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Structural effects of the *Solanum* steroids solasodine, diosgenin and solanine on human erythrocytes and molecular models of eukaryotic membranes

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ABSTRACT

This report presents evidence that the following *Solanum* steroids: solasodine, diosgenin and solanine interact with human erythrocytes and molecular models of their membranes as follows: a) X-ray diffraction studies showed that the compounds at low molar ratios (0.1–10.0 mol%) induced increasing structural perturbation to dimyristoylphosphatidylcholine bilayers and to a considerable lower extent to those of dimyristoylphosphatidylethanolamine; b) differential scanning calorimetry data showed that the compounds were able to alter the cooperativity of dimyristoylphosphatidylcholine, dimyristoylphosphatidylethanolamine and dimyristoylphosphatidylserine phase transitions in a concentration-dependent manner; c) in the presence of steroids, the fluorescence of Merocyanine 540 incorporated to the membranes decreased suggesting a fluidization of the lipid system; d) scanning electron microscopy observations showed that all steroids altered the normal shape of human erythrocytes inducing mainly echinocytosis, characterized by the formation of blebs in their surfaces, an indication that their molecules are located into the outer monolayer of the erythrocyte membrane.

1. Introduction

Solanum plants are a widespread distributed family with more than 3000 species of trees, shrubs and herbs, native from Central and South America. Their study is a topic of great interest for botanists and pharmaceutical industries interested in novel molecules as potential drugs. Among the most representative plants of this family can be mentioned: potatoes (*Solanum tuberosum*), tomatoes (*Solanum lycopersicum*), and eggplants (*Solanum melongena*) [1]. *Solanum* plants are recognized for containing in the bark, fruit and roots a wide variety of lactones, glycosides, alkaloids and flavonoids [2]. These compounds are secondary metabolites, and their content can vary greatly depending on the growing conditions of the plant. The role of steroids and glycoalkaloids in plants is not fully understood; they probably belong to the chemical armory of plants that protects them from insects and herbivorous attacks. These metabolites are considered important ingredients responsible for several health benefits in traditional medicine [3–5]. Steroid alkaloids generally occur as glycosides, the aglycones which possess the C27-carbon skeleton of cholestane and belong to the following five groups: spirosolanes, e.g. solasodine, epiminocholestanes, e.g. solacongestidine, solanidanes, e.g. solanidine, solanocapsine, e.g. solanocapsine, and 3-aminospirostanes, e.g. jurubidine. Steroidal sapogenins such as diosgenin contain steroid or other triterpene frameworks as their key organic feature. Sapogenins are the aglycone portion of the family of natural products known as saponins.

Our interest focused on assessing the interaction of three natural occurring steroids present in *Solanum* plants with cell membranes: diosgenin (Fig. 1A), a steroid sapogenin with a spirostan ring, found in *Dioscorea* and other *Solanum* plants that have been traditionally used for the treatment of diabetes [6,7], hypercholesterolemia [8,9] and gastrointestinal ailments [10], solasodine (Fig. 1B), a natural derivative of diosgenin, in which the oxygen in the spiro-ring is replaced by nitrogen; it is the aglycone of several glycoalkaloids such as solasonine and solamargine, and has a great demand in pharmaceutical industries as a precursor in the production of other steroidal compounds like cortisone and progesterone [11]. It has been reported that solasodine has embryotic, teratogenic, antifungal, antiviral and molluscicidal effects

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPS, dimyristoylphosphatidylserine; SEM, scanning electron microscopy; DSC, differential scanning calorimetry; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; RFU, relative fluorescence units

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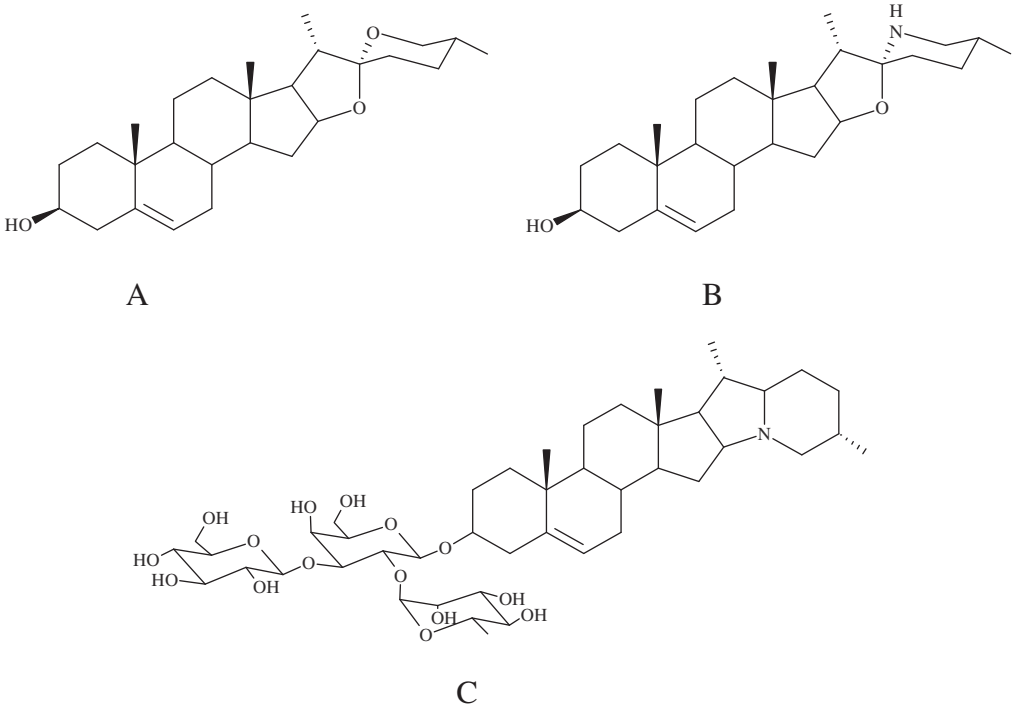

Fig. 1. Structural formula of A) solasodine B) diosgenin and C) solanine.

[12]. Solanine (Fig. 1C), is a glycoalkaloid with a branched α-solatriose (α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-galactopyranose) side chain attached to the 3-OH group of solanidine. It has antifungal activity and inhibitory effects on the growth of insects and other phytopathogens [13].

Despite several reviews about the properties of these molecules, there is a lack of information about the molecular mechanism of their biological activity [14]. Pharmacological activity of a biologically relevant molecule frequently involves an initial interaction with cell membranes, even if they are not the final targets. Cell membrane is a diffusion barrier which protects the interior of the cell. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with chemical species. With the aim to better understand the molecular mechanism of the interaction of solasodine, diosgenin and solanine with biological membranes we utilized human erythrocyte membranes and molecular models of red cell membranes. Human erythrocytes were chosen because its membrane has been extensively studied [15,16], they do not have internal organelles, which make them an ideal cell system for studying basic drug–membrane interactions [17]. On the other hand, although less specialized than many other cell membranes, they carry on enough functions in common with them such as active and passive transport, and the

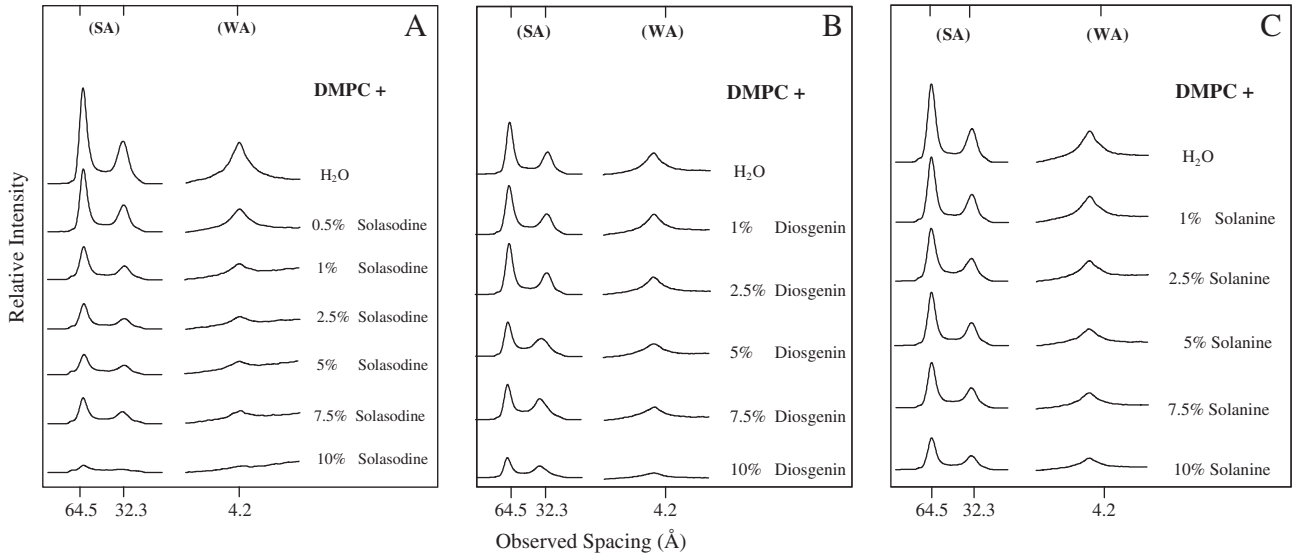

Fig. 2. X-ray diffraction patterns of dimyristoylphosphatidylcholine (DMPC) in water and aqueous suspensions of (A) solasodine, (B) diosgenin and (C) solanine; (SA) small-angle and (WA) wide-angle reflections.

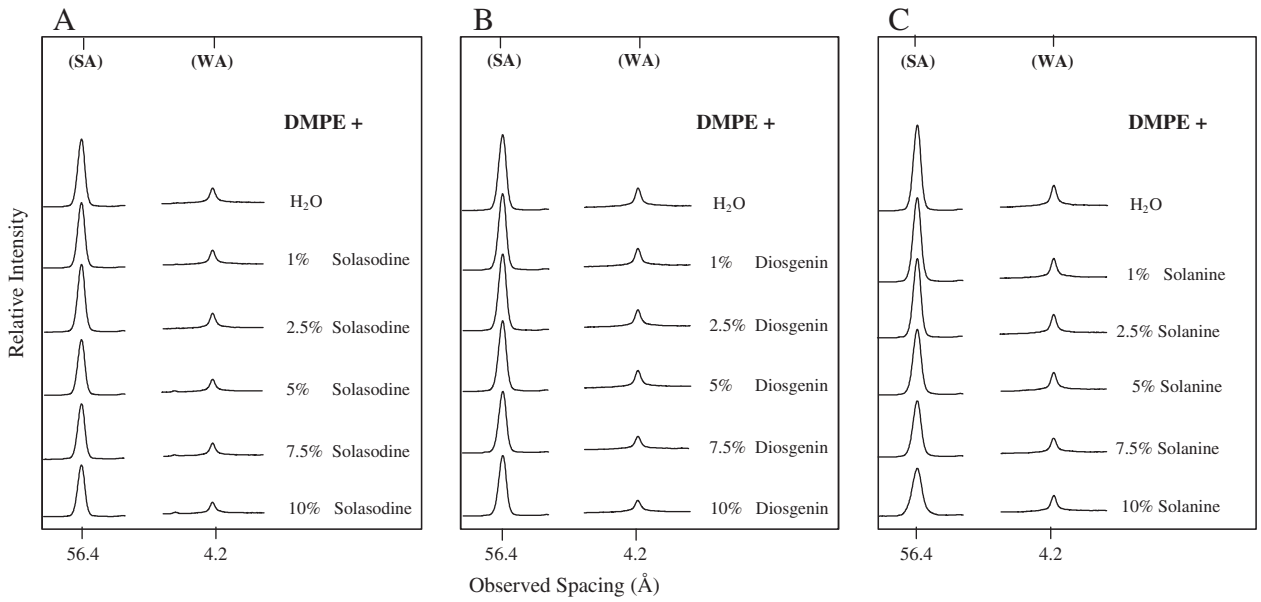

Fig. 3. X-ray diffraction patterns of dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous suspensions of (A) solasodine, (B) diosgenin and (C) solanine; (SA) small-angle and (WA) wide-angle reflections.

production of ionic and electric gradients, to be considered representative of the plasma membrane in general. Because cell membranes are highly complex systems, there is an ample use of molecular models of membranes consisting in lipid bilayers with controlled composition and concentration which are simpler to study [18]. The use of model membranes that mimic the complexity of natural cell systems provides the opportunity to investigate how exogenous molecules interact with cells to carry out their physiological activity. With this aim we utilized well-established molecular models of the human erythrocyte membrane consisting in dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidylserine (DMPS) bilayers, representative of phospholipids classes located in the outer and inner monolayers of eukaryotic cell membranes, particularly of the human erythrocyte [19,20]. The capacity of solasodine, diosgenin and solanine to perturb the bilayer structure of DMPC and DMPE was evaluated by X-ray diffraction; DMPC multilamellar vesicles (MLVs) were studied by fluorescence spectroscopy; DMPC, DMPE and DMPS multilamellar vesicles (MLVs) were studied by differential scanning calorimetry (DSC), and intact human erythrocytes were observed by scanning electron microscopy (SEM). These systems and techniques have been used in our laboratories to study the membrane-perturbing effects of other biologically relevant molecules [21,22].

2. Material and methods

2.1. X-ray diffraction studies of DMPC and DMPE multilayers

Synthetic DMPC (lot. 140PC-236, MW 677.9), DMPE (lot. 140PE-58, MW 635.9) and DMPS (lot. 140PS-81, MW 701.8) from Avanti Polar

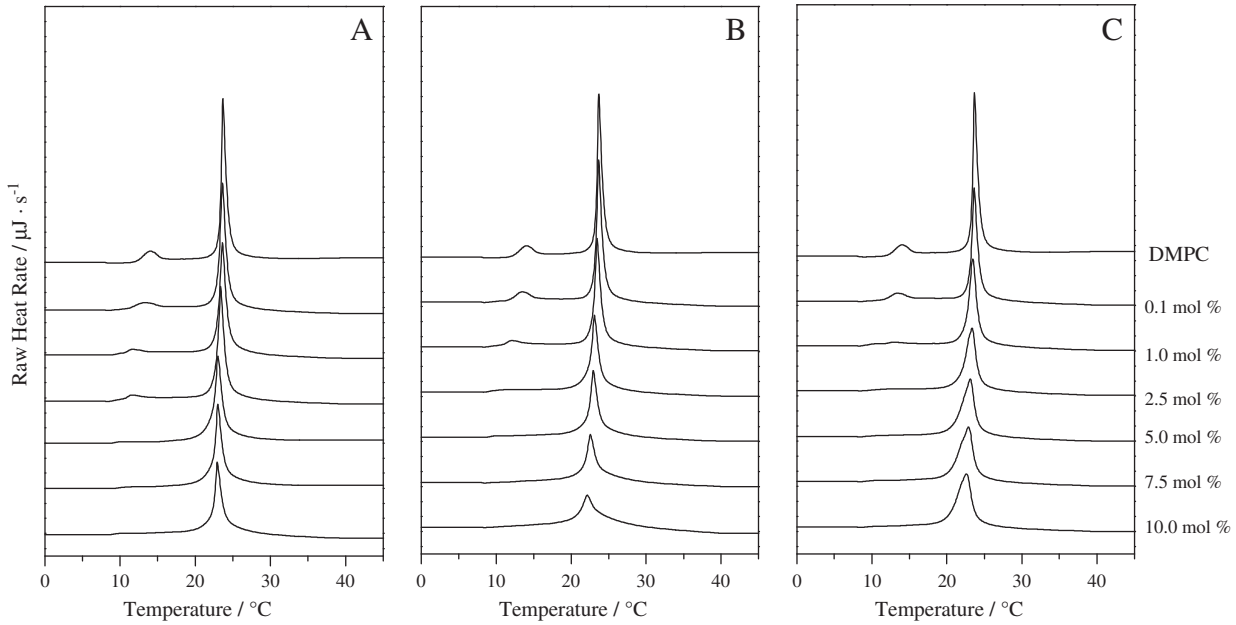

Fig. 4. Representative DSC thermograms obtained for multilamellar DMPC liposomes in the presence of (A) solasodine, (B) diosgenin and (C) solanine. The numbers at the right side represent the steroid content.

Lipids (Alabaster, AL, USA), solasodine (lot. 70192, MW 413.3) from ICN Biomedicals (South Chillicothe, OH, USA), solanine (lot. 108 K5054, MW 868.1) and diosgenin (lot. 129K1800V, MW 414.6) from Sigma (St. Louis, MO, USA) were used without further purification. The capacity of solasodine, diosgenin and solanine to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. About 2 mg of each phospholipid was mixed in Eppendorf tubes with 200 μl of (a) distilled water and (b) appropriated amounts of solasodine, solanine and diosgenin in a range molar proportion (0.5% to 10%). The specimens were incubated for 30 min at 37 °C and 60 °C with DMPC and DMPE respectively. The samples were then transferred to 1.5 mm dia special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany), centrifuged for 45 min at 1459 *g* and X-ray diffracted using a 0.25 mm inner diameter glass collimator. Specimen-to-detector distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray generator was used. The relative reflection intensities were obtained in an MBraun PSD-50M linear position-sensitive detector system (Garching, Germany) and no correction factors were applied. All experiments were performed at 18 ± 1 °C, which is below the main phase transition temperature of both DMPC and DMPE. Higher temperatures would have induced transitions to more fluid phases making the detection of structural changes harder. Each experiment was performed in triplicate.

2.2. Differential scanning calorimetry (DSC) on DMPC, DMPE and DMPS

Solanine stock solution was prepared in a solvent mixture of ethanol:methanol (3:1, v/v), and diosgenin and solasodine were dissolved in chloroform. Chloroform solutions of phospholipid (DMPC, DMPE or DMPS) and the appropriate amount of steroids were dried under a stream of nitrogen in order to obtain a thin film at the bottom of the test tubes. Last traces of solvents were removed by keeping the samples under reduced pressure (about 13.3 Pa) for 30 min. Dried lipids were hydrated with buffer (10 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 7.4) and MLVs were formed by vortexing the samples above the main phase transition temperature of the lipids for at least 15 min. The final phospholipid concentration in buffer was 1 mM. DSC measurements were performed using a Nano DSC device (TA Instrument). The sample cell was filled with 400 μl of MLV suspension and, as a reference an equal volume of buffer was used. Cells were sealed and equilibrated for about 10 min at starting temperature. Heating/cooling rates were 1 °C per minute and the scans were recorded within a range of 5 °C to 65 °C. Heating scans were previously carried out. The reference scan was subtracted from the sample scan. Each data set was analyzed and the values of transition temperatures were calculated using a software package supplied by TA Instruments. At least three independently prepared samples were measured to check the reproducibility of the DSC experiments. The accuracy was ±0.1 °C for the main phase transition temperature and ±1 kJ mol^{-1} for the main phase transition enthalpy.

2.3. Fluorescence measurements of DMPC large unilamellar vesicles (LUVs)

Liposomes were prepared by film hydration procedure [23]. Briefly, DMPC (1 mg/ml) and the steroids (1 to 10 mol%) were dissolved in chloroform, evaporated under a nitrogen flow and the resulting film was dried under vacuum to remove the residual solvents. Liposomes were obtained by adding to the film phosphate buffer (pH 7.4) and then heating at a temperature above that of the gel–liquid crystalline phase transition (23.5 °C) and vortexing four times. Samples were shaken for 1 h in a water bath at 37 °C to hydrate the liposomes. Liposome size was homogenized through 0.22 μm polycarbonate membranes (Avanti, Alabaster, AL, USA). Fluorescence spectra were obtained using a Synergy HT spectrophotometer (Biotek, Winooski, VE, USA) equipped with a thermostatic cell holder and a slit ±3 nm. The fluorophore merocyanine 540 (MC540) (Sigma (St. Louis, MO, USA) (0.1 μg/ml) in 0.01% (v/v) ethanol (final concentration of ethanol in the sample) was added to liposomes. Samples were maintained at low temperature and then heated through the whole temperature range incubating for 15 min at each point. After that, the fluorescence emission at 585 nm (excited at 540 nm) was determined.

2.4. Scanning electron microscopy (SEM) studies of human erythrocytes

Blood was obtained from healthy human male donor not receiving any pharmacological treatment. Blood samples (0.1 ml) were obtained by puncture of the ear lobule and received in an Eppendorf tube containing 10 μl of heparin (5000 UI/ml) in 0.9 ml of saline solution (NaCl 0.9%, pH 7.4). The sample was centrifuged at 1013 *g* for 10 min and the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. The sedimentary red blood cells were suspended in 0.9 ml of saline solution and fractions of this stock of red blood cells

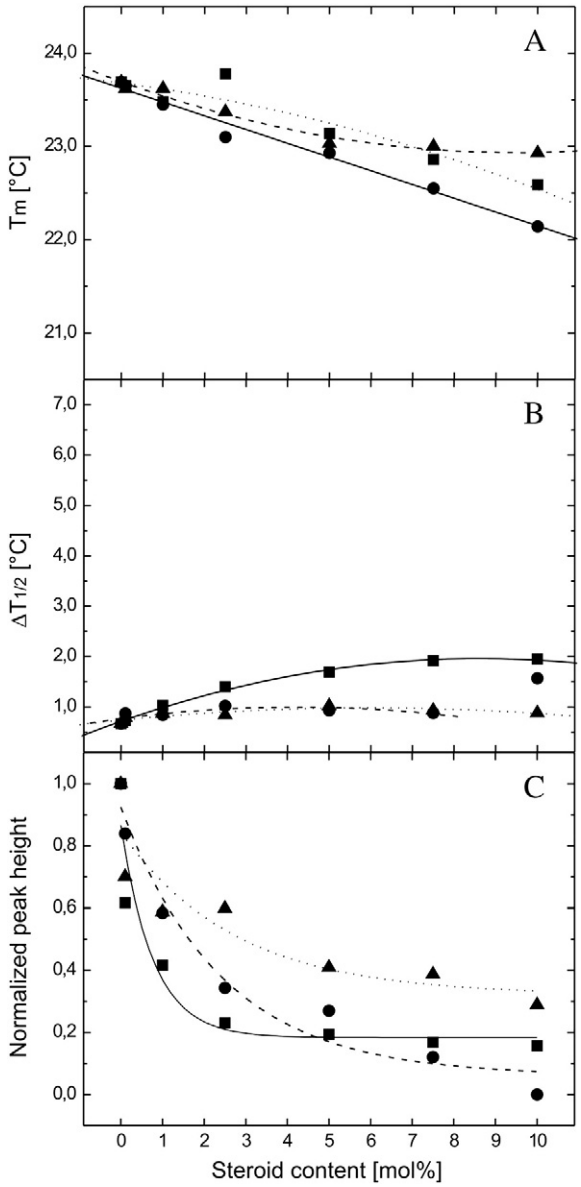

Fig. 5. A plot of main phase transition temperature (A), half peak width (B) and normalized peak height (C) of DMPC multilamellar liposomes as a function of solasodine (▲), diosgenin (●) and solanine (■) content.

suspension (RBCS) and saline solution were placed in Eppendorf tubes to prepare (a) the control, by mixing 100 μl of saline solution plus 100 μl RBCS, and (b) a range of molar proportions of solasodine, diosgenin and solanine (0.5–10%) by mixing 100 μl of RBCS with a known amount of each glycoalkaloid. All samples were then incubated for 1 h at 37 °C. After the incubation, samples were centrifuged (1013 *g* for 10 min) and the supernatant was discarded. Then, they were fixed overnight at 4 °C by adding 500 μl of 2.5% glutaraldehyde to each one. The fixed samples were washed with distilled water, placed over Al glass cover stubs, air dried at 37 °C for 30 min to 1 h, and gold-coated for 3 min at 13.3 Pa in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in a Jeol SEM (JSM 6380 LB, Japan).

3. Results

3.1. X-ray diffraction studies of DMPC and DMPE multilayers

The molecular interactions of solasodine, diosgenin and solanine with DMPC and DMPE multilayers were determined by X-ray diffraction experiments at 18 ± 1 °C. Fig. 2 exhibits the results obtained by incubating DMPC with water and solasodine, diosgenin and solanine. As expected, water altered the DMPC structure: its bilayer repeat (bilayer width plus the width of the water layer between bilayers) increased from about 55 Å in its dry crystalline form to 64.5 Å when immersed in water (gel phase) and its small-angle reflections (SA), which corresponds to the bilayer repeat, were reduced to only the first two orders of the bilayer width [24]. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region (WA), which corresponds to the distance between the neighboring planes in the nearly hexagonal packing of the fully extended acyl chains. These results were indicative of the changes in layer and chain packing structure reached by DMPC bilayers. Fig. 2A discloses that after exposure to 0.5% solasodine there was a weakening of the small- and wide-angle lipid reflection intensities; addition of solasodine in increasing molar proportions caused a monotonically decrease in the phospholipid reflection intensities, until they practically disappeared at a concentration of 10%, condition under which a disruption of the in-plane structure and the bilayers stacking occurred. Fig. 2B shows the results of DMPC incubated with diosgenin. Increasing molar proportions of the steroids also caused a weakening in DMPC reflection intensities, but at about ten times higher molar concentration (5%); however, with 10% diosgenin still remained DMPC bilayer structure. Fig. 2C clearly shows that solanine was the steroid that produced the lowest structural perturbation of DMPC bilayers. X-ray diffraction patterns of DMPE bilayers incubated with water, solasodine, diosgenin and solanine are shown in Fig. 3. As reported elsewhere, water did not significantly affect the bilayer structure of DMPE [24]. Fig. 3 also shows that even at the highest assayed molar proportion (10 mol%) of all three steroids none induced a significant structural perturbation of DMPE bilayers.

3.2. Differential scanning calorimetry (DSC) on DMPC, DMPE and DMPS bilayers

DSC heating profiles of pure DMPC multilamellar vesicles and these containing steroids are presented in Fig. 4. In the temperature range of 5–40 °C, fully hydrated DMPC liposomes, in the absence of any additives, underwent a sharp main-transition at about 24 °C, with an enthalpy change (ΔH) of 23 kJ mol^{-1}. This transition corresponds to the conversion of the rippled gel phase ($P_{\beta'}$) to the lamellar liquid-crystal (L_α) phase. The smaller transition, called pretransition, occurred at 14 °C and it was characterized by a ΔH of 3 kJ mol^{-1}. Here, the transition temperatures corresponded to the transition peak at the maximal peak heat, and transition enthalpies corresponded to the integrated area under the peak divided by the lipid concentration. Data are in agreement with previous reports [25,26]. The interaction of the steroids with DMPC clearly altered the thermotropic behavior of the bilayers as illustrated in Fig. 4. The pretransition was entirely abolished in DMPC vesicles containing 5 mol% of solasodine and even less content (2.5 mol%) of diosgenin or solanine. This suggests that the steroids displayed very strong distortions of DMPC packing in the gel state. Moreover, upon addition of increasing amounts of steroids, the temperature of main phase transition shifted to lower values, the peak broadened and its height decreased (Fig. 5A–C).

The representative high-sensitivity DSC heating thermograms obtained for pure DMPE multibilayer vesicles and binary mixtures of DMPE and the steroids at various concentrations are shown in Fig. 6. Dispersions of DMPE exhibited a single thermotropic phase

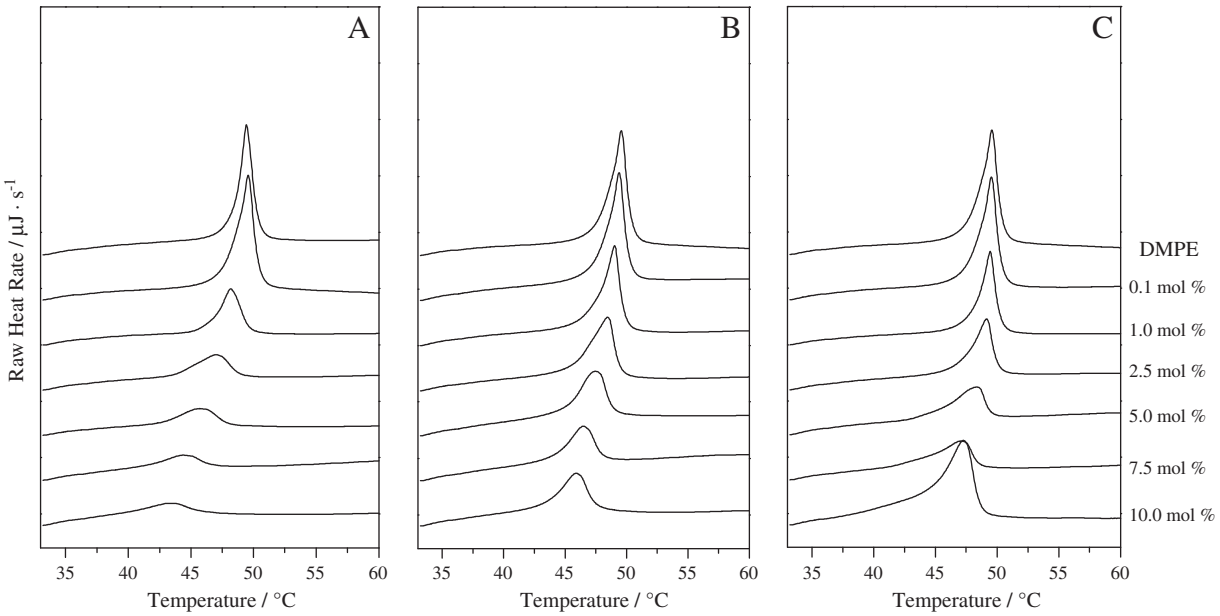

Fig. 6. Representative DSC thermograms obtained for multilamellar DMPE liposomes in the presence of (A) solasodine, (B) diosgenin and (C) solanine. The numbers at the right side represent the steroid content.

transition in the temperature range from 25 °C to 80 °C. It corresponds to the highly cooperative $L_\beta \rightarrow L_\alpha$ phase transition at 49.6 °C with a ΔH of 28 kJ mol^{-1} (gel-to-liquid-crystal transition). Thermodynamic parameters found for DMPE are concurrent with the literature data [27]. Considering the DSC profiles depicted in Fig. 6, it can be concluded that the thermotropic behavior of DMPE bilayers was significantly influenced by the three steroids. Generally, they induced a broadening of the transition (Fig. 7B), which may reflect a reduction of the cooperativity between the lipid acyl chains. This, together with the lowering of the transition temperature (Fig. 7A) is an indication that the presence of steroids perturbed DMPE multibilayers, both in the gel L and in the liquid-crystalline L phases. This effect is much more pronounced than in the case of DMPC bilayers.

The influence of the steroids on the thermotropic transition of DMPS is presented in Fig. 8. In the absence of the steroids, DMPS exhibited a single, fully reversible and relatively energetic transition from the gel phase to the liquid-crystalline phase. It was found that transition occurred at 38.7 °C with an enthalpy change of 27.63 kJ mol^{-1}, in agreement with previous results [28,29]. The presence of increasing amounts of steroids resulted in shifting the phase transition temperature to lower values and a decrease of the cooperativity of this transition (Fig. 9A–C). These results suggest that hydrophobic moieties of the steroids incorporated into the phosphatidylserine acyl chains palisade with its consequent perturbation. Moreover, in the presence of all three steroids, the thermograms were fairly asymmetric, suggesting the presence of steroid-enriched domains. Additionally, in the case of solasodine (at content higher than 5 mol%), there is evidence of a new shoulder in the lower part of the thermograms.

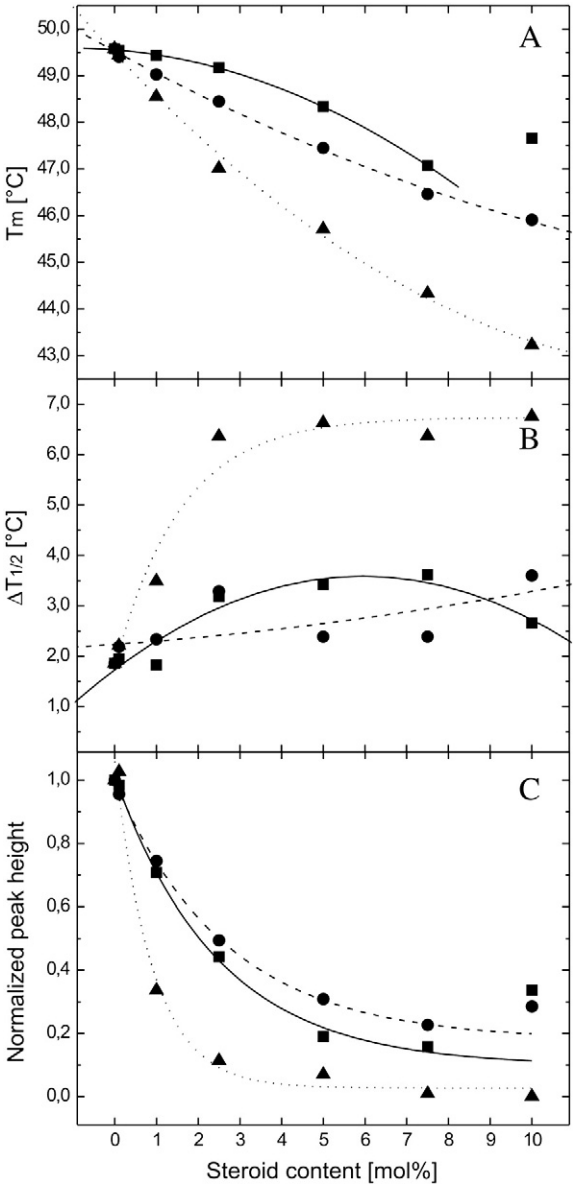

Fig. 7. A plot of main phase transition temperature (A), half peak width (B) and normalized peak height (C) of DMPE multilamellar liposomes as a function of solasodine (▲), diosgenin (●) and solanine (■) content.

3.3. Fluorescence measurements of DMPC large unilamellar vesicles (LUVs)

It has been proposed that the merocyanine 540 fluorophore resides slightly above domain of the glycerol backbone of phospholipid liposomes, and it has been shown that MC540 binding is very sensitive to lipid packing of phospholipid bilayers [30]. We made use of the dye sensitivity to lipid packing to measure the changes in DMPC vesicles. The dependence of the fluorescence intensity of MC540 on temperature, under the presence of the three steroids (at 10 mol%), is shown in Fig. 10. In empty vesicles (control), fluorescence of MC540-liposomes showed a typical increase with inflection change close to DMPC main transition temperature (≈24 °C). The presence of solasodine resulted in loosely packed membranes when compared to solanine, diosgenin and empty vesicles.

3.4. Scanning electron microscope (SEM) studies of human erythrocytes

The effects of the in vitro interaction of solasodine, diosgenin and solanine with human erythrocytes were followed by SEM. Under physiological conditions, normal human red blood cells assume a flattened biconcave disk shape (discocyte) ~8 μm in diameter. Red blood cells were incubated with the three steroids in a range of molar proportions (0.1–10 mol%) and in each case one sample was incubated with saline solution as control. Results of incubation of erythrocytes with solasodine are shown in Fig. 11. As it can be observed, the steroid induced changes in the erythrocyte normal shape: 1% solasodine produced knizocytes (a triconcave red-cell shape) and some echinocytes (a spiny configuration with blebs in the cell surface); increasing solasodine concentrations up to 10% increased the presence of echinocytes. SEM observations of red blood cells incubated with diosgenin are shown in Fig. 12. The analysis revealed that erythrocytes treated with 1 mol% of diosgenin underwent slight changes over the cell surfaces. At higher molar proportions, diosgenin induced a polymorphic cell population with presence of echinocytes, knizocytes, stomatocytes (cup-shaped cells), elliptocytes (abnormally elongated shaped red cells), spherostomatocytes (spherically shaped cells with lightly or minor cup profiles) and microcytes (abnormally small-sized erythrocytes). SEM examinations of erythrocytes in the presence of solanine (Fig. 13) revealed that its lowest molar proportion (1%) induced initial echinocytosis, whereas higher concentrations provoked generalized echinocytic deformation of the red blood cells.

4. Discussion

The study of *Solanum* steroid–cell membrane interactions is essential for understanding the molecular mechanism of their physiological properties. Amazingly, studies on the effects of solasodine, diosgenin and solanine on cell membranes are not too frequent, but a few of them have indicated that these compounds produced some important effects. Thus, solasodine and diosgenin have been tested for their biological activity on human 1547 osteosarcoma cells. Solasodine induced an important DNA fragmentation, while diosgenin is an effective cell death inductor in the human osteosarcoma 1547 cell line [31], inhibiting cell growth and apoptosis induction by p53 activation. Diosgenin has been previously reported to induce apoptosis in different human cancer cell lines [32,33]. However, it has not fully understood the molecular effects induced by

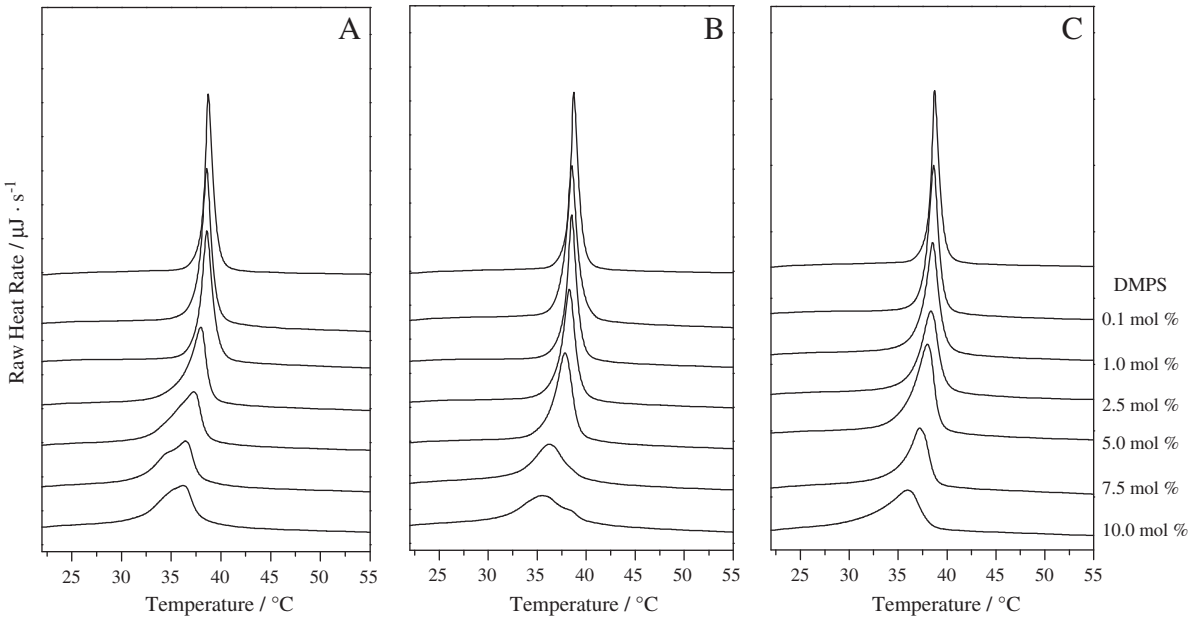

Fig. 8. Representative DSC thermograms obtained for multilamellar DMPS liposomes in the presence of (A) solasodine, (B) diosgenin and (C) solanine. The numbers at the right side represent the steroid content.

solasodine, diosgenin and solanine in human erythrocytes. This study presents the following evidence that the three compounds affect the membrane bilayers and therefore the proper functioning of human erythrocytes. X-ray diffraction experiments performed on DMPC and DMPE, classes of the major phospholipids present in the outer and inner erythrocyte membrane, respectively showed that the three steroids interacted mainly with DMPC, affecting both its polar head and acyl chain regions. The strongest effect was observed with solasodine and the lowest with solanine. These results can be explained on the basis of the different nature of DMPC and DMPE, and of solasodine, diosgenin and solanine. DMPC and DMPE differ only in their terminal amino groups, being these $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their dry crystalline phases [24] with the hydrocarbon chains mostly parallel and extended and the polar head groups lying perpendicularly to them. However, DMPE molecules pack tighter than those of DMPC. This effect, due to DMPE smaller polar group and higher effective charge, stands for a very stable multilayer arrangement that is not significantly perturbed by the presence of water. The strong hydrogen network of DMPE bilayers is certainly a reason for the reduced penetration of the compounds into its interfacial head group region. On the other hand, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces with the resulting width increase. This phenomenon allows the incorporation of the compounds into DMPC bilayers with the resulting disruption of DMPC bilayer structure.

Experiments performed by Keukens et al. with red blood cells demonstrate that glycoalkaloid solanine induced hemolysis at a concentration close to 100 μM. They also evaluated the effects of solanine on human epithelial colon carcinoma cell line (CaCo-2), reporting that similar concentrations of the glycoalkaloid tested in the hemolysis experiments induced membrane disruption effects on CaCo-2 cells. Their results suggested that solanine bounded to the membrane bilayer during the incubation process, being the lipids of the membrane the primary target of glycoalkaloid [34]. Moreover, cytotoxity studies on rat and human intestinal mucosal epithelium cells proved the ability of solanine to alter the membrane integrity [35].

The lipophilicity of steroids, and thereby their potential for interaction with the hydrophobic part of the phospholipid bilayer can be evaluated by the corresponding octanol/water partition coefficient. The logarithm of this value (log *P*) is used as a measure of the hydrophobicity and in the case of neutral molecules it is accepted as the value satisfactorily modeling their uptake into membranes. In fact, as it can be appreciated in Fig. 2, the most lipophilic steroids solasodine (log $P = 6.83$ and diosgenin (log $P = 6.34$) [36]) induced the higher structural perturbation to DMPC. However, with its relatively high partition coefficient (log $P = 2$, [36]) even solanine, although the least lipophilic steroid among studied ones, is in dispersion of multilamellar liposomes almost exclusively present in the lipid phase. This was furthermore found to be supported by our DSC data. Taken together steroid-induced decrease of T_m and the reduction of the phase transition cooperativity observed in the presence of any of the three steroids, it constitutes a proof that these bioactive compounds are intercalated into the hydrophobic matrix of the membrane. Moreover, it indicates that steroids act as spacers disordering the lipid molecules within the bilayer and thus bringing about its fluidization. At the gel to liquid crystalline phase transition the gauche conformers of the lipid fatty acyl residues appear which bring about the decrease of hydrophobic thickness of PC bilayers by approximately one third. Thus, the introduction of guest molecule oriented with the long axis perpendicular to the bilayer plane might result in disturbing the relative stability of the phases, according to the degree of mismatch between the effective hydrophobic length of the molecule and the phospholipid hydrocarbon chains. Actually, the aglycone portion of the three compounds under our studies has similar structure, as solasodine is an analogue of diosgenin formed by replacing oxygen in the spiro-ring with nitrogen, while solanine is a glycoalkaloid with a branched carbohydrate residues (α-solatriose) attached to the aglycone solanidine. Correspondingly, T_m shifts observed in the DSC experiments for DMPC bilayer in the presence of any of these steroids are fairly alike. Nevertheless, the capability of particular compounds to modify thermotropic behavior of DMPC bilayer differs markedly, with the strongest effect of diosgenin. It is also noteworthy that although, the shifts of the thermogram peaks are rather small when compared to PE or PS counterparts, steroids are capable to affect DMPC phase transitions at relatively low concentrations in contrast to cholesterol as well as other substances. The DMPC pretransition is diminished when solasodine or solanine content exceeds 2.5 mol% and even less for diosgenin. Moreover,

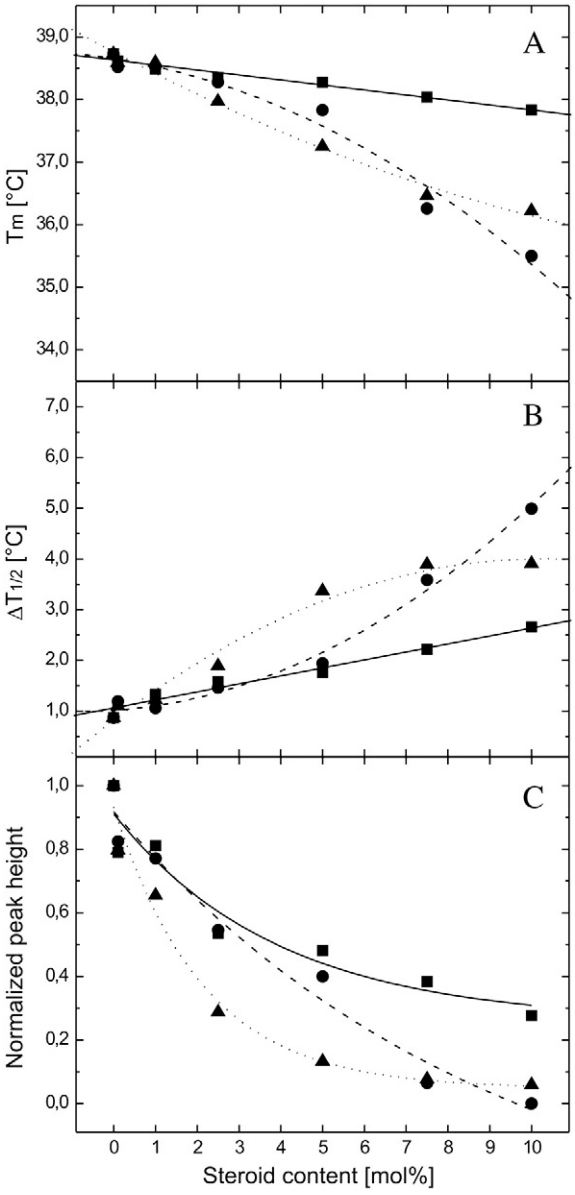

Fig. 9. A plot of main phase transition temperature (A), half peak width (B) and normalized peak height (C) of DMPS multilamellar liposomes as a function of solasodine (▲), diosgenin (●) and solanine (■) content.

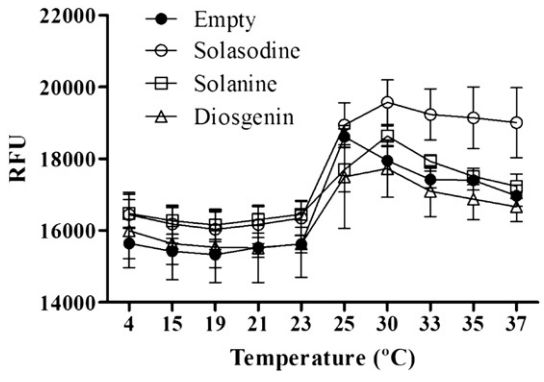

Fig. 10. Fluorescence intensity of MC540 in DMPC vesicles with steroids (10 mol%). Data are from three independent assays shown as RFU (relative fluorescence units).

the extent of suppression of gel to liquid crystalline transition indicates that even though rather large, rigid and bulky, the steroids studied in this paper can penetrate into the DMPC bilayer and induce loose packing of the lipid molecules in the gel state, which was also observed in the X-ray and fluorescence experiments. In fact, Williamson et al. observed that fluorescence of MC540 increased in the presence of loosely packed membranes when compared to that in the presence of lipid vesicles in the gel phase [36]. Furthermore, Wilson-Ashworth et al. showed that MC540 fluorescence, presumably reflecting lipid spacing, varied more extensively with temperature compared to that of the other probes (prodan, bis-pyrene and nystatin) [37]. In our case, the enhanced fluorescence intensity of MC540 with solasodine suggests decreased organization of lipids. The effect of the molecular packing on the fluorescence of MC540 is best illustrated when the membrane experienced gel–fluid phase transition, where the fluorescence of the dye increased in a step-like fashion. Meanwhile, above the main transition the increase of fluorescence intensity is affected by both temperature and lipid molecular packing, and decreasing when temperature rises [38]. As it can be seen in Fig. 10, only solasodine was able to maintain the fluorescence intensity above the main transition, which would indicate increased membrane surface area accessible for the binding of the dye due to loss of lipid packing as reported by Langner et al. [38].

Our DSC data are in line with the findings by Bae et al. who showed the very profound effect of low concentrations of clover saponins on DMPC liposomes [39]. The transition peak of DMPC liposomes was broadened and shifted to lower temperatures and proportional to the concentration of saponins. The reduction of the phase transition temperature was related with the penetration of the saponins into the lipid bilayers, inducing loose packing of the lipids. This means that clover saponin is markedly effective in permeating into lipid bilayers. Finally, Mbadugha and Menger [4] examined the importance of carbohydrate residues of steroids for their effectiveness in perturbing DPPC thermal behavior. Taking advantage of DSC, they showed that while bisdesmosidic saponin molecule might span a bilayer, which via interdigitation locally contracts to accommodate the guest molecule, a compound with only a single galactose fixed to a cholesteryl aglycone can slip its steroid moiety between the hydrophobic fatty acid chains without inducing a bilayer contraction. Accordingly, in the presence of the latter, monodesmonic saponin, DPPC main phase transition peak almost disappears.

Among the structural features of phospholipids that can influence their interactions with steroids, apart from hydrocarbon chain length and the conformation and degree of saturation, which are beyond the scope of this work, a key role is played by net charge on the headgroup and the inter headgroup hydrogen bonding. Incorporation of increasing amounts of solasodine or diosgenin into DMPE bilayer progressively decreased the cooperativity and enthalpy of the chain melting transition, which is almost completely abolished at 10 mol% of solasodine. While solasodine appears to be fully miscible in both gel and liquid-crystalline states of DMPE, gradual broadening and lowering of the peak corresponding to the transition as well as T_m shift are observed for DMPE in the presence of solanine only up to 7.5 mol%. Further increment of solanine content results in the increase of T_m and ΔH; the behavior that was previously described for the cholesterol/DMPE mixtures [40] and discussed in terms of the limited miscibility in gel state or the tendency to facilitate the lateral phase separation in these systems. However, in contrast to the studies by McMullen [41] we have not observed the decrease of the temperature and enthalpy of the chain melting transition for these mixtures on subsequent cooling scans (data not shown).

The limited lateral miscibility of steroids in PE relative to PC bilayers can be explained on the basis of relatively strong inter headgroup hydrogen bonding and electrostatical attractions that takes place in PE and gives priority to lipid–lipid over steroid–lipid interactions. Thus, the insertion of steroid molecules is unfavorable, especially at low temperatures, when PE molecules are packed tightly. Consequently, even at

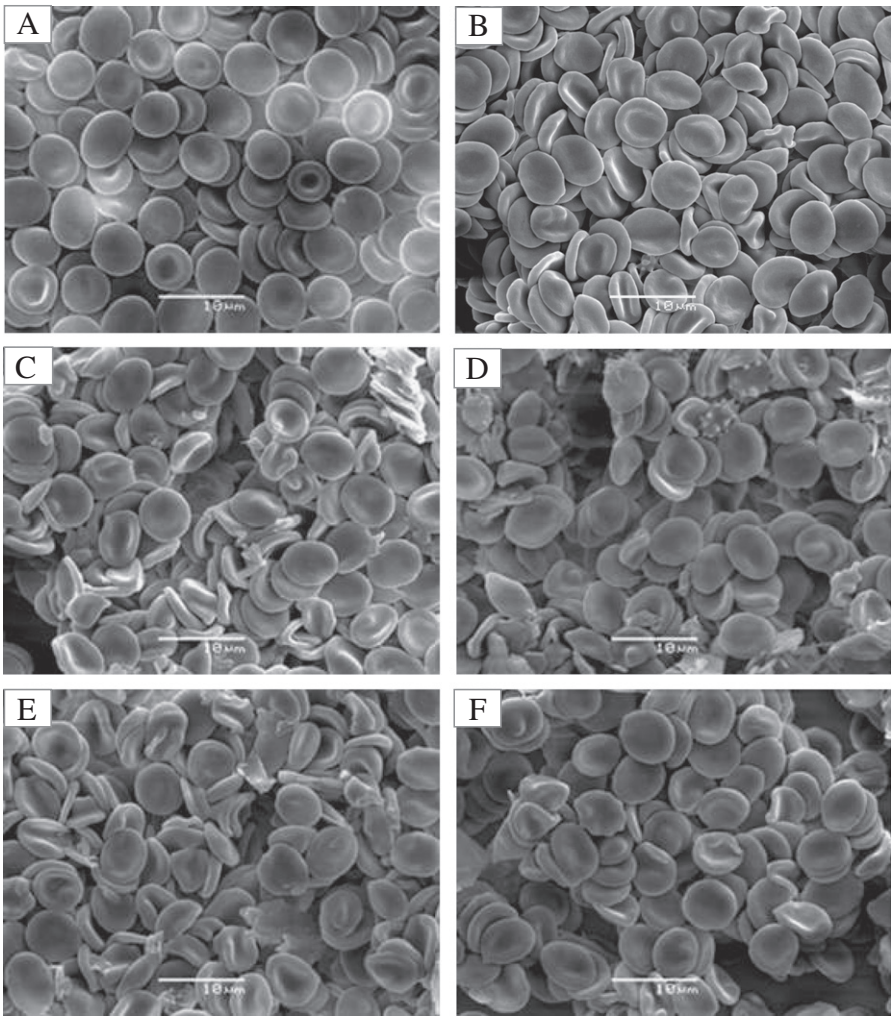

Fig. 11. Effects of solasodine on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 1.0 mol%; (C) 2.5 mol%; (D) 5.0 mol%; (E) 7.5 mol%, and (F) 10.0 mol% of solasodine.

low concentrations, the observed calorimetric profiles (Fig. 5) appear to be superposition of the two components: a sharp and a broad one. The former, identified as s-poor, decreases in proportion to the latter (s-rich) as steroid concentration increases. Similar findings were reported by McMullen et al. who studied the effect of cholesterol on PC and PE lipid bilayers [40,41]. Such differential affinity of cholesterol for different lipids prompted them to suggest the formation in membranes of phospholipid domains selectively enriched or depleted in cholesterol.

X-ray diffraction experiments performed on DMPE were unable to reveal any changes induced by steroids on bilayer structure, while the effect on thermotropic phase behavior was pronounced and generally showed concentration-dependence. The explanation lies in that in the case of X-ray experiments DMPE molecules packed very tightly in a very stable multibilayer arrangement which is not easily perturbed. On the other hand, in DSC experiments DMPE was in the form of multilamellar vesicles which were more accessible to steroid interactions.

DMPS bilayers are an especially interesting model system due to its well defined fully saturated hydrocarbon chains (C14) and to the fact that PS headgroup not only carries a net charge of (−1) but also participates in inter headgroup hydrogen bonding. In the light of Bach et al. findings [42], the hydration of PS is similar to PC, with the number of water molecules bound per lipid molecule estimated as 3.8 and 3.5, respectively. Although phosphatidylserine is negatively charged at neutral pH, the headgroups are connected by intermolecular bonding which prevents additional hydration. PE, being zwitterionic as PC, is less hydrated than PC due to the hydrogen bond interactions. However, many more water molecules per lipid (up to 17) are required to attain the hydrated state with transitions and thermotropic profiles characteristic for the fully hydrated phospholipids [43]. The strongest effect displayed by diosgenin, solasodine and solanine on the thermal behavior of DMPS bilayer observed in our DSC studies is in line with the conclusion drawn by Bach for cholesterol. Based on the findings for mixtures with bilayer forming phospholipids, the averaged solubility of cholesterol in disaturated C14 liposomes decreased in the following order: PC > PE > PS [44]. Correspondingly, the presence of either a net charge on a headgroup or inter headgroup hydrogen bonding acts to intensify steroid influence on phase transitions of phospholipids while the presence of both structural features has a reinforcing effect.

SEM observations of human erythrocytes showed that the three steroids affected to different extents their normal morphology inducing the formation of several different shapes. However, the prevalent one was that of spiculated echinocytes, particularly in the case of solanine (Fig. 13). According to the bilayer couple hypothesis [45,46], shape changes are induced in red cells due to insertion of foreign species in either the outer or inner monolayer of the erythrocyte membrane. Thus, spiculated echinocytes are observed in the first place while cup shapes

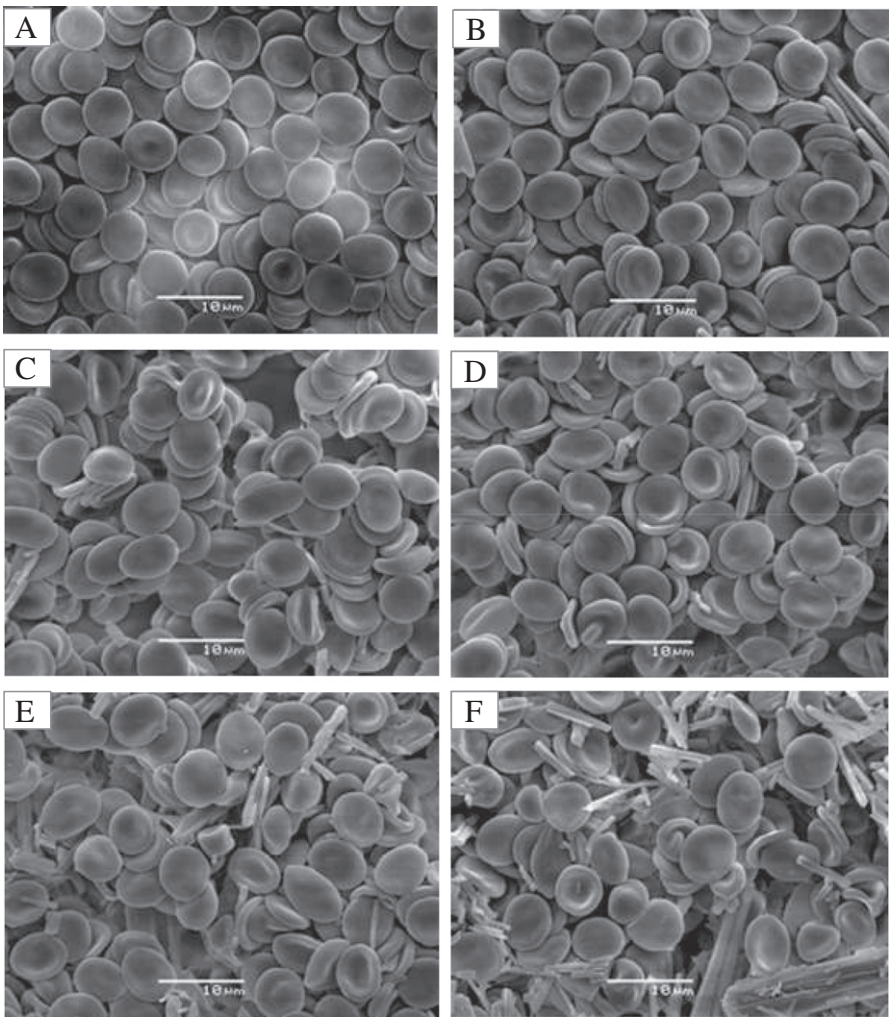

Fig. 12. Effects of diosgenin on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 1.0 mol%; (C) 2.5 mol%; (D) 5.0 mol%; (E) 7.5 mol%, and (F) 10.0 mol% of diosgenin.

(stomatocytes) are produced in the second due to the differential expansion of the corresponding monolayer. The observed formation of echinocytes might imply that the studied steroids preferentially located into the outer monolayer of the erythrocyte membrane. Thus, it is very likely that these molecules interacted mainly with lipids located in the outer moiety. However, interactions with cholesterol and/or proteins cannot be disregarded. In fact, in natural membranes steroids interact through their OH groups not only with polar heads of membrane phospholipids but also with the OH group of cholesterol. This results in their ability to form micelle-like aggregates. Additionally, since hydrophobic aglycone backbone tends to intercalate into the hydrophobic interior of the bilayer, both these factors account for alteration of the lipid environment around proteins. It seems to be well documented that the lipid environment of ion channels, transporters and receptors is of great importance for their function. Interestingly, it has been suggested that alteration of the function of proteins in the plasma membranes is the main reason of the secondary biochemical responses induced by steroids [14]. On the other hand, the glycoalkaloid α-solanine caused most generalized deformation of the erythrocytes, probably due to its interaction with membrane associated cholesterol resulting in the formation of specific complexes which caused disruption of the membrane, which do not occur for aglycones solasodine and diosgenin [14].

5. Concluding remarks

Our experimental findings are certainly of interest as they demonstrated that solasodine, diosgenin and solanine interact with lipid bilayers and the human erythrocyte membrane affecting the cell morphology. It must be considered that an alteration of the normal biconcave shape of red blood cells increases their resistance to enter into capillaries, which could contribute to a decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion [47,48]. Functions of ion channels, receptors and enzymes immersed in cell membrane lipid moieties also might be affected. These findings may also provide a new insight into the possible mechanism of action of these three compounds.

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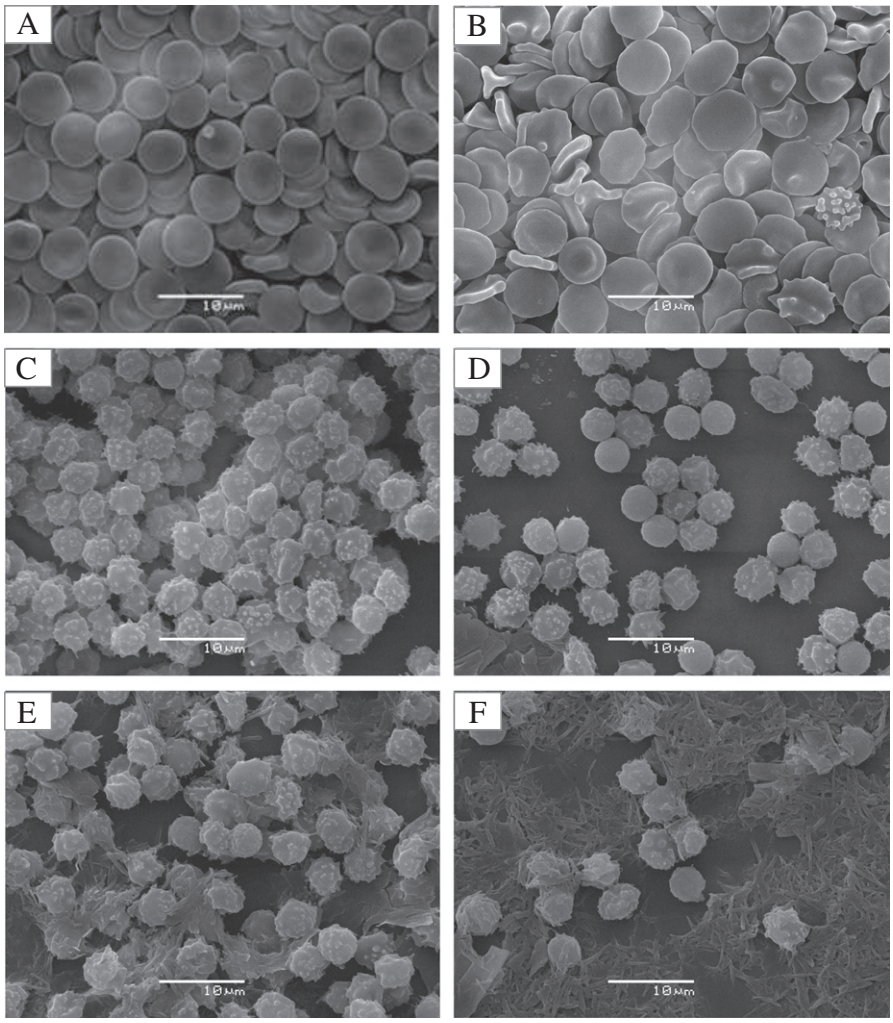

Fig. 13. Effects of solanine on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 1.0 mol%; (C) 2.5 mol%; (D) 5.0 mol%; (E) 7.5 mol%, and (F) 10.0 mol% of solanine.

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