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ORIGINAL CONTRIBUTION

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Prevention of brain protein and lipid oxidation elicited by a water-soluble oryzanol enzymatic extract derived from rice bran

Summary *Background* The antioxidant capacity of rice bran (RB) (due mainly to its γ-oryzanol content) is very well known. We have recently developed a water-soluble oryzanol enzymatic extract (WSOEE), which shows a greatly increased functionality. *Aim of the study* The aim of our study is the evaluation of the antioxidant potential of WSOEE in an *ex vivo* model to compare its protective capacity against oxidative damage by active-oxygen substances and free radicals (mainly the peroxyl radical) to biomolecules (such as proteins and lipids) with that of antioxidants, such as Trolox (a water-soluble derivative of vitamin E), melatonin, and folic acid. *Methods* WSOEE γ-oryzanol content and composition were determined by HPLC. Free-radical-scavenging capacity was evaluated using the assay based on phycoerythrin fluorescence. Antioxidant capacity against hydroperoxide-caused oxidative injury to proteins and lipids was evaluated using an *ex vivo* model: a rat brain homogenate. The effectiveness was determined by assessing protein damage (measured as carbonyl group content by Western blot immunoassay) and lipid peroxidation (measured as malondialdehyde content). *Results* The WSOEE γ-oryzanol composition profile was similar to that of RB (cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates), but with two major differences: WSOEE γ-oryzanol concentration was five times higher than that of RB, and WSOEE was water soluble. WSOEE total antioxidant capacity to trap the peroxyl radical was high, and similar to that of Trolox. The capacity to inhibit lipid peroxidation induced by cumene hydroperoxide in rat brain homogenate yielded a protection similar to that of Trolox. WSOEE also showed the capacity to protect protein from oxidation phenomena in rat brain homogenate, with a behavior similar to that of melatonin. This is of particular importance, since the loss of protein function caused by oxidative modification may affect the activity of enzymes, receptors, and membrane transporters, among other functions. *Conclusion* WSOEE is a new potential antioxidant agent from rice bran that shows a high free-radical-scavenging capacity and prevents protein oxidation and lipid peroxidation when cells *ex vivo* are exposed to free radicals.

Key words rice bran – antioxidants – γ-oryzanol – protein oxidation – lipid peroxidation

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Introduction

The role of dietary plant constituents and their derivatives in the prevention and treatment of a wide variety of diseases is long established [1]. Rice bran (RB) has been shown to have promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidaemia, fatty liver, hypercalciuria, kidney stones, and heart disease [2]. RB is a rich source of γ-oryzanol, which is a mixture of 10 ferulate esters of triterpene alcohols [3], and is being increasingly fo-

cussed on as an ingredient for drugs, nutraceuticals, functional foods, and feeds, as well as cosmetics.

The main potential properties of γ-oryzanol are its anti-hyperlipidaemic effects – reducing total plasma cholesterol and triglyceride concentration, and increasing the high-density lipoprotein cholesterol level – in rodents, rabbits, non-human primates, and humans [4]. Its antiulcerogenic [5] and antitumoural properties [6] have also been investigated. All these activities are correlated with antioxidant activity. γ-oryzanol possesses antioxidant functions because its structure includes ferulic acid. This constituent is responsible for the strong antioxidant activity [7, 8]. The high antioxidant activity of γ-oryzanol, which has been demonstrated against cholesterol oxidation [3] and lipid peroxidation in retinal homogenates under oxidative stress [9], shows superoxide-dismutase-like activity in that it inhibits pyrogallol autoxidation, which is catalysed by the superoxide radical [10].

The preparation of products enriched with the antioxidant components of RB can be of great importance for the treatment of diseases associated with an increased generation of active-oxygen substances and/or free radicals, such as atherogenesis, neurodegeneration, and cancer. In a previous work we described the physicochemical characterisation, stabilisation, and production of a water-soluble oryzanol enzymatic extract (WSOEE) from RB [11]. We now report the *in vitro* and *ex vivo* antioxidant activity of WSOEE, and propose its use as a functional food or nutraceutical for the prevention and/or treatment of pathologies caused by active-oxygen substances and/or free radicals.

Material and methods

Chemicals

Cumene hydroperoxide (CH), melatonin, Trolox, and 2,4-dinitrophenylhydrazyne (DNPH) were obtained from Sigma (Madrid, Spain); electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA, USA).

Chemical characterisation

WSOEE was prepared as we described previously [11]. WSOEE was chemically characterised using the AOAC standard protocols [12]: total protein content was determined by the Kjeldahl method (multiplying the total nitrogen content by the factor 5.5), total fat content by Soxhlet method, carbohydrates by HPLC, as described elsewhere [13], and starch by the method described by Vasanthan [14].

Extraction and quantification of γ-oryzanol

Lipids containing γ-oryzanol were extracted from WSOEE using hexane. The γ-oryzanol was semi-purified using a low-pressure silica column to remove the triglycerides and other lipids. The components of γ-oryzanol were separated and quantified by analytical reversed-phase HPLC [3].

Preparation of "fat-free WSOEE" and "protein-free WSOEE"

The lipid fraction of WSOEE was extracted with hexane. The lipid components constituted the "protein-free WSOEE", and the sediment originated during the extraction process, after washing and drying, constituted the "fat-free WSOEE".

Measurement of antioxidant activity of WSOEE

The antioxidant capacity of WSOEE was assayed using the method described by Glazer [15]. The assay uses the protein phycoerythrin as a free-radical-sensitive fluorescent indicator to monitor the effectiveness of WSOEE in protecting phycoerythrin from free-radical damage. The working solution containing 1.7×10^{-2} mM of phycoerythrin in 50 mM phosphate buffer pH 7.0 was preincubated at 37 °C for 30 min, in both the absence and presence of the appropriate dose of the antioxidants Trolox, WSOEE, "protein-free WSOEE", or "fat-free WSOEE". Oxidation was initiated by adding the water-soluble free-radical initiator 2-2'-azobis-(2-amidinopropane)-hydrochloride (AAPH) to a final concentration of 4 mM. After an additional incubation for 30 min at 37 °C, fluorescence was measured in rectangular (1 cm) fluorimeter cuvettes at $\lambda_{ex} = 540$ nm and $\lambda_{em} = 575$ nm.

Preparation of tissue homogenates

Wistar rats (3 months old) were used in all the experiments. Animals were handled according to guidelines established by the Animal House of this university. They were housed in a pathogen-free environment in groups of three or four in a temperature- and light-controlled room, with free access to food and water. Rats were decapitated and the brain was rapidly removed. The brain was homogenised in 3 volumes of homogenisation buffer: 50 mM Tris/HCl, 1 mM NaCl, and 0.25 M sucrose, pH 7.5. A cocktail of protease inhibitors (0.2 mM phenylmethylsulphonyl fluoride, 50 mM EDTA, chymostatin, leupeptin, and pepstatin: 1 μg/mL of each) was added to the incubation mixture to prevent proteolysis. The ho-

mogenates were centrifuged at 1000 × g. All operations were performed at 0–4 °C.

Treatment with oxidant

Brain homogenates (4 mg proteins/mL) were preincubated at 37 °C for 30 min in homogenisation buffer, in both absence and presence of the appropriate dose of the antioxidants Trolox, WSOEE, "protein-free WSOEE" or "fat-free WSOEE". Oxidation was initiated by adding CH to a final concentration of 2 mM. After an additional incubation for 30 min at 37 °C, 10 % w/v of ice-cold TCA was added, and the samples were centrifuged in an Eppendorf centrifuge for 5 min at 8,000 × g. The pellet was used for carbonyl group quantification, and the supernatant for malondialdehyde (MDA) quantification.

Quantification of protein carbonyl groups

Protein carbonyl groups were quantified by reaction with DNPH as described previously by Levine et al. [16]. Proteins (pellets), after dissolution in 50 mM phosphate buffer, pH 7.5, were treated with 10 mM DNPH in 2 M trifluoroacetic acid, washed three times with 10 % TCA, and then washed three times by resuspension in ethanol/ethyl acetate (1:1). Proteins were solubilised in 400 µL of 6 M guanidine and 50 % formic acid, and centrifuged at 16,000 × g for 10 min to remove any trace of insoluble material. Carbonyl groups were measured spectrophotometrically at 366 nm ($\varepsilon = 21{,}000\ M^{-1} x\, cm^{-1}$).

Immunoblot analysis of protein carbonyls

Protein carbonyl profile was performed as described previously [17]. Briefly, proteins – dissolved in 6 % (w/v) SDS – were mixed with an equal volume of DNPH solution and incubated at room temperature. The solution was neutralised and prepared for loading onto SDS gels by addition of 2 M Tris/HCl and 300 g/L achieve final concentrations of 0.52 M and 78 g/L, respectively. Samples were separated by SDS-polyacrylamide (12 %) gel electrophoresis, and transferred to a nitrocellulose membrane. The membranes were then incubated with a mouse anti-DNPH antibody at a dilution of 1:1,500 for 1 h at 4 $_{o}$C. After extensive washing, the membranes were incubated with peroxidase-conjugated mouse anti-rabbit IgG (Boehringer Mannheim) at a dilution of 1:5,000 for 2 h, and washed with blocking buffer. The oxidised proteins were detected by chemiluminescence (Boehringer Mannheim), and immunoblots were quantified using an image analysis program (ImageQuant for Windows 2000, Amersham Bioscience) which allows the measurement of the optical density, directly correlated to the amount of carbonyl groups in the sample.

Determination of malondialdehyde

An aliquot (0.5 mL) of the supernatant obtained after the treatment with oxidant was used for MDA determination [18]. Samples were treated with TBA reagent (20 mM TBA in 50 % v/v glacial acetic acid), and then heated at 100 °C for 1 h. After a cooling period, butanol was added, the organic layer was removed, and end-point fluorescence was measured at $\lambda_{ex} = 515$ nm and $\lambda_{em} = 585$ nm.

Statistical analysis

Statistical analyses of data were performed using the SAS system [19]. To determine statistical differences between means, a one-way ANOVA was used. P values < 0.05 were considered significant.

Results

WSOEE chemical characterisation

The chemical composition of WSOEE is shown in Table 1. As these data show, the main component is protein (50 %) – in the form of peptides and free amino acids, due to the use of proteases for RB stabilisation and in the extraction – and showing no differences in amino acid composition with respect to RB [11]. The second component is carbohydrate (19 %), free of simple sugars and starch. The fatty acid composition of the fat content present in the extract (14 %) is similar to that present in RB: mainly unsaturated and polyunsaturated fatty acids (oleic and linoleic as the main components) [11], with other minor components such as γ-oryzanol (1.2 mg/g). These data also show that the qualitative composition of γ-oryzanol is similar to that found in RB (see Table 2), and includes cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates as the main components.

Table 1 Physicochemical characterisation of WSOEE

WSOEE	(g/100 g)
Protein	50.0 ± 2.3
Fat	14.0 ± 0.9
Carbohydrates	19.0 ± 1.1
Ash	5.1 ± 0.2

Data are means ± SD of three experiments
WSOEE water-soluble oryzanol enzymatic extract; *RB* rice bran

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Table 2 γ-oryzanol concentration and composition of WSOEE and RS

	WSOEE	RB
γ-oryzanol (mg/g)	1.26±0.12	0.25±0.10
Stearyl ferulate esters composition (%):		
2.4-methylene cycloartenyl ferulate	22.1±1.1	25.0±1.7
Campesteryl ferulate	28.1±1.3	24.2±0.9
Cycloartenyl ferulate	21.3±0.8	23.5±1.1
Sitosteryl ferulate	14.1±0.5	13.3±0.6

Data are means ± SD of three experiments
Ferulate compositions are expressed as percent of γ-oryzanol
WSOEE water-soluble oryzanol enzymatic extract; *RB* rice bran

WSOEE free-radical-scavenging activity

Taking into account that different products derived from RB have been reported to possess antioxidant activity [3, 7–10], we tested the antioxidant properties of WSOEE by measuring its free-radical-scavenging activity, using a fluorimetric procedure. The assay is based on phycoerythrin fluorescence, using the water-soluble free-radical initiator 2-2'-azobis-(2-aminopropane)-hydrochloride (AAPH) as a source of peroxyl radicals. Fig. 1 shows the rate of phycoerythrin oxidation produced by the generation of peroxyl radicals, in the absence and presence of an antioxidant. The protection given by WSOEE (with an inhibition of oxidation of 98 % at 250 μg/mL) (0.3 μg/mL of γ-oryzanol) is similar to that by Trolox (95 % at 10 μg/mL). The data of Fig. 1 also show that the protection of the phycoerythrin structure by Trolox and WSOEE is dose-dependent.

The inclusion of WSOEE protected phycoerythrin from damage caused by the peroxyl radical. At 250 μg/mL WSOEE (which corresponds to 0.3 μg/mL γ-oryzanol), the protection is complete, with all the peroxyl radicals neutralised. The scavenging capacity was significantly less effective than that of Trolox, an antioxidant whose trapping protection property is well known, and which gave complete protection at 1/25th the dose (10 μg/ml) (Fig. 1). Nonetheless, the protective capacity of WSOEE against the peroxyl radical is considerable, and similar to that of other less potent antioxidants such as β-carotene (Cremades et al., personal communication).

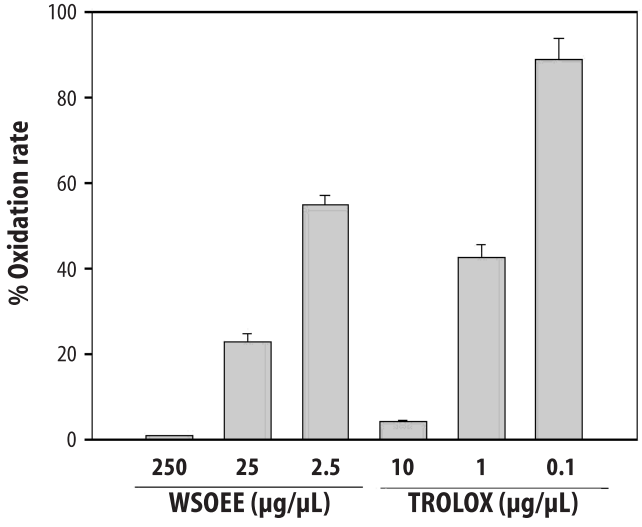

Fig. 1 Evaluation of free-radical-scavenging capacity of WSOEE using the phycoerythrin-fluorescence-based assay for peroxy-radical scavengers. Oxidation rate is defined in relation to phycoerythrin fluorescence (absence of antioxidant = 100 % oxidation rate). The results are mean ± SD of five independent experiments

To test whether one of the WSOEE components (and if so, which) is mainly responsible for this protective activity, or if it is a synergistic result of the integral product, we prepared "fat-free WSOEE" and "protein-free WSOEE", and tested their antioxidant activity as described above for the integral WSOEE. Compared with the 100 % protecting activity of integral WSOEE, that of "fat-free WSOEE" was 10 % and that of "protein-free WSOEE" 73 %. The blend of "fat-free WSOEE" and "protein-free WSOEE" practically restored (98 %) the total protection against peroxyl radicals afforded by "integral WSOEE". These data show that the main antioxidant activity of this product is associated with its lipophilic components, and that the proteinaceous components contributed only to a minor extent (10 %). However, the presence of the two components, lipids and proteins, produces a synergistic action, increasing the protective capacity of the product.

Effects of WSOEE on protein oxidation and lipid peroxidation induced by CH

The previous data show only that WSOEE has antioxidant activity, like that shown by Trolox, and that this activity is associated with its lipid fraction. To find out how specific the protection is, we evaluated the capacity to protect proteins and lipids against damage (oxidation) caused by the peroxyl radical, using an *ex vivo* model (a brain homogenate). We used this model since brain tissue is very vulnerable to oxidative damage because of its relative lack of antioxidant enzymes, such as catalase and glutathione peroxidase, and a high abundance of oxidisable substrates, such as polyunsaturated fatty acids, catecholamines, etc. [20, 21]. We used CH as lipid oxidising agent, because it has been used to assess the effects of free radicals and reactive oxygen intermediates on various biological molecules [20, 22]. In the present study, the protective effect of WSOEE against the damage caused by CH has been evaluated measuring MDA and carbonyl group content in a brain extract, as an assessment of oxidative damage to lipid and protein, respectively. Aldehydes are always produced as products of lipid peroxidation when lipid hydroperoxides break

down in biological systems, with MDA as the most abundant aldehyde [18]. Fig. 2 shows the effect of CH on brain homogenate lipids. CH produced a significant increase in MDA content (64.3 nmol/mg) in the absence of a protective substance. However, the inclusion of WSOEE or Trolox (control antioxidant) had a marked protective effect, lowering the production of MDA. These data also show that the protection was concentration dependent: at 20 mg WSOEE/mL (24 ng γ-oryzanol/mL), MDA production was reduced to 11.4 ± 0.7 nmol/mg, while 10 mg Trolox/mL reduced MDA production to 2.5 ± 0.2 nmol/mg.

Carbonyl groups are introduced into proteins by oxidative mechanisms occurring in a variety of physiological and pathological processes, and are an established marker of protein oxidation [23]. CH reaction with brain proteins in the homogenate resulted in a rapidly increasing yield of carbonyl groups, whose formation was quantified using DNPH [16]. The process was essentially complete 15 min after addition of CH, at levels of 18 ± 1.3 nmol of carbonyl groups per mg of protein. The inclusion of WSOEE prevented oxidation of the brain proteins, as reflected in the absence of statistically significant difference between the values obtained for the samples treated with 10 mg/mL WSOEE and that found in the controls (unoxidised samples): 1.40 ± 0.2 and 1.28 ± 0.3 nmol of carbonyl group per mg of protein, respectively. The measurement of total carbonyl content does not allow the determination of whether a specific protein becomes oxidised in this process. To locate specific oxidation of particular proteins, we measured the distribution of the carbonyl groups on brain proteins by labelling carbonyl groups with DNPH and subjecting the proteins to SDS-PAGE and Western blotting. The total protein was detected in parallel blots by Coomassie-blue staining. The protein molecular-weight profile does not show any differences in amounts or in molecular-weight profile between the samples (Fig. 3A); in contrast, the protein-oxidation profile changes strongly. After incubation of brain homogenate with CH in absence of a protective substance, protein oxidation increased dramatically, becoming very patent and extensive (Fig. 3B, lane 2). We quantified the immunoblot, measuring the optical density (which is related to the levels of protein oxidation). The data are shown in Fig. 3. The inclusion of WSOEE (Fig. 3B, lane 3), as we show above, prevented protein oxidation. From the immunoblot, we observed a band of about 60 kDa with weak anti-DNPH reactivity. In order to compare this protection of protein oxidation with that of other antioxidants, we chose melatonin (Fig. 3B, lane 4) – one of the most potent and versatile cellular natural antioxidants [24] – and folic acid (Fig. 3B, lane 5) – which has been reported to produce antioxidant activity *in vivo* [25] and *in vitro* [26], although its antioxidant potential is controversial.

Melatonin showed a significant protein protection, lowering the protein carbonyl background even in untreated samples (Fig. 3B, lane 1). However, in this model of oxidation, folic acid showed no protein protection against free-radical attack. Thus, only the treatment with WSOEE or melatonin (control antioxidant) showed the capacity to diminish the protein oxidation induced by CH. This could be of great importance for the treatment and prevention of a series of diseases, as we will discuss below.

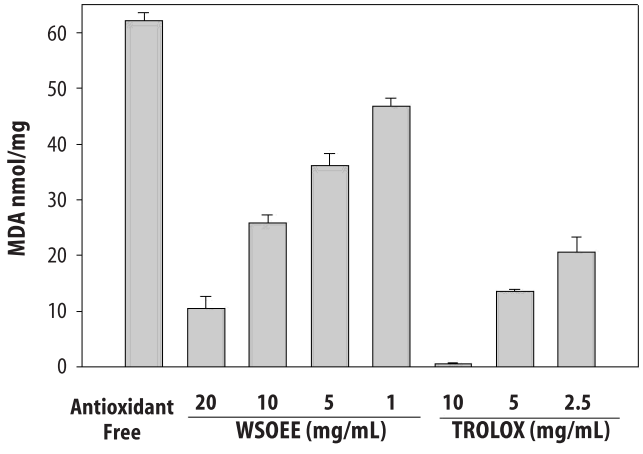

Fig. 2 WSOEE inhibitory capacity against lipid peroxidation in brain homogenate. Lipid peroxidation was performed by induction with cumene hydroperoxide, and antioxidant (WSOEE and Trolox) inhibitory capacity was evaluated by malondialdehyde production. The results are mean ± SEM of five independent experiments. The levels in samples with WSOEE and Trolox are significantly different ($p < 0.05$) from those in antioxidant-free samples

Discussion

The primary aim of our study was to evaluate the antioxidant potential of WSOEE, a new source of water-soluble oryzanol. The main functional feature of its chemical composition is that all its components are water soluble. The major components are proteins. These are mainly in the form of peptides and free amino acids [11], improving protein bio-absorption over that of the original protein, which is insoluble due to extensive disulphide bonding and aggregation [27, 28]. The carbohydrates of WSOEE comprise mainly slow-absorption carbohydrates. The fat components present in WSOEE are soluble due to interactions with proteins, and are extracted using an enzymatic procedure [11]. The WSOEE γ-oryzanol composition profile was similar to that of RB (cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates) but with two main differences, deriving from physical properties: i) the fat components are in a soluble form, and ii) there is a specific enrichment in γ-oryzanol content, which is five-fold that in RB.

WSOEE shows *in vitro* antioxidant capacity due to the extensive scavenging of peroxyl radicals, a similar

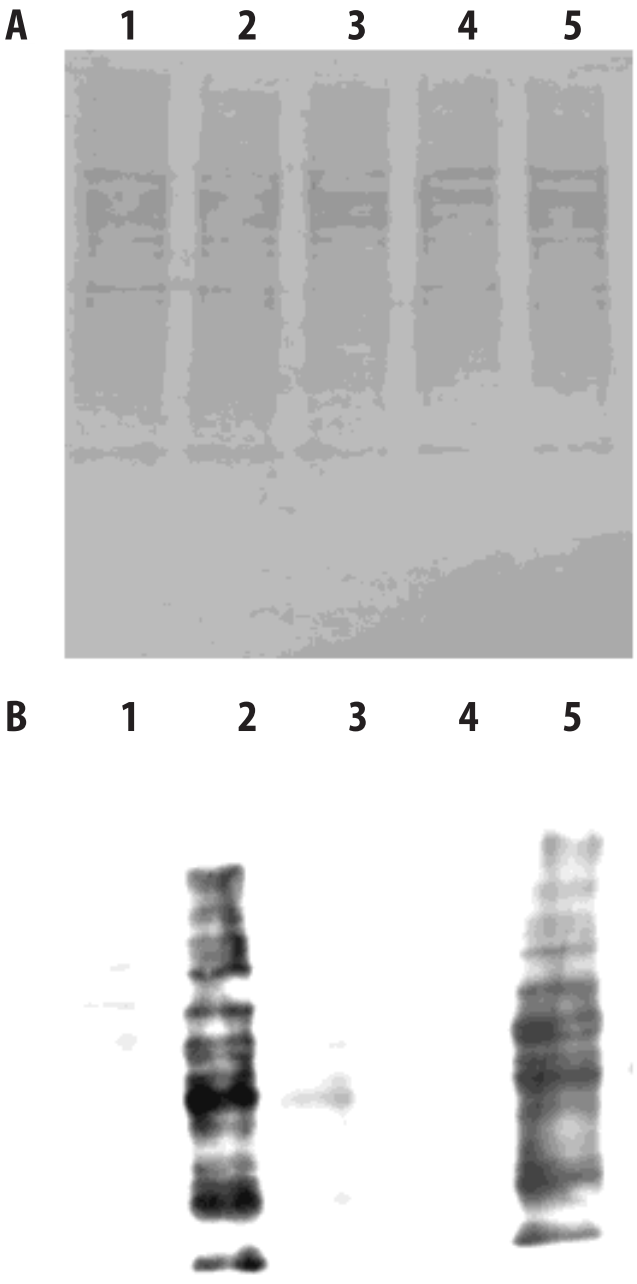

Fig. 3 Detection of oxidatively modified proteins of brain homogenate after treatment with cumene hydroperoxide alone (2) or in the presence of WSOEE (3), melatonin (4), and folic acid (5). **Panel A**: Western blot stained with Coomassie blue. **Panel B**: After incubation of a hepatic homogenate with CH alone or in the presence of antioxidants, aliquots of the reaction mixture were subjected to SDS-PAGE followed by immunoblotting with anti-DNPH antibody. Optical density of the immunoblot was measured using PCBass software. *Lane1, 0.17; Lane 2, 12.78; Lane 3, 0.50; Lane 4, 0.04.; Lane 5, 11.99. Arbitrary values*

behaviour to that of Trolox (a water-soluble derivative of vitamin E). Nevertheless, the capacity of WSOEE, as an extract, is lower than that of pure antioxidant. More valuable physiological information comes from the interaction of the WSOEE components with cell biomolecules under oxidative stress, whereby the antioxidant function is improved as a result of distribution and localisation of the components in the cell. We have used measurements in an *ex vivo* model to study the preventive capacity against oxidative damage by active-oxygen substances and free radicals (mainly the peroxyl radical) to biomolecules, such as proteins and lipids. We also compared the effect of WSOEE with that of antioxidants such as Trolox, melatonin, and folic acid (a substance with antioxidant properties). We chose to use brain tissue because *in vitro* models have implicated oxidative insults in various forms of brain damage or altered neuronal functions [29, 30]. The role of oxidative stress in the genesis of various neurodegenerative diseases is also well established [31, 32], as are cytotoxic aldehydes in the pathogenesis of Parkinson's disease [31]. Oxidative modification of proteins and lipids by reactive species is implicated in the aetiology or progression of a panoply of disorders and diseases, such as Alzheimer's disease, Huntington's disease, prion disorders, Parkinson's disease, atherosclerosis, and the ageing process [23].

The capacity to inhibit lipid peroxidation and protein oxidation induced by CH in rat brain homogenate was also tested. WSOEE was found to be protective, but significantly less effective than Trolox, in preventing lipid peroxidation phenomena that have been associated with important pathophysiological events. Such toxicity is largely attributable to the unsaturated aldehydes that are produced. There is also abundant evidence showing that lipid peroxidation products are capable of modifying proteins [33–35]. WSOEE also showed the capacity to protect protein from oxidation phenomena in rat brain homogenate under oxidative conditions induced with CH, showing a behaviour similar to that of melatonin. Melatonin has been reported to play an important role in the protection of DNA and cell membranes from oxidative stress [36, 37], and this effect is a consequence of its ability to scavenge hydroxyl [37] and peroxyl radicals [38].

This is of particular importance, since the loss of protein function caused by oxidative modification may affect the activity of enzymes, receptors, and membrane transporters, among other functions. Under oxidative stress, proteins suffer various structural modifications, which have been extensively studied [39, 40]. The physiological consequences of these modifications depend on whether the alteration occurs in the active site of the enzyme, and on the number of affected molecules and the relevance of the protein. WSOEE has been proved to protect against damage caused by active-oxygen substances, lowering lipid peroxidation and protein oxidation induced by CH in brain extracts.

WSOEE is a new potential antioxidant agent from rice bran, being a new source of water-soluble oryzanol. This soluble product shows a high free-radical-scavenging capacity, preventing protein oxidation and lipid per-

oxidation when cells *ex vivo* are exposed to active-oxygen substances and/or free radicals. This property makes it potentially useful in the formulation of solid and liquid food for treatment and prevention of chronic pathological states associated with a high generation of active-oxygen substances and/or free radicals – such as atherosclerosis, neurodegeneration, and cancer – and for elderly persons and practitioners of sports.

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