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# *Hyperici herba* extract interaction with artificial lipid bilayers

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## Abstract

*Hyperici herba* (Hyp) is the aerial part collected during the flowering period from the well-known herb, *Hypericum perforatum*. Black lipid membrane experiments were performed to investigate the effect of the ethanolic Hyp extract on the electrical properties (capacitance and conductance) of artificial lipid bilayers. Hyp extract (1–10 $\mu$g mL$^{-1}$) induced a concentration-dependent increase of both specific transmembrane capacitance and conductance in phosphatidylcholine (PC) membranes. The effect on conductance was enhanced when the Hyp extract (3 $\mu$g mL$^{-1}$) was present on both sides of the membrane ($G_m = 77.89 \pm 8.81$ nS cm$^{-2}$, n = 5) compared with single-sided application ($G_m = 36.48 \pm 2.41$ nS cm$^{-2}$, n = 5). In bilayers containing PC and phosphatidylserine (PS), PC:PS, the Hyp extract effect was greater than on pure PC bilayers, although the surface charge was not the determining factor of this enhanced activity. Adding cholesterol to the PC:PS mixture reverted the conductance increase induced by the Hyp extract in a dose-dependent manner. The specific pattern of the Hyp extract interaction with lipid bilayers has possible consequences concerning its absorption and bioavailability, as well as its pharmacodynamic effects on neuronal excitability.

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## Introduction

*Hypericum perforatum*, also known as St John's wort, has been used as a medicinal herb since ancient times (Payk 1994), in both allopathic and homeopathic preparations. It is extensively administered in clinical trials as well as in current medical practice for the treatment of depression in countries such as Germany, UK and the USA. Industrial preparations include Jarsin/-300 (LI 160), Hyperforat, Psychotonin, Psychotonin M, Neuropas and Esbericum. Most of these products are standardized relative to the total amount of hypericins, although hyperforin is now known to be an important constituent for antidepressant activity (Muruganandam et al 2001; Cervo et al 2002). Clinical trials suggest that hypericum extract is a more potent antidepressant than placebo (Kaufeler et al 2001; Whiskey et al 2001; Lecrubier et al 2002). In randomized controlled trials, hypericum extracts have been shown to be as effective as certain standard antidepressants, for example imipramine (Vorbach et al 1997; Woelk 2000; Alkhenizan 2001; Kaufeler et al 2001; Volp 2001), amitriptyline (Wheatley 1997), maprotiline (Harrer et al 1994; Johnson et al 1994), fluoxetine (Fornal et al 2001; Kaufeler et al 2001; Behnke et al 2002), and sertraline (Fornal et al 2001), in the treatment of mild to moderate depression. Although not completely understood (for review see Butterweck 2003), this therapeutic effect has been attributed to inhibition of monoamine oxidase A and B, inhibition of synaptosomal serotonin (5-HT), dopamine, norepinephrine (Roz & Rehavi 2003), GABA and glutamate (Kaehler et al 1999; Wonnemann et al 2000; Gobbi et al 2001; Kientsch et al 2001; Muller et al 2001) reuptake, as well as to interactions with purinergic, GABA and glutamate receptors (Krishtal et al 2001; Langosch et al 2002; Watkins et al 2003). Long-term administration of hypericum extract significantly increases 5-HT concentrations in the hypothalamus (Butterweck et al 2002) and improves spatial learning and memory (Widy-Tyszkiewicz et al 2002). Clinical doses of hypericum do not impair attention, sensorimotor function or information processing (Timoshanko et al 2001).

Side-effects of the hypericum extract include photosensitivity (Schempp et al 2002a), gastrointestinal symptoms, dizziness/confusion and tiredness/sedation (Ernst et al 1998). Special precautions should be taken when co-administered with HIV protease inhibitors, ciclosporin, warfarin, digoxin, theophylline, anticonvulsants, selective serotonin reuptake inhibitors, triptans and oral contraceptives (Barnes et al 2001). These effects can be explained, at least in part, by interactions leading to increased activity of cytochrome P450 enzymes 3A4 and 2C9 (Komoroski et al 2004) via activation of the pregnane X receptor (Goodwin et al 2001; Chen et al 2004). Inhibitory effects on another cytochrome P450 isoform (1A1) may limit carcinogen formation from benzopyrene derivatives (Schwarz et al 2003). Induction of apoptosis in various malignant cell lines by activation of several caspases (Hostanska et al 2003) may prompt a different therapeutic use of hypericum extract active compounds as cytostatic agents (Schempp et al 2002b). Other pharmacological effects reported to date include antioxidant (Hunt et al 2001; Heilmann et al 2003) and antimicrobial activity (Avato et al 2004), inhibition of DNA topoisomerase II $\alpha$ (Peebles et al 2001), of cyclooxygenase-1 and 5-lipoxygenase (Albert et al 2002; Fischer et al 2003), and of NF-$\kappa$B (Bork et al 1999).

The crude drug, *Hyperici herba* (Hyp), consists of the aerial parts of *H. perforatum*, collected just before or during the flowering stage (Nahrstedt & Butterweck 1997). *H. perforatum* extract contains approximately seven groups of bioactive natural products: phenylpropanes, flavonol derivatives (quercetin), biflavones, oligomeric proanthocyanidins, xanthones, naphthodianthrones (e.g. hypericin, pseudohypericin) and phloroglucinols (e.g. hyperforin) (Nashrstedt & Butterweck 1997). Different patterns of interaction with lipid bilayers have been demonstrated for several of these compounds. Hyperforin salt decreases fatty acid tail flexibility in the membrane hydrocarbon core, but fluidizes the hydrophilic region of membrane phospholipids (Eckert & Muller 2001). Hypericin salt partitions into the liposomal phospholipid bilayer, experiencing a gradient of solvent polarities, with atoms C10–C11 protruding deeper between the acyl chains (Weitman et al 2001). Quercetin penetrates the lipid bilayer by intercalating between the flexible acyl chains of phospholipids, the deepest insertion occurring in acidic conditions (Movileanu et al 2000).

As hypericum extract is already prescribed on a large scale, its bioavailability after oral intake is important for pharmaceutical formulations. Using the black lipid membranes technique, we studied the effectiveness of insertion of Hyp extract in model membranes, setting different membrane compositions and negative surface charge densities.

## Materials and Methods

### Chemicals

Hyp extract was kindly provided by Dr Gudrun Werner (Bionorica, Germany) together with its HPLC analysis. *Hypericum* is a member of the *Clusiaceae* (alternative names: *Guttiferae*, *Hypericaceae*) family. According to the HPLC analysis, this extract contains 0.19% hypericin, 0.25% pseudohypericin and 3.4% hyperforin, as the main active components. The ethanolic Hyp extract was obtained as previously described by Butterweck et al (2002). Briefly, the plant extract was suspended in 10 $\mu$L ethanol and diluted with deionized water up to 1 mg mL$^{-1}$. It was then sonicated for 5 min at 5 $\mu$A in a Soniprep 150 ultrasonic desintegrator (Sanyo, Gallenkamp PLC, UK), followed by centrifugation at 8000 *g* for 10 min. Filtration of the extract was performed using 0.22-$\mu$m GV membrane filters (Millipore, Ireland). The stock solution was kept in the dark at 0–2°C. *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), NaOH, sodium dodecyl sulfate (SDS), and *n*-hexane were purchased from Merck (Darmstadt, Germany). *n*-Decane, NaCl, L-$\alpha$-phosphatidylcholine (PC) from soybean, L-$\alpha$-phosphatidylserine (PS) from bovine brain, and cholesterol (CHOL) were purchased from Sigma (St Louis, MO, USA).

### Electrical measurements on black lipid membranes

We obtained black lipid membranes (they were maintained for at least 3 h) using the method of painted bilayers (Hanke & Schlue 1993), with a capacitance over the range of 0.15–0.30 $\mu$F cm$^{-2}$ and a conductance of 10–250 nS cm$^{-2}$, depending on the lipid composition of the bilayer. A bilayer membrane was painted across a 1-mm aperture. Each of the two compartments of a Teflon chamber was filled with 1.5 mL electrolyte buffer (100 mM NaCl, 20 mM HEPES, pH 7.00 with 1 M NaOH). The aperture was pre-treated with a solution of 5 mg lipid mL$^{-1}$ *n*-hexane. Subsequently, the lipid bilayer was formed with a solution of 20 mg lipids mL$^{-1}$ *n*-decane. Membranes with different weight ratios of phospholipids PC:PS:CHOL (w/w) were used.

Electrical measurements were performed as previously described by Fendler et al (1985). The transmembrane current was recorded with Ag/AgCl electrodes connected to the bathing electrolyte through agar salt bridges (1% agar in 1 M KCl). The signals were I/V converted and amplified using a current amplifier with a gain of $10^7$ V/A, followed by a voltage amplifier (with a gain and bandwidth of 100 and 1 kHz, respectively). The signals were then lowpass-filtered at 500 Hz and recorded with a digital oscilloscope (model 54603B; Hewlett Packard, USA). All the experiments were performed at room temperature (24 $\pm$ 1°C) and in darkness.

The transmembrane conductance and capacitance were recorded by applying a 1-s rectangular voltage pulse of $\pm$50 mV or a triangular voltage pulse of +20 mV, respectively, and measuring the elicited current. In all experiments, Hyp extract was added after ~30 min, when a stable plateau of the recorded parameters was reached. The electrical parameters were normalized to the unit surface area of the bilayer.

### Statistical analysis

Data are reported as means $\pm$ s.e.m., with n indicating the number of replicates. To assess the statistical significance of

the time course of transbilayer capacitance and conductance in various experimental settings, we performed one-way analysis of variance for repeated measures followed by post-hoc Tukey's honestly significant difference pairwise comparisons, using scripts generously provided by Richard Lowry (http://faculty.vassar.edu/lowry/corr3.html and http://faculty.vassar.edu/lowry/hsd.html). The effects of Hyp extract on different bilayer compositions were analysed using two-way analysis of variance (http://faculty.vassar.edu/lowry/anova2X3.html) and Bonferroni post-tests (http://graphpad.com/quickcalcs/posttest1.cfm). A level of $P < 0.05$ denoted significance in all cases.

## Results

### Characteristics of ethanolic HYP extract insertion in lipid membranes

We carried out dose–response experiments to investigate the characteristics of Hyp extract interaction with lipid bilayers. Capacitance and conductance were monitored in the presence of different Hyp extract concentrations (Figure 1). We noticed a narrow range of Hyp extract activity: from 0.1 to 5 $\mu$g mL$^{-1}$ there was very little increase in conductance, and at 10 $\mu$g mL$^{-1}$ there was a strong destabilizing effect. The bilayers exposed to 10 $\mu$g mL$^{-1}$ usually broke before the end of the experiment, therefore the corresponding values were recorded until bilayer breakdown. Additionally, we performed control experiments with the black lipid membranes alone in the presence of ethanol in the bath (0.6 $\mu$L added to 1500 $\mu$L bathing electrolyte). Ethanol alone did not influence the physical parameters of lipid bilayers (data not shown).

Hyp extract induced an increase in capacitance in a concentration-dependent manner (Figure 1A). This increase in capacitance occurs as a result of insertion of molecules into bilayers, which leads to the extension of the surface area (Movileanu et al 2000). The normalized conductance increase was also dose-dependent (Figure 1B) in PC bilayers.

We also performed experiments where 3 $\mu$g mL$^{-1}$ Hyp extract was added on either one side or both sides of the bilayer (Figure 2). The specific transmembrane conductance was 2-fold less for single-sided application ($G_m = 36.48 \pm 2.41$ nS cm$^{-2}$, n = 5) compared with application on both sides ($G_m = 77.89 \pm 8.81$ nS cm$^{-2}$, n = 5) (Figure 2B). The specific transmembrane capacitance variation was greater for single-sided application ($C_m = 0.24 \pm 0.02$ $\mu$F cm$^{-2}$, n = 5) than for application on both sides ($C_m = 0.21 \pm 0.01$ $\mu$F cm$^{-2}$, n = 5) (Figure 2A), as noted previously for quercetin (Movileanu et al 2000). We attribute this effect to asymmetrical tension in the bilayer leaflets owing to asymmetrical distribution and interaction between Hyp extract and the lipid matrix.

### Composition of the lipid bilayer modulates its interaction with the HYP extract

In binary systems containing PC and PS, the Hyp extract produced greater increases in conductance than in pure PC bilayers, indicating a more efficient insertion of different Hyp extract components. In both binary and ternary systems, the Hyp extract effect was sensitive to the CHOL content (Figure 3A). The increase in capacitance after Hyp extract addition was reduced when the percentage of CHOL in the lipid mixture was increased (PC:CHOL = 1:4 or PC:PS:CHOL = 1:1:4), showing that high CHOL content hinders Hyp extract insertion in the lipid membrane. In binary systems (PC:CHOL), the Hyp extract also induced a significant increase in conductance in a CHOL-dependent manner (Figure 3B).

As the increase in conductance of PC:PS = 1:1 (w/w) bilayers after Hyp extract addition was greater than that of PC bilayers, we went further to test if this effect could be attributed to the increase in negative surface charge density, or to the conformational changes of the lipid bilayer, which occur when PS is present in the mixture. To test the influence of the negatively charged surface on

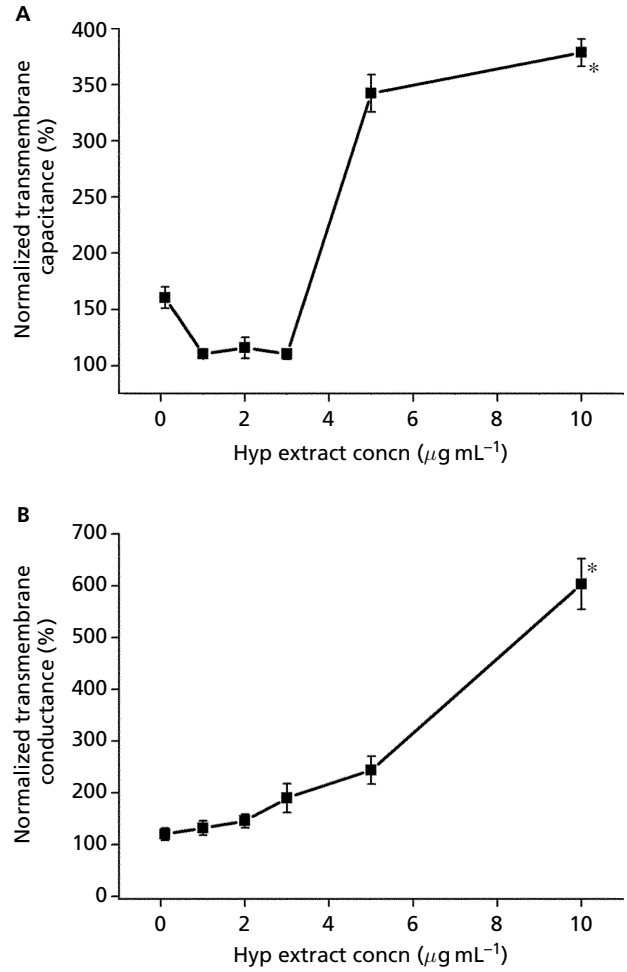


**Figure 1** Dose-dependent increase of phosphatidylcholine (20 mg PC/1 mL *n*-decane) bilayers capacitance (A) and conductance (B) induced by *Hyperici herba* (Hyp) extract under dark conditions (mean ± s.e.m., n = 5). Results were normalized as the ratio between the values recorded at t = 150 min and at t = 30 min. *Data were recorded until the breakdown of the bilayer.

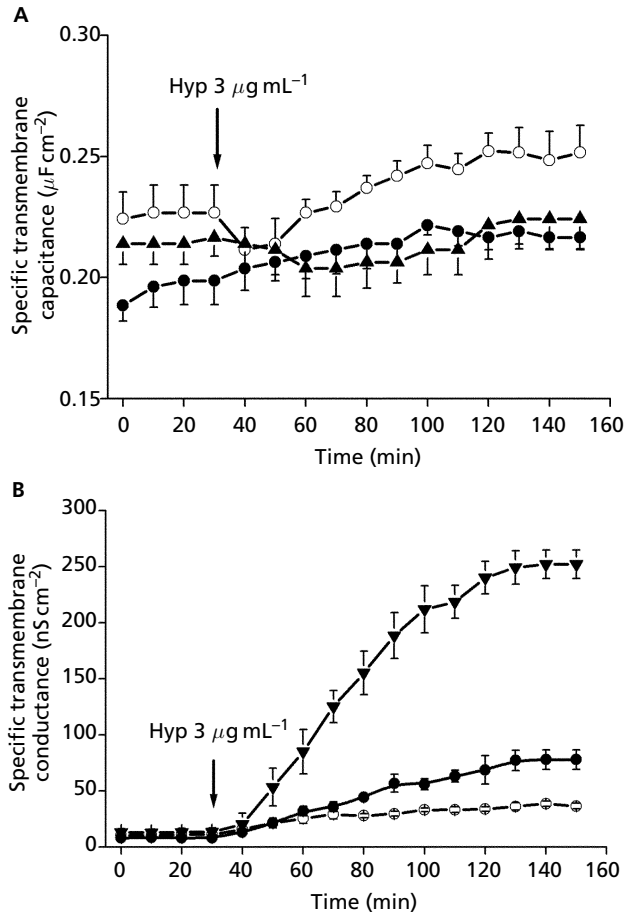


**Figure 2** Variation of specific transmembrane capacitance (A) and conductance (B) of phosphatidylcholine bilayers in the presence of 3 $\mu$g mL$^{-1}$ *Hyperici herba* (Hyp) extract (mean ± s.e.m., n = 5). The Hyp extract was added on one side of the bilayer (○), on both sides of the bilayers (●), and on both sides of the bilayers containing 20 $\mu$mol sodium dodecyl sulfate (▼). Time series were significantly different ($P < 0.01$).

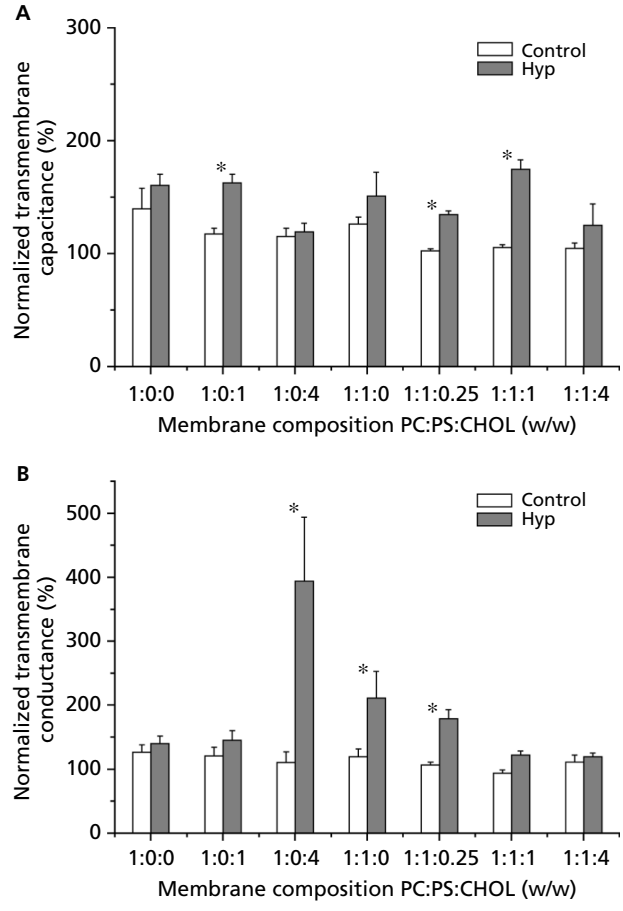


**Figure 3** Changes of the normalized capacitance (A) and normalized conductance (B) induced by 3 $\mu$g mL$^{-1}$ *Hyperici herba* (Hyp) extract relative to the control in lipid bilayers with different compositions (mean ± s.e.m., n = 5 or 6). The content of the phosphatidylcholine, phosphatidylserine and cholesterol mixture (PC:PS:CHOL) is given as parts of lipids added to 1 mL *n*-decane in order to give a final concentration of 20 mg lipids/1 mL *n*-decane. The results were normalized as the ratio between the values recorded at t = 150 min and at t = 30 min. The compositions where Hyp extract addition exerted significant changes are marked with an asterisk ($P < 0.05$).

the insertion in the lipid bilayer, we added SDS in the bath before addition of the extract as previously described (Turnheim et al 1999). SDS is a charged lipid-like amphiphile molecule with negatively charged headgroups, which can insert into the bilayer and alter its surface charge. Adding 20 $\mu$M SDS alone in the bath had no effect on the electrical capacitance or conductance of the membrane (data not shown). The conductance variation of PC bilayers containing SDS ($G_m = 270.06 \pm 12.7$ nS cm$^{-2}$, n = 5) showed that they were very sensitive to Hyp extract (Figure 2B). The time course of capacitance of PC bilayers with SDS ($C_m = 0.22 \pm 0.01$ $\mu$F cm$^{-2}$, n = 5) was similar to that of pure PC bilayers ($C_m = 0.21 \pm 0.01$ $\mu$F cm$^{-2}$, n = 5) (Figure 2A). The negative surface charge accounts for the enhanced bilayer permeabilization but not for a faster insertion of Hyp extract.

To further assess whether an electrostatic attraction mediated by the surface charges was involved in the Hyp extract effect on lipid bilayers, we studied ternary systems with PC, PS and CHOL. An increase in CHOL content decreased the negative surface charge via charge screening. It is known that PS lipid chains pack inefficiently around CHOL molecules, leading to the formation of voids at the bilayer interface (Barnes & Freed 1998). These voids would be expected to lower the negative surface charge density at the membrane aqueous interface.

In ternary systems, the Hyp extract induced an increase in the normalized capacitance, which was larger in bilayers with low CHOL content (PC:PS:CHOL = 1:1:0.25 and 1:1:1 w/w). In a high cholesterol mixture (PC:PS:CHOL = 1:1:4 w/w), the normalized capacitance did not change significantly, indicating a low rate of Hyp extract insertion. When the CHOL content of ternary mixtures increased, the Hyp extract induced a lower increase in conductance.

## Discussion

The pronounced increase in transmembrane conductance observed after addition of the Hyp extract suggests a more complicated interaction mechanism than the bilayer fluctuations postulated by the model of anaesthetics interaction with the lipid matrix proposed by Mouristen et al (1995). The increase in conductance with concentration did not show any saturation until the breakdown of the bilayer. A permeabilization effect is suggested by the fact that a double increase in the specific transmembrane conductance was induced when Hyp extract bathed both sides of the membrane as compared with single-sided application.

As the presence of SDS makes no difference in the variation of the specific capacitance of PC bilayers, we conclude that the number of molecules inserted in pure PC bilayers and PC bilayers with SDS is the same. Therefore, the increase in specific conductance of PC bilayers with SDS after the addition of Hyp extract cannot be attributed to an increased negative surface charge. If the membrane negative surface charge is not directly responsible for improved insertion of Hyp extract in lipid bilayers, the enhanced insertion in PC:PS = 1:1 (w/w) bilayers may be owing to the peculiarities of acyl chain packing arising, very likely, from the reduced surface tension coefficient in the presence of PS.

Hyp extract presents a specific pattern of interaction with binary systems, being sensitive to the percentage of CHOL. In CHOL-rich bilayers (PC:CHOL = 1:4 w/w) its insertion was decreased compared with CHOL-poor bilayers (PC:CHOL = 1:1 w/w). As the bilayer organization cannot be the sole cause of this preference, we conclude that Hyp extract insertion in binary systems implies formation of aggregates, a process that may be hindered at high percentages of CHOL ( > 33%).

In ternary systems, CHOL can associate with phospholipids in highly ordered domains, the so-called condensed complexes or lipid rafts (Simons & Ikonen 1997), their formation obeying a specific stoichiometry (Radhakrishnan & McConnell 1999; Xu & London 2000; Feigenson & Buboltz 2001). Increasing the CHOL percentage above that required for complex formation may induce the disappearance of the rigidly ordered phase. The CHOL concentration where this shift in membrane organization occurs depends on the membrane composition and on lipid saturation (Xu & London 2000; Feigenson & Buboltz 2001). The tight packing of acyl chains in the presence of CHOL hardens Hyp extract insertion and cannot account for the increased bilayer specific capacitance in low CHOL systems (PC:PS:CHOL = 1:1:1 and 1:1:0.25 w/w). We consider that condensed complexes facilitate the insertion of active molecules from the Hyp extract; it is plausible that they easily find places for penetration in the disorganized regions between the ordered complexes. Hyp extract interaction with the bilayer is favoured by the presence of PS. Thus, the systems with high PS and low cholesterol (PC:PS:CHOL = 1:1:1 and 1:1:0.25 w/w) allow better insertion of the Hyp extract components in the lipid membrane.

The main finding of our study is an increased bilayer conductance induced by Hyp extract. Hyp extract interacts with the lipid bilayer, this interaction being modulated by the membrane composition and not directly by the negative charge of the membrane surface. The specific pattern of interaction between the Hyp extract and bilayers can be explained by insertion of each of its components into the lipid phase. Previous studies have demonstrated the insertion of hyperforin (Eckert & Muller 2001) and hypericin (Weitman et al 2001) into lipid bilayers. In our study, a synergistic effect of these compounds can explain the effects produced by the Hyp extract on mixed lipid systems. As the plasma membrane and mitochondrial membranes have different compositions (Hanke & Schlue 1993), our study offers some clues about the complex pattern of Hyp extract interaction with different types of membranes encountered during the distribution of the drug inside the body.

### Conclusions

Hyp extract (1–10 $\mu g\,mL^{-1}$) induced a concentration-dependent increase of both specific transmembrane capacitance and conductance in PC membranes. The effect on conductance was enhanced when the Hyp extract (3 $\mu g\,mL^{-1}$) was present on both sides of the membrane ($G_m = 77.89 \pm 8.81\,nS\,cm^{-2}$, $n = 5$) compared with single-sided application ($G_m = 36.48 \pm 2.41\,nS\,cm^{-2}$, $n = 5$). The Hyp extract effect was greater on PC:PS bilayers than on pure PC bilayers; the surface charge was not directly responsible for this increase. Adding cholesterol to the PC:PS mixture reverted the conductance increase induced by the Hyp extract in a dose-dependent manner. The specific pattern of the Hyp extract interaction with lipid bilayers has possible consequences on its absorption in the body, bioavailability, and can partly account for the depolarizing effects in excitable cell membranes, leading to the well-known but poorly understood antidepressant action of this complex drug.

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