acid (BAA)

BA (2 g) in a round bottom flask was added acetic anhydride (5 ml) and pyridine (5 ml), the mixture was then refluxed for 2 h. Then stirred at room temperature for 24 h, after which water (10 ml) was added and stirring continued for another 30 min. The mixture was then filtered under suction and washed thoroughly with 10% hydrochloric acid to give a white powder. NMR analysis and comparison with literature (Mahato and Kundu, 1994) confirmed the structure of BAA (Figure 2).

Animals

Ethical clearance for the use of animals in this study was obtained from the research animal ethics committee of the University of Zululand, South Africa. Adult rats (Sprague-Dawley) of either sex were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand, South Africa. The animals were maintained under standard conditions and had free access to standard pellet feed and drinking water, for a minimum of 7 days before use.

Preparation of blood platelets

The blood platelet was prepared according to the method described by Tomita et al. (1983). Briefly, blood was surgically collected from

the heart immediately after the rat has been rendered unconscious. The blood was transferred into a centrifuge tube and mixed (5:1 v/v) with an anticoagulant, acid-dextrose-anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose). The platelets were obtained by series of centrifugation at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively, the supernatant was collected and discarded and the sediment (platelets) was re-suspended in 5 ml washing buffer (pH 6.5). This was centrifuged again at 300 rpm for 15 min after which the supernatant was discarded and the platelets were finally suspended in a (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose buffer). The platelet was diluted in the re-suspending buffer (1:10), and the resulting solution was mixed with calcium chloride (0.4 ml; 10 μl $CaCl_2$).

Anti-platelet aggregation study

The method of Mekhfi et al. (2004) was adopted with some modifications. Various concentrations (1, 3 and 10 mg/ml) of the test compounds were made, by separately dissolving the compounds in dimethyl sulfoxide (DMSO) and making up the volume with a buffer (pH 7.4) solution. The antiplatelet aggregation activity of the compounds were separately tested on thrombin (5 U/ml), ADP (5 mM) and epinephrine (10 mM) induced platelet aggregation; the platelets (150 μl) were incubated in a 96-well micro-titre plate at 37°C for 5 min, with 50 μl of different concentrations of the compounds (1, 3, and 10 mg/ml) and an aggregation inducer (20 μl)
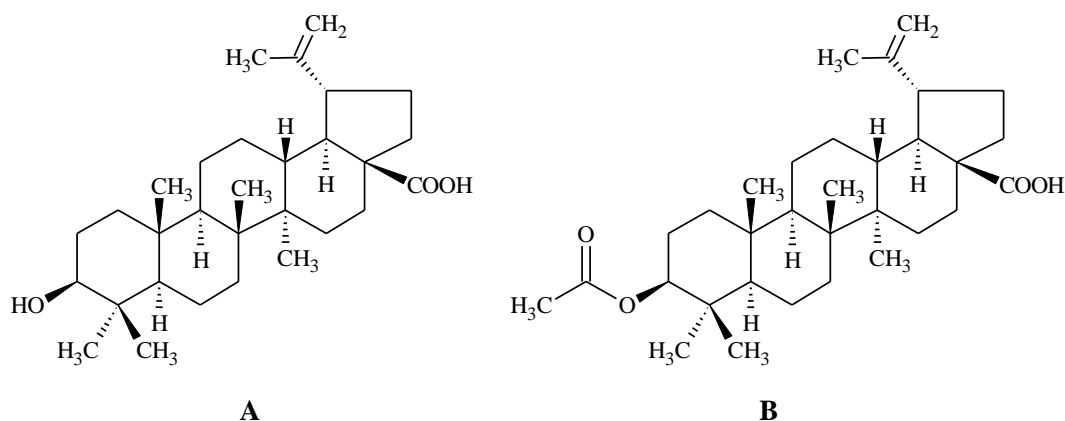
Figure 3. Structure of isolated and modified compounds: (A) Betulinic acid; (B) 3-β acetoxy betulinic acid.
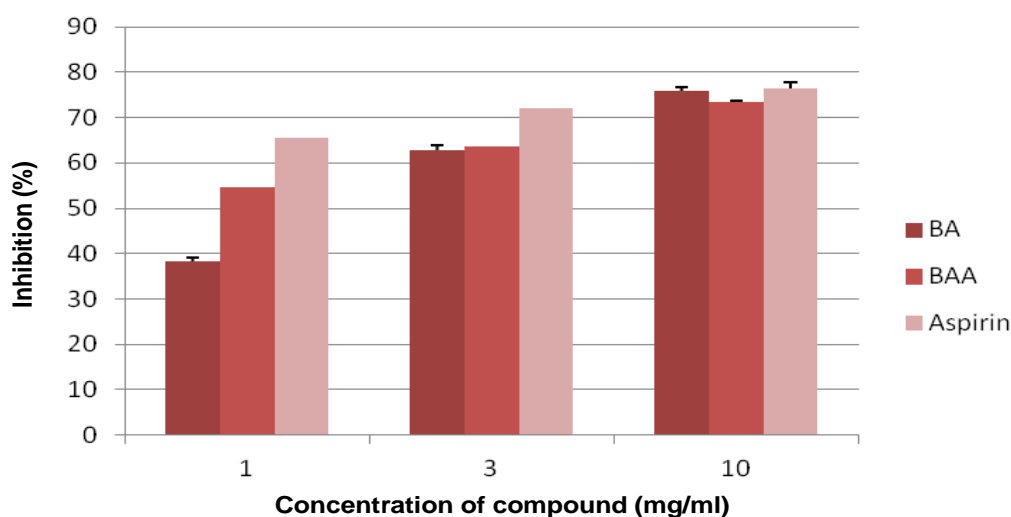
Figure 4. Percentage inhibitory activity of test compounds on thrombin-induced platelet aggregation.

was introduced to the mixtures. Aggregation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm for 20 min at 30 s intervals. DMSO (1%) and aggregation inducer were used as negative control; aspirin was used as positive control.

The inhibitory effects of the compounds on each agonist were calculated as:

Inhibition (%) = {(1-(At/Ac) × 100}

The inhibitory concentration providing 50% inhibition (IC_{50}) was determined using statistical package (Origin 6.1), where At is the absorbance of the mixture containing the compounds and Ac is absorbance of the control without the test compounds.

Statistical analysis

All assays were performed in triplicates and the results are expressed as mean ± standard deviation (SD). P ≤ 0.05 were considered to be statistically significant.

RESULTS

The results of the NMR analysis, ^{13}C, DEPT-90 and DEPT-135 for both BA and BAA are shown in Figures 1 and 2, respectively, were in agreement with those reported by Mahato and Kundu (1994). The results presented in Table 1 showed the inhibitory concentration providing 50% inhibition (IC_{50}) of the test compounds and standard, on platelet aggregation for all the three agonist used (thrombin, ADP and epinephrine). While the results of the percentage inhibition of platelet aggregation of the various concentration of the test compounds on each of the agonist are shown in Figures 3, 4 and 5.

DISCUSSION

Platelet aggregation is one of the major causes of
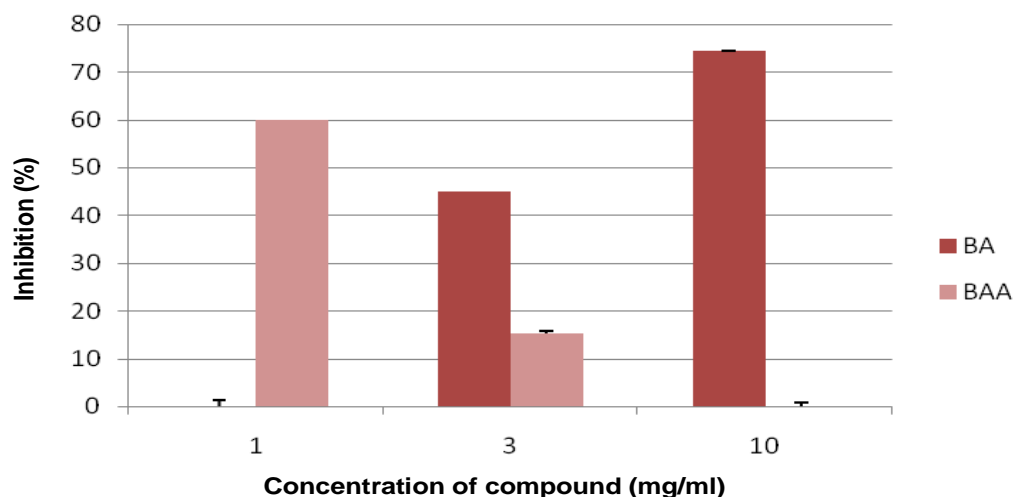
Figure 5. Percentage inhibitory activity of test compounds on ADP-induced platelet aggregation.
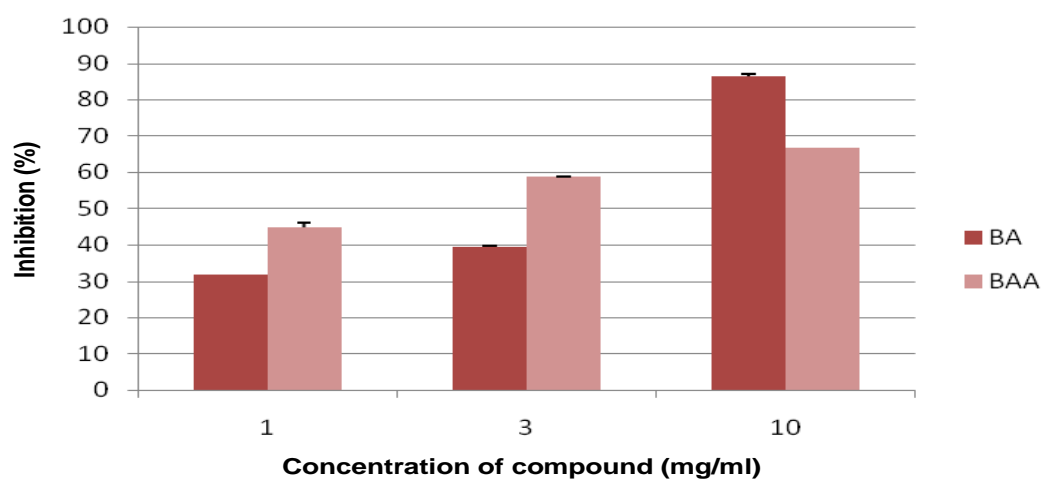
Figure 6. Percentage inhibitory activity of test compounds on epinephrine-induced platelet aggregation.

Table 1. IC_{50} values (mg/ml) of the isolated and modified compound on rat platelet aggregation.

Agonist/Compound	BA	BAA	Aspirin
Thrombin	1.99	0.81	0.33
ADP	4.20	1.39	-
Epinephrine	4.63	1.81	-

artherothrombotic dysfunction, and the commonly used drugs for the treatment of these disorders are becoming undesirable, because of their associated side-effects. This has fuelled the search for new class of effective anti-platelet agents from nature. In this study, the effect of functional group modification of BA to BAA led to enhanced

anti-platelet aggregation activity against thrombin, ADP and epinephrine induced rat platelet aggregation.

The spectral data defined BA and BAA as triterpenoids, triterpenes and their various derivatives have been shown to inhibit platelet aggregation induced by thrombin, ADP and epinephrine (Jin et al., 2004; Yang et al., 2009; Sankaranarayanan et al., 2010; Habila et al., 2011; Mosa et al., 2011). The results of our investigation showed that anti-platelet aggregation inhibitions of BA and BAA against thrombin, ADP and epinephrine-induced platelet aggregation (Figures 3 to 5) showed a dose dependent increase in percentage inhibition, with increase in concentration. BA and BAA were more potent in the thrombin-induced platelet aggregation with IC_{50} of 1.99 and 0.81 mg/ml, respectively, which were comparable to that of aspirin (0.33 mg/ml). BAA showed more enhance

activity in all the three agonist used (IC$_{50}$: thrombin, 0.81 mg/ml; ADP, 1.39 mg/ml; epinephrine, 1.81 mg/ml), as compared to BA (IC$_{50}$: thrombin, 1.99 mg/ml; ADP, 4.20 mg/ml; epinephrine, 4.63 mg/ml) the parent nucleus. The observed activity was attributed to the modification of the 3β-hydroxyl (-OH) of BA to 3β-acetyl (OCOCH$_3$) functional group. The overall result of the anti-platelet aggregation studies reveals that both compounds, BA and BAA inhibit platelet aggregation at a significant difference (p<0.05) between the different concentrations used in all the three agonist. Our findings are in agreement with Tzakos et al. (2012) who reported the potency of betulinic acid to inhibit human platelet aggregation. The results of this study showed that the isolated (BA) and modified (BAA) compounds are good candidates in the search for anti-platelet aggregation agents from nature.

Conclusion

The results showed that structural modification of the isolated compound (BA) to mimic the functionality of aspirin, led to enhanced platelet aggregation inhibition, induced by thrombin, ADP and epinephrine. The findings suggest that BA and BAA may be considered as lead candidates, in the search for anti-platelet aggregation agents from nature that may offer a better alternative to aspirin.

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