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Pharmacological *in vivo* Test to Evaluate the Bioavailability of some St John's Wort Innovative Oral Preparations

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In this study, the optimisation of biopharmaceutical properties of a dried commercial extract of St John's Wort were evaluated employing the *in vivo* forced swimming test (FST). Three new dosage forms containing β-cyclodextrin and surfactants (SDS, ASC8) were compared in the FST with the commercial extract. The commercial extract showed antidepressant activity in mice after 60 min at a dosage of 100 mg/kg. The same antidepressant activity appeared in 30 min with a micellar solution of SDS containing the same quantity of extract (100 mg/kg), while with micelles of ASC8 the effect appeared at 15 min and with a dosage of 30 mg/kg. In the case of β-cyclodextrin the best results were obtained at 30 min, administering 60 mg/kg of the extract. Finally, the influence of the formulations on the water solubility of the constituents of the extract is reported. The tensides dramatically enhanced solubility, in particular that of the more lipophilic compounds, in the case of β-cyclodextrin this effect was very pronounced for flavonoids and biapigenin, lower for hypericins and practically insignificant for hyperforins. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: St John's Wort; micelles; β-cyclodextrin; *in vivo* 'Porsolt test'; water solubility of formulations.

INTRODUCTION

The World Health Organization ranks depression as fourth among the world's greatest health problems, with psychiatric pain most often diagnosed (Chatterjee *et al.*, 1998a,b).

Hypericum perforatum L. (St John's Wort, SJW) has been used since antiquity mainly for external use, but the quantified extract has become increasingly popular in the last ten years in Europe and the USA for the treatment of mood disorders, especially conditions of mild to moderate depression according to ICD-10 (ESCOP 1997; Harrer *et al.*, 1994; Rasmussen, 1998).

Generally, the herbal medicinal products are formulated from the quantified extracts which contain a variety of compounds including a broad range of flavonols, based on quercetin aglycone; naphthodianthrones (hypericin and pseudohypericin) and phloroglucinol derivatives such as hyperforin, adhyperforin and others. In the literature, many papers demonstrate the synergic effects between different constituents of *Hypericum perforatum* L. (Butterweck *et al.*, 1998; Noldner and Schotz, 2002; Butterweck, 2003). All the constituents of the extract are considered active markers and, in general, the effects of these preparations are assumed to arise from the whole mixture of these main constituents (Gaedcke and Steinhoff, 2003; Council of Europe, 2005).

Thus, flavonols and naphtodiantrones are polyphenols and quite polar derivatives but their water solubility is very scarce; phloroglucinols are highly lipophilic constituents. The bioavailability of the extract's active principles is very low, as shown by some studies reported in the literature (Kubin *et al.*, 2005; Schulz *et al.*, 2005; Wurglics and Schubert-Zsilavecz, 2006) and the maximum plasma levels were reached after a lag time of 3–6 h depending on the constituents (Biber *et al.*, 1998; Agrosì *et al.*, 2000). To our knowledge, a single report appears concerning an alternative formulation to the widely diffused tablets or hard capsules of the dried extracts; it is represented by the soft gelatin capsule technology containing *inter alia* the dried extract and soya oil. This new dosage form exhibited a higher individual absorption of both hyperforin and hypericin when compared with the corresponding data for hard gelatin capsules of the dried extract (Agrosì *et al.*, 2000).

In the present study the effect of SDS and ASC8 surfactants and β-cyclodextrin on the biopharmaceutical properties of SJW extract were evaluated. A pharmacological approach, instead of the pharmacokinetic one, was used in order to evaluate the improvement of the properties of the new developed formulations. The mouse forced swimming test (Porsolt test, FST) (Porsolt *et al.*, 1977) – a behavioural model where antidepressant drugs clomipramine and amitriptyline are active – was used to test the antidepressant-like activity of the different dosage forms and the data were compared with those obtained with the widely used dried extract.

The aim of this work was to develop new oral formulations with a better pharmacokinetic, a more rapid onset and prolonged action with respect to the extract alone and to verify the possibility of reducing the dosage of total extract while obtaining the same antidepressant-like effect with minor potential side-effects. Finally, the influence of β-cyclodextrin and micelles on the

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solubility of the constituents of the extract within the formulations was evaluated to better elucidate the results obtained from the pharmacological tests.

MATERIALS AND METHODS

Materials. Acetonitrile and MeOH (HPLC grade) were purchased from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA).

Indena Research Laboratories (Settala, Milan, Italy) offered a commercial sample of *Hypericum perforatum* L. dried extract – Lotto 28662/M1 – B.A. 84204 and kindly provided the reference rutin trihydrate (Batch no. K12408717, standard purity 96.25% considering the content of residual solvents, moisture and amount of impurities).

KLEPTOSE® β-cyclodextrin (Roquette Frères, S.A. Lestrem, France); sodium dodecyl sulfate (SDS) (Sigma Chemical Co., St. Louis, USA); ottanoic acid 98+% (Aldrich, Steinheim, Germany); and L-ascorbic acid 99% (Aldrich, Steinheim, Germany). Octanoyl-6-O-ascorbic acid (ASC8) was synthesised according to the procedure reported in literature (Capuzzi *et al.*, 1996); water content and purity were checked by elemental analysis (theoretical C 55.62%, H 7.33%; found C 53.80%, H 7.45%). Ethanol was analytical reagent grade from Riedel-de Haen (Seelze, Germany). Carboxymethylcellulose Sodium Salt, (CMC) (Fluka Chemie GmbH, Steinheim, Germany); clomipramine hydrochloride, amitriptyline hydrochloride (Sigma, Milan, Italy); and amphetamine (St Louis, MO, USA).

Animals. Male Swiss albino mice (23–25 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Fifteen mice were housed per cage (26 × 41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12-h light/dark cycle, light on at 7 a.m. Animals were naïve and used only once. All experiments were carried out in accordance with the Animal Protection Law of the Republic of Italy, DL n. 116/1992, based on the European Community's Council Directive of 24 November 1986 (86/609/EEC).

HPLC-DAD and HPLC-MS drug analyses. To evaluate the constituents' content, an HPLC analysis was performed using a method described in the literature (Bergonzi *et al.*, 2001). Identification of the constituents was performed using combined HPLC-diode array detection (DAD) analysis and HPLC-thermospray mass spectrometry. Quantification of the constituents was performed using rutin as an external standard and considering each constituent and the relative response factor (RRF) with respect to rutin, as previously reported (Brolis *et al.*, 1998).

All the samples were analysed in triplicate and a calibration graph with six data points of external standard was used. The content of each constituent of the drug substance is reported in Table 1.

The HPLC system consisted of a HP 1100 L instrument with a diode array detector managed by a HP 9000 workstation (Agilent Technology, Palo Alto, CA, USA). The reverse-phase column was a 201 TP 54 (250 × 4.6 mm, 6 mm, 5 µm, Vydac Separation Group, Hesperia, CA, USA) maintained at 26 °C. The mobile phase was a linear solvent gradient $CH_3CN/CH_3OH/H_2O$ (pH 3.2, HCOOH) over a 60-min period at a flow rate of 1 ml/min, as previously reported (Bergonzi *et al.*, 2001). Before the HPLC analysis, each sample was filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane (d = 13 mm, porosity 0.45 µm, Lida manufacturing Corp.) and immediately injected. The injected volume of sample was a 25-µl solution. UV–Vis spectra were recorded in the range 200–590 nm, and chromatograms were acquired at 230, 254, 270, 350 and 590 nm.

Table 1. Dried extract composition (mg/100mg)

Constituent	Content % (mg/100 mg), ±S.D.
Rutin	4.28 ± 1.23
Hyperoside	6.35 ± 1.02
Isoquercitrin	0.61 ± 0.08
Quercitrin	0.65 ± 0.08
Quercetin	0.83 ± 0.13
I3,II8-Biapigenin	0.62 ± 0.08
Total flavonols	12.72 ± 1.08
Total hyperforins	4.23 ± 0.01
Total hypericins	0.32 ± 0.03

The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technology, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of the HPLC-DAD analysis. The same column, mobile phase, time period and flow rate were used. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values; gas temperature 350 °C at a flow rate of 10 l/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from m/z 100–800 in the positive ion mode were obtained (scan time 1 s).

Forced swimming test (FST). The FST used was the same as described by Porsolt (Porsot *et al.*, 1977). Briefly, mice were dropped individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 6 cm of water maintained at 22–23 °C and left there for 6 min. A mouse was judged to be immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect. Twenty-four mice per group were tested. The experiments reported in each figure were performed on the same day. Each treated group was compared to the corresponding control group. The observers were unaware of the treatments. The experiments were performed blind. To evaluate the antidepressant-like activity of a compound in the FST not only a dose-response but also a time-course study must be performed in order to determine the most appropriate administration schedule. The reference drugs were tested concomitantly with the unformulated

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extract. Time-course experiments performed in our laboratory indicated that the amitriptyline maximum antidepressant-like effect was reached 30 min after administration (data not shown); amitriptyline 10 mg/Kg s.c. reduced time immobility to 102.5 ± 10.4 ($P < 0.001$); clomipramine 25 mg/Kg s.c. reduced time immobility to 139.1 ± 12.3 ($P < 0.001$).

Hole-board test. The hole-board test consisted of a 40-cm square plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 5 min each. Two electric eyes, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. Twenty-four mice per group were tested. Amphetamine was used as reference drug.

Rota-rod test. The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught (1985). Those mice scoring more than six falls in the pre-test were rejected (20%). The performance time was measured before (pre-test) and 15, 30 and 45 min after the beginning of the test. Twenty-four mice per group were tested.

Drugs. Clomipramine and amitriptyline were dissolved in water solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 ml kg^{-1}. Doses and administration schedule were chosen on the basis of time-course and dose–response experiments previously performed in our laboratory. Furthermore, literature data confirm the selectivity and efficacy of the above mentioned treatments at the times and concentrations used.

SJW dried commercial extract was dissolved in a 1% CMC solution immediately before use and administered by gavage. Different extract concentrations from 10 to 1000 mg kg^{-1} were tested.

New developed formulations. Aqueous formulations of the SJW dried commercial extract (100 mg kg^{-1}) were prepared using ASC8 and SDS 40 mM solutions by sonication for 15 min.

The β-cyclodextrin formulation (β-Cd/extract 2:1 w/w) was prepared by colyophilized method (Hedges, 1998).

Solubility test. Solubility studies of the extracts were carried out in water at 26 ± 0.5 °C. Saturated solutions of *Hypericum perforatum* L. extract in water or in micellar solution of SDS and ASC8 40 mM and a saturated solution of colyophilized with β-Cd were prepared. After 24 h, an aliquot of the four suspensions was filtered (pore size 0.45 μm) and the constituents' concentration was determined by HPLC-DAD-MS analysis. Each experiment was performed in triplicate.

Statistical analysis. All experimental results are given as the mean ± SEM. An analysis of variance ANOVA, followed by Fisher's Protected Least Significant Difference procedure for post hoc comparison, was used to verify the significance between the two means of behavioural results. Data were analyzed with the StatView software for the Macintosh (1992). P values of less than 0.05 were considered significant.

RESULTS

As a first step of our investigation, we evaluated the dose of total extract to obtain a significant reduction in time immobility. Dried commercial extract, dispersed in a CMC 1% solution, showed an antidepressant-like effect in the mice after 60 min with a dosage of 30, 60 and 100 mg/Kg administered orally (p.o.) which reduced time immobility to 143.1 ± 10.3 ($P < 0.01$), 133.7 ± 14.6 ($P < 0.01$) and 142.4 ± 10.2 ($P < 0.01$) s respectively (bell-trend). The doses of 10, 150, 300 and 1000 mg/kg p.o. were devoid of any effect (Fig. 1).

A dose of 100 mg/Kg was chosen as reference for the innovative forms because it gave the most reproducible data (Fig. 1). The *time-course* of the commercial extract at a dosage of 100 mg/Kg p.o. showed a brief range of activity, after only 60 min (immobility time = 136.4 ± 15.4 s., $P < 0.001$) (Fig. 2). The observed antidepressant-like effect disappeared 90 min after administration.

The behaviour of three tested formulations, containing all the same quantity of extract corresponding to a dosage of 100 mg/Kg was reported in Figs 3, 4 and 5.

A micellar solution of SDS showed an antidepressant activity similar to that of the extract alone after 30 min (immobility time = 150.7 ± 8.2 sec., $P < 0.05$), subsequently the effect disappeared (Fig. 3).

A micellar solution of ASC8 containing the same quantity of extract corresponding to a dosage of 100 mg/Kg reduced immobility time after 15 and 30 min to 136.4 ± 11.2 s ($P < 0.05$) and 129.6 ± 11.4 s, respectively ($P < 0.05$) (Fig. 4).

Finally, we tested the preparation with β-cyclodextrin in ratio by weight 1:2. With a dosage of 100 mg/Kg, the antidepressant-like effect was obtained after 30 min, reducing significantly immobility time to 130.9 ± 11.3 s ($P < 0.01$); the effect remained also after 60 min (immobility time = 128.9 ± 11.7 s, $P < 0.01$) (Fig. 5).

Then, we tested the same formulations with lower content of extract, trying to reduce the dosage of the *Hypericum perforatum* L. The more significant results were reported in Figs 6 and 7, where we reported the dose-response curve for antidepressant-like effect of ASC8 and β-cyclodextrin formulations after 15 (A), 30 (B) and 60 (C) min after administration. The lower dosage utilizable with a micellar solution of ASC8 was 30 mg/Kg. In this case the formulation resulted still active and reduced immobility time after only 15 min to 126.2 ± 11.3 s ($P < 0.05$) (Fig. 6A) and after 30 min to 140.8 ± 14.1 s ($P < 0.05$) (Fig. 6B). The antidepressant-like effect with β-cyclodextrin was obtained with a dosage

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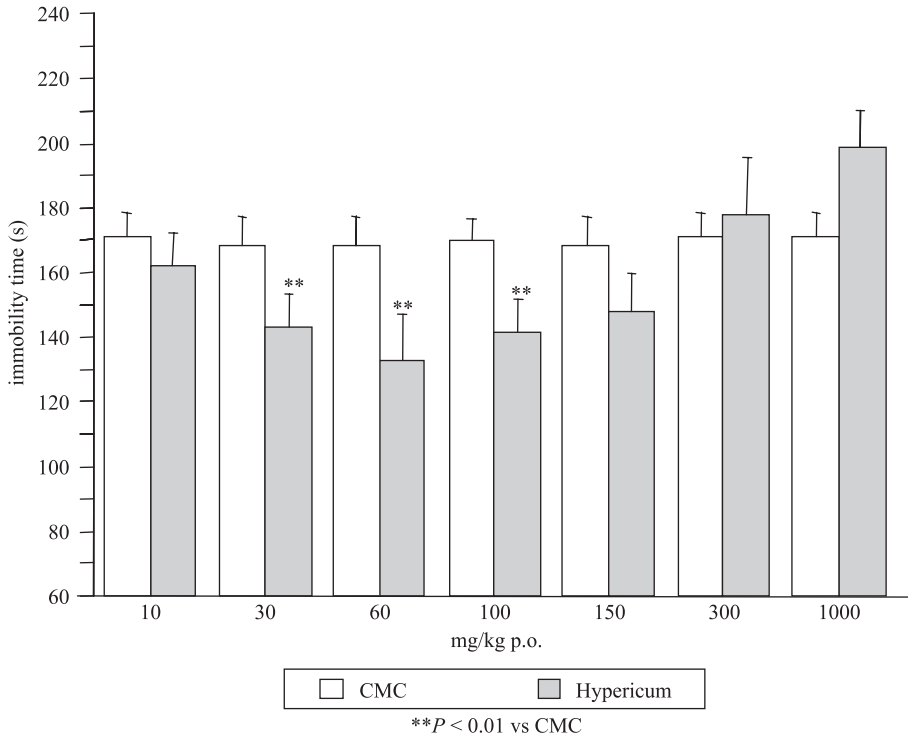

Figure 1. Curve dose-response of different dosages of St. John's wort commercial extract after 60 minutes.

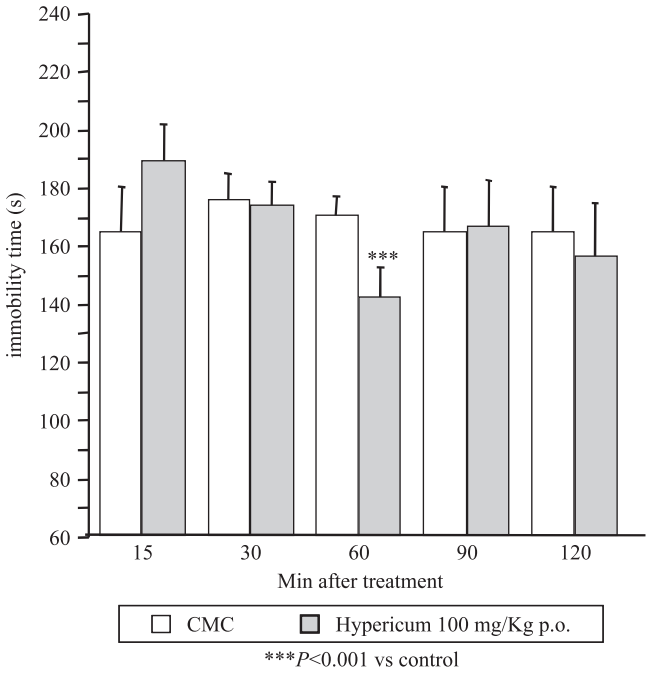

Figure 2. *Time course* of St. John's wort commercial extract with at a dosage of 100 mg/Kg p.o.

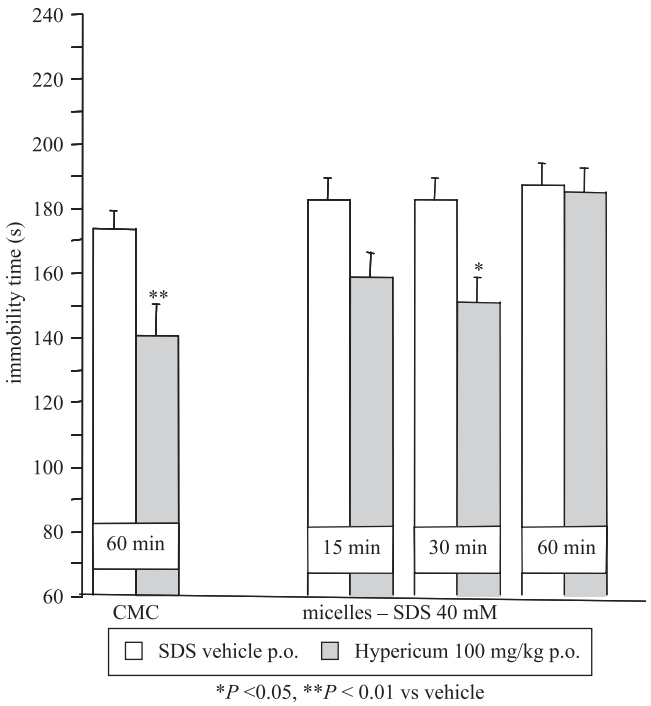

Figure 3. *Time course* of micellar solution of SDS 40 mM with at a dosage of 100 mg/Kg p.o.

of 60 mg/Kg after 30 min (Fig. 7B) and it reduced significantly immobility time to 151.3 ± 9.2 s ($P < 0.01$); the effect remained also after 60 min (immobility time = 145.4 ± 8.6 s, $P < 0.01$) (Fig. 7C).

However, drugs that increase motor activity may produce false positives in the Porsolt's test (Borsini and Meli, 1988; Detke and Lucki, 1995) thus it was necessary to conduct the hole-board test in parallel to evidenced possible alterations of spontaneous motility and explorative activity and the Rota-rod test to evaluate the influence on motor coordination of the extract and the other vehicles investigated.

No statistical differences were noted between controls and mice pre-treated with the highest effective dose of extract (100 mg/Kg), SDS, ASC8 and β-cyclodextrin. The spontaneous motility as well as the exploratory

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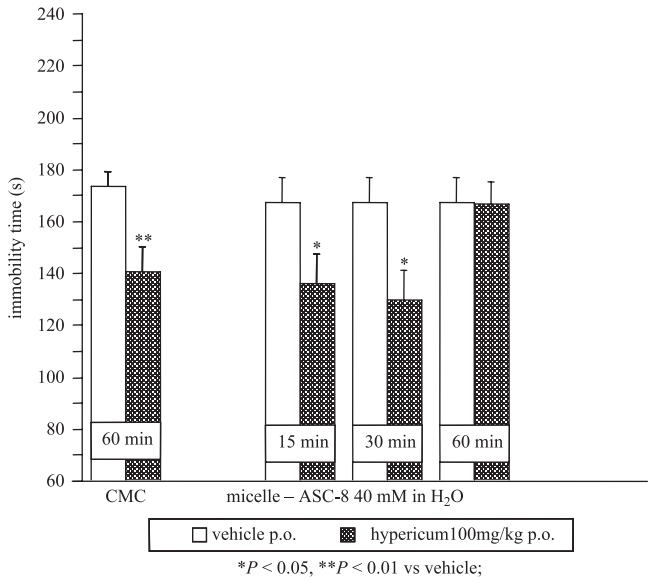

Figure 4. *Time course* of micellar solution of ASC-8 40 mM with at a dosage of 100 mg/Kg p.o.

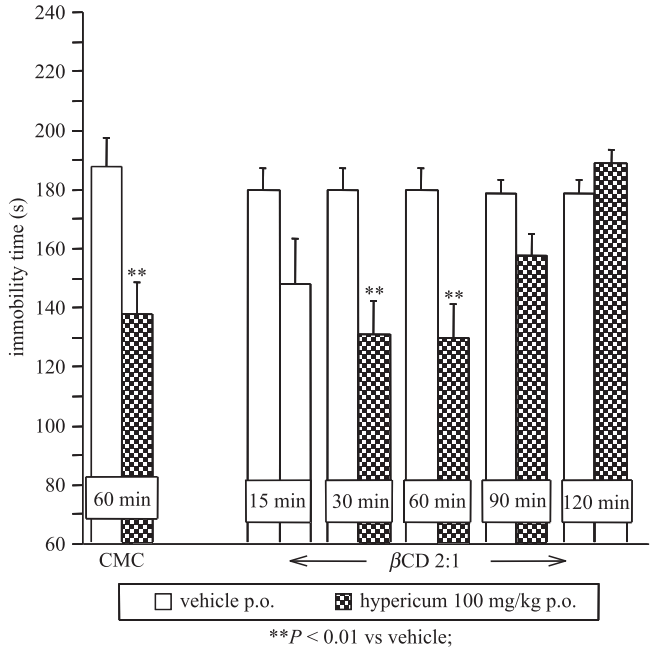

Figure 5. *Time course* of the colyophilized St. John's wort at 100 mg/Kg:β-CD 1:2 w/w p.o.

activity of mice was unmodified by treatment with the above-mentioned compounds in comparison with control groups (Fig. 8).

The number of falls from the rotating rod showed a lack of any impairment in motor coordination of animals treated with all substances in comparison with the control group. During each session there would have been a decrease in the number of falls if motor coordination was not altered (Fig. 9).

The solubility of three main classes of compounds of *Hypericum perforatum* L., flavonoids, hyperforins and hypericins, was affected by the different excipients of the formulations. The results obtained are reported in Table 2.

Concerning the extract, water solubility at 26 °C was 353.66 μg/ml for flavonoids and 10.40 μg/ml for hypericins, while that of hyperforins was $\cong 10^{-1}$ μg/ml.

The solubility of flavonoids in the presence of β-cyclodextrin and SDS was increased three times and with ASC8 six times. Also the solubility of biapigenin increased in the formulations ranging from 6.87 μg/ml to 150.34 μg/ml; the highest was obtained in the micellar solution of ASC8.

The total amount of hypericins was 21.26 μg/ml in the case of β-cyclodextrin, 44.31 μg/ml with SDS micelles and 72.18 μg/ml with ASC8 micelles.

The most interesting results concern the phloroglucinol solubilities. The water solubility and solubility in the β-cyclodextrin formulation was $\cong 10^{-1}$ μg/ml. Micelles had a high influence on hyperforins: their solubility was 15.85 μg/ml with SDS and 94.99 μg/ml with ASC8.

DISCUSSION

In this study oral dosage forms containing surfactants (SDS, ASC8) and β-cyclodextrin were evaluated by *in vivo* Porsolt's test and the resulting data were compared with those obtained with the extract alone. Antidepressant-like activity was evaluated in the conventional conditions of the FST; this is a good experimental model where antidepressant drugs used in clinical therapy showed their action reducing time immobility. The decrease in the immobility time induced by the extract was compared to that induced by the antidepressant drug amitriptyline used as reference compound.

The micellar solution of SDS 40 mM showed a rapid onset compared to the extract alone, but with this anionic surfactant it was not possible reduce the dosage.

Better results were obtained with the micelles of ASC8. In addition to behaving as a surfactant, with a low CMC (8.1 mM), ASC8 retains the antioxidant properties of vitamin C and acts as a powerful radical scavenger both in aqueous and in non-aqueous media. It is under consideration for human use, and may be suitable both for oral and parenteral formulation. The micelles of ASC 8 represent a valid formulation to decrease the dosage active of the extract until 30 mg/Kg, with a better pharmacokinetic profile.

Table 2. Solubility of constituents (μg/ml) of *Hypericum perforatum* L. extract in water and in three different dosage forms: β-Cd colyophilized, SDS and ASC8 micelles.

Constituents	H_2O	β-Cd	SDS 40 mM	ASC-8 40 mM
Flavonoids	353.66 ± 11.72	1172.77 ± 18.22	1021.39 ± 12.56	1841.02 ± 13.42
I3,II8-biapigenin	6.87 ± 0.27	59.68 ± 0.78	137.47 ± 1.42	150.34 ± 6.31
Total hyperforins	$\cong 10^{-1}$	$\cong 10^{-1}$	15.85 ± 0.95	94.99 ± 3.93
Total hypericins	10.40 ± 0.30	21.26 ± 1.06	44.31 ± 3.00	72.18 ± 2.62

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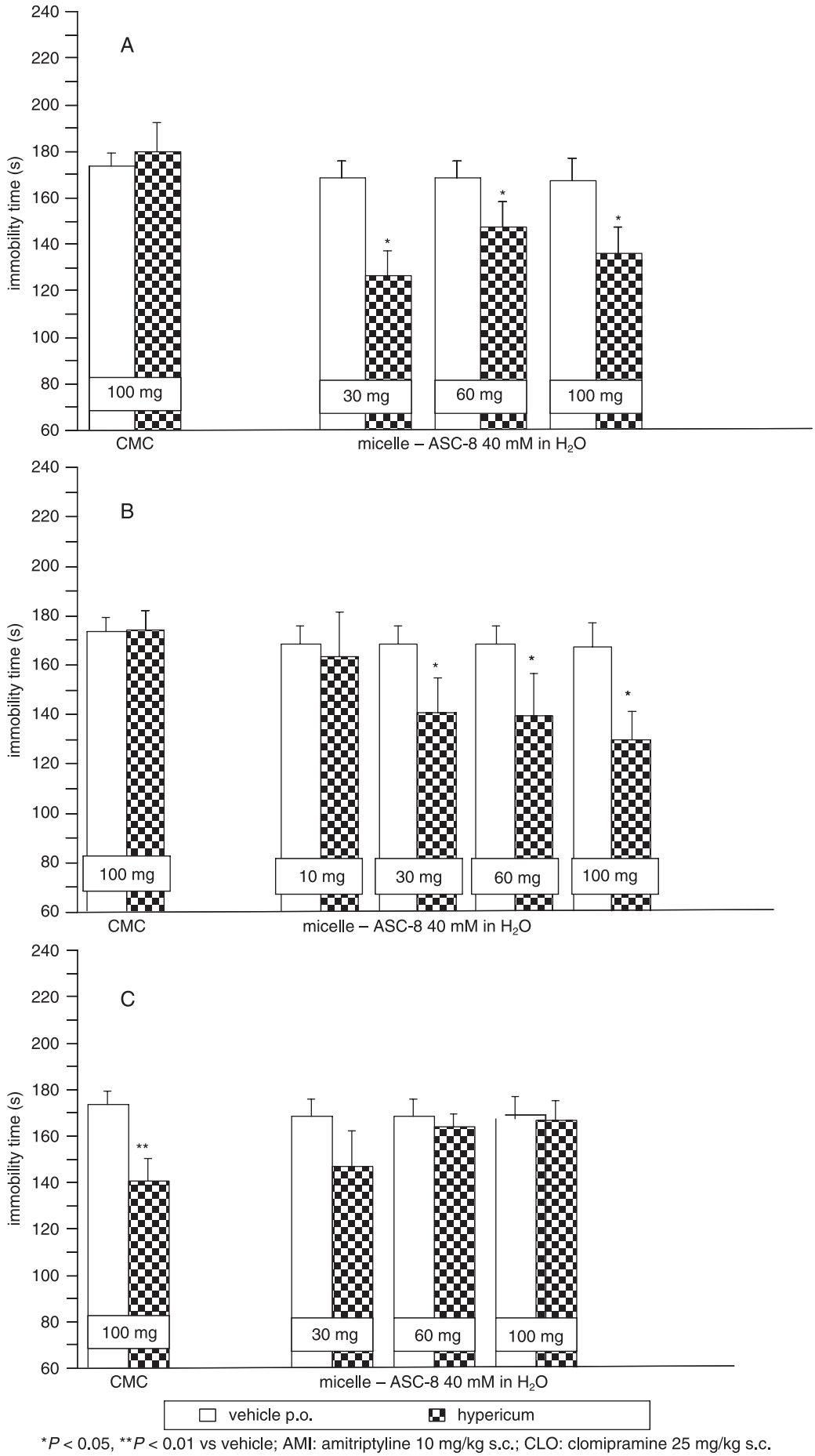

*$P < 0.05$, **$P < 0.01$ vs vehicle; AMI: amitriptyline 10 mg/kg s.c.; CLO: clomipramine 25 mg/kg s.c.

Figure 6. Dose-response curve for the antidepressant effect of ASC8 40 mM micelles of St. John's wort commercial extract A: 15 min after administration, B: 30 min after administration, C: 60 min after administration.

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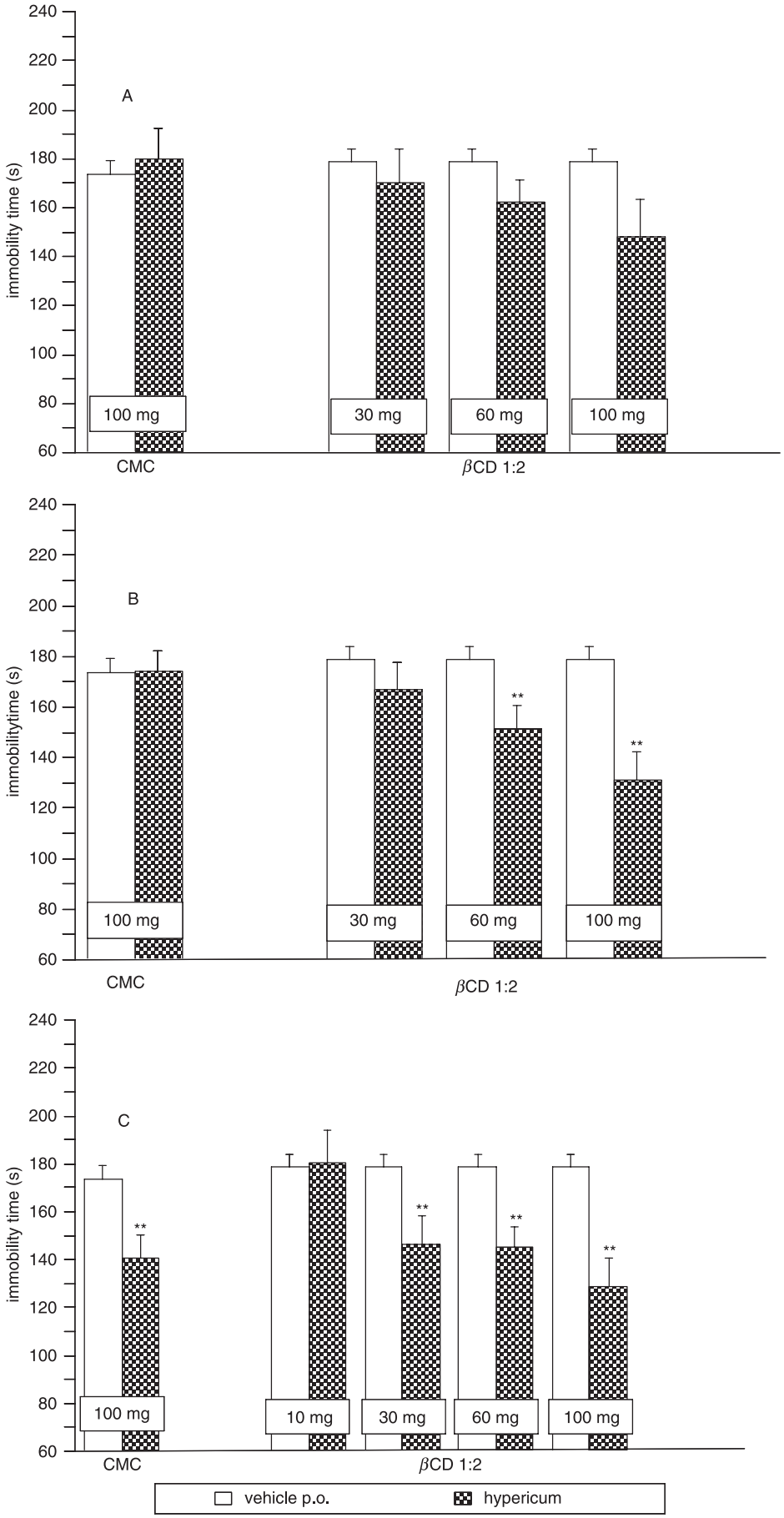

**$P < 0.01$ vs vehicle; AMI: amitriptyline 10 mg/kg s.c.; CLO: clomipramine 25 mg/kg s.c.

Figure 7. Dose-response curve for the antidepressant effect of β-CD formulation of St. John's wort commercial extract A: 15 min after administration, B: 30 min after administration, C: 60 min after administration.

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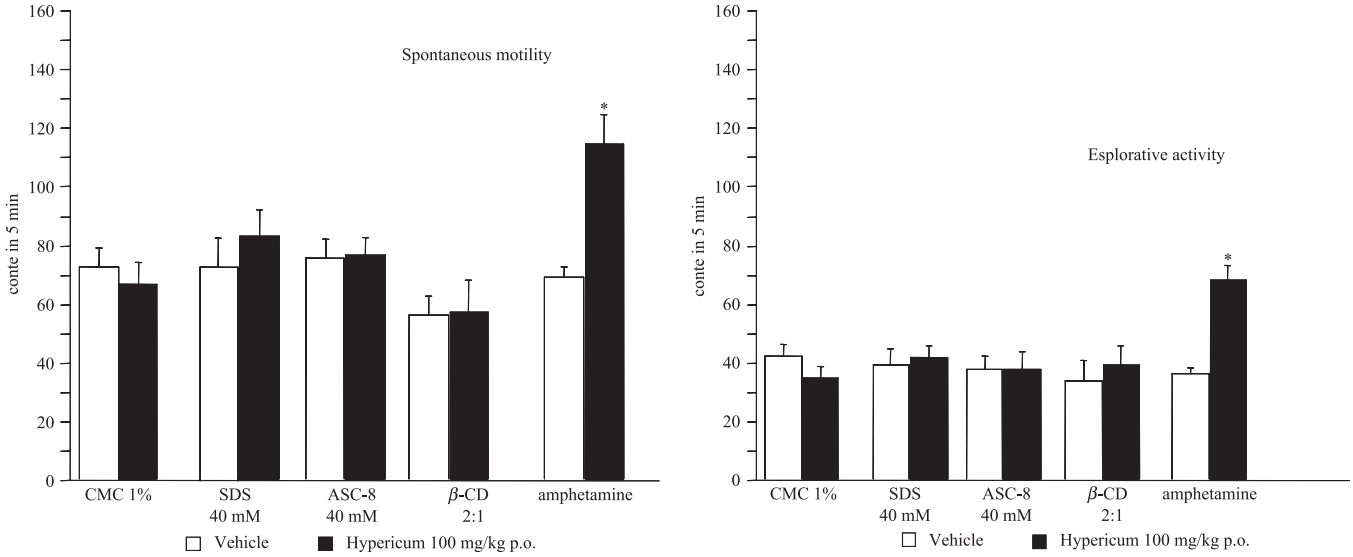

Figure 8. Evaluation of the effect of St. John's wort and other vehicles regarding spontaneous motility and explorative activity through the hole board test.

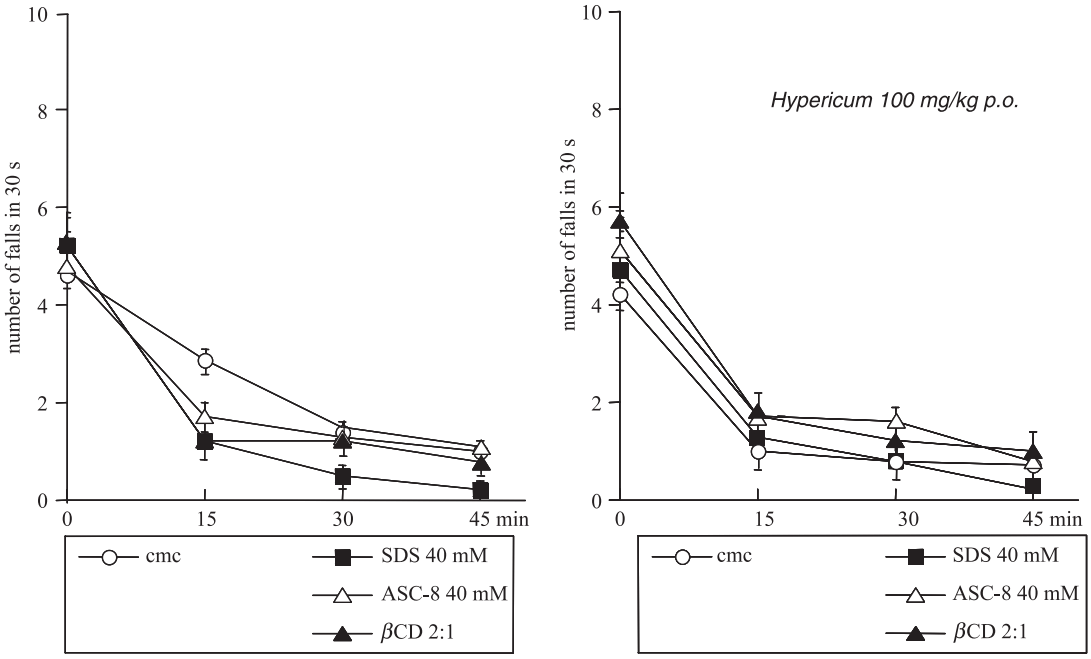

Figure 9. Evaluation of the effect of vehicles (a) and vehicles with St. John's wort (b) regarding motor coordination with the Rota rod test.

The motility observed in the Porsolt's test really represented the measure of the antidepressant-like effect and it was not to be considered a side-effect, as evidenced by Rota-rod test and hole-board test. Moreover pharmacological modulators did not produce any alteration in motor coordination, spontaneous motility and exploratory activity at all doses tested (data not shown). Neither alteration of animals' gross behaviour nor signs of toxicity/death of mice treated with all the investigated compounds was observed up to seven days following treatment (data not shown).

A second part of our work concerned the influence of the dosage forms on the water solubility of the extract's constituents. This parameter could be useful to interpret the pharmacological behaviour of the extract formulated with the technological excipients.

If we compare the results obtained within the pharmacological assays with those obtained from the water solubility studies, ASC8 micelles resulted the best formulation for *Hypericum perforatum* L., decreasing the active dosage and showing a better pharmacokinetic profile. These results can be explained by a notable influence of surfactant on the solubility of all the constituents of the extract, particularly regarding hyperforin solubility.

Also the formulation with β-cyclodextrin had a more rapid onset in the Porsolt test, having a maximum of peak of activity after 30 min. This excipient influences

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the solubility of flavonoids and hypericins, but not with regards to hyperforins. In particular, for such compounds it can act as an enhancer of penetration and increases the abortion of all constituents, as reported in the literature (Rajewski and Stella, 1996; Hirayama and Uekama, 1999).

In summary, all these new oral formulations showed in the Porsolt test a more rapid onset and prolonged action as well as the possibility of reducing the dosage of total extract of SJW to obtain the same antidepressant-like effect with minor potential side-effects.

The extract alone demonstrated antidepressant activity in mice after 60 min with a dosage of 100 mg/Kg. A similar antidepressant-like activity appeared in 30 min with a micellar solution of SDS containing the same quantity of extract (100 mg/Kg), while with micelles of ASC8 the effect appeared after 15 min with a dosage of 30 mg/Kg. In the case of preparation with β-cyclodextrin, the best results were obtained at 30 min administering 60 mg/Kg of the extract.

To interpret the pharmacological behaviour of the extract formulated with the technological excipients, the effect of the dosage forms on the water solubility of the extract's constituents was evaluated. If tensides can dramatically enhance solubility, in particular that of the more lipophilic compounds, in the case of β-cyclodextrin this effect is very pronounced for flavonoids and biapigenin, lower for hypericins and practically insignificant for hyperforins. The pharmacological results have pointed out the possibility of this excipient to ameliorate drug availability through a facilitation of the absorption.

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