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Protective Effect of *Scoparia dulcis* on Brain and Erythrocytes

[1]Ahmed Y. Coulibaly, [1]Pierre A.E.D. Sombie, [2]André Tibiri, [1]Martin Kiendrebeogo,
[1]Moussa M.Y. Compaore and [1]Odile G. Nacoulma
[1]Laboratoire de Biochimie & Chimie Appliquées (LABIOCA), UFR/SVT,
Université de Ouagadougou, 09 BP 848 Ouagadougou 09, Burkina Faso.
[2] Institut de Recherche en Sciences de la Santé (IRSS),
03 BP 7192 Ouagadougou 03, Burkina Faso

Abstract: In order to evaluate the protective effect of *Scoparia dulcis* L. (Scrophulariaceae), a medicinal plant known to have medico-magic power, different extracts were prepared by successive extraction with hexane (HE), chloroform (CE) and methanol (ME) and by a soaking in acetone (80%). These extracts were checked for their protective role against neuroinflammation and erythrocytes haemolysis. They exhibited a significant neuroprotective effect on both brain neuronal cells and neurotransmitter enzyme as acetylcholinesterase. By their cytoprotective ability the extracts prevented rat erythrocytes haemolysis between 56% and 83% at 300 µg/mL. These observed protective effects were related to the extracts antioxidant components.

Key words: Acetylcholineterase, brain, erythrocytes, lipid peroxidation, *Scoparia dulcis*

INTRODUCTION

Scoparia dulcis L. (Scrophulariaceae), commonly known as sweet broomweed, is a perennial and widespread herb in tropical and subtropical regions. In these areas, it is well known as a folk-medicinal plant with medico-magic power (Dalziel, 1955).

Previous investigations evidenced its use to help with the symptoms of several diseases such as arterial hypertension and diabetes mellitus (Satyanarayana, 1969) related to inflammation and oxidative stress.

A number of the medicinal properties of *S. dulcis* was previously studied including its anti-diabetic, anti-inflammatory and antioxidant capacity *in vivo* (Adaikpoh *et al.*, 2007; Freire *et al.*, 1993), its impact on lipid peroxidation (Pari and Latha, 2005; Ratnasooriya *et al.*, 2005), its *in vivo* anti-anemic properties (Orhue and Nwanze, 2009) and its protective role on insulinoma cell line RINm5F (Latha *et al.*, 2004) and on kidney, heart and liver of rats exposed to cadmium (Adaikpoh *et al.*, 2007).

A few phenolic and terpenic compounds isolated from *S. dulcis* were pointed to justify these medicinal properties and various biological activities (Hayashi *et al.*, 1988, 1990, 1991).

The brain and nervous system have limited antioxidant capacity and are most vulnerable to oxidative stress (Vega-Naredo *et al.*, 2005). They cannot synthesize glutathione, a fundamental component of antioxidant machinery (Peng *et al*., 2007). Immunohistochemical data revealed many of the hallmark modifications of oxidative damage in Alzheimer's disease brain and increased amounts of lipid peroxidation (Markesbery and Carney, 1999; Ando *et al.*, 1998). ß-amyloid peptides in Alzheimer's disease brain were also pointed to induce inflammatory process with subsequent liberation of free radicals (Vina *et al.*, 2004; Stuchbury and Munch, 2005). Acetylcholine (Ach) is a neurotransmitter in neuronal cells that deficiency in cerebral cortex was found to be a major cause of neurodegenerative disorder such as Alzeimer's disease (Bierer *et al.*, 1995). Its action is stopped by acetylcholinesterase (AChE), a key enzyme catalyzing the hydrolysis of acetylcholine (ACh) in the nervous system of animals and insects. The use of AChE inhibitors can help for neuroprotection in neurodegenerative disorders by enhancing the Ach level in the brain (Enz *et al.*, 1993).

Reactive oxygen species induce lipid peroxidation in bio-membranes phospholipids (Sarada *et al.*, 2002), damaging tissues in the body. The oxidation of erythrocyte membranes by free radicals induces the oxidation of lipids and proteins and eventually causes haemolysis (Sato *et al.*, 1995).

A growing interest is being focused on natural products that provide food supplements or specific pharmaceutics for human well being. So, information about their protective role on central nervous system neuro-inflammation and cell integrity could be highly benefited. This study report the protective role of *S. dulcis* on cellular and enzymatic models in brain and blood, two most vulnerable compartments in the body.

Corresponding Author: Ahmed Y. Coulibaly, LABIOCA, UFR/SVT, Université de Ouagadougou, 09 BP 848 Ouagadougou 09, Burkina Faso. Tel: (00226)76698802/(00226)50470559

MATERIALS AND METHODS

Plant material: *Scoparia dulcis* L (whole plant) was collected at Gampela (25 km, East of Ouagadougou, Burkina Faso) in July, 2010. Taxonomic identification was verified by the Laboratoire de Biologie et Ecologie Végétales (University of Ouagadougou, Burkina Faso) where a voucher specimen (SD-ca 001) has been deposited for archive.

Extraction: Air-dried grounded *Scoparia dulcis* (25 g) was sequentially extracted with 250 mL of hexane, chloroform and methanol using a Soxhlet apparatus. The extracts were then concentrated to dryness in a vacuum evaporator and stored for the different investigations.

Another powder of *Scoparia dulcis* plant (25 g) was soaked in 250 mL of acetone containing 20% of water for 36 h. The mixture was filtered and evaporated to dryness to obtain the aqueous-acetone extract.

Chemicals: All chemicals were of analytical grade. Aluminium trichloride ($AlCl_3$), Bovine serum albumin (BSA), Quercetin, Sodium phosphate dibasic (Na_2HPO_4), Sodium phosphate monobasic (NaH_2PO_4), Thiobarbituric acid were purchased from Sigma-Aldrich (Germany). Trichloroacetic acid was supplied by Fluka Chemika (Buchs, Switzerland). Ascorbic acid, Iron dichloride ($FeCl_2$) was provided from Labosi (France). Acetylthiocholine iodide (ATCI), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), Acetylcholinesterase (AChE) type VI-S from electric eel, tris-hydroxymethane, magnesium chloride hexahydrate, sodium chloride, galanthamine hydrobromide, p-nitrophenyl acetate and carboxylesterase from porcine liver were supplied from Sigma (USA).

Flavonols quantification: Flavonol content was estimated as described by Almaraz-Abarca *et al.* (2007). Aluminium trichloride (0.75 mL, 20% in ethanol) was mixed with the extract solution (0.75 mL, 1 mg/mL) and incubated for 10 min. Then the absorbance was read at 425 nm against a blank containing ethanol and the extract solution without aluminium chloride. Quercetin was used to produce the standard curve calibration and the results are expressed as mg quercetin equivalent (QE)/100 mg of extract.

Carboxylesterase inhibition: The enzymatic activity of carboxylesterase from porcine liver was determined as described by Djeridane *et al.* (2008). p-nitrophenyl acetate was used as substrate. The reaction mixture consisted in 400 µL of Tris-HCl buffer (50mM, pH 8), 100 µL of enzyme solution (0.27 U/mL in Tris-HCl buffer) and 100 µL of the extract solution (1 mg/mL in Tris-HCl buffer). The mixture was allowed to stand in room temperature (30ºC) for 5 min. The reaction was then initiated by adding 400 µL of the substrate solution (p-nitrophenyl acetate, 1mM). The liberated p-nitrophenol was monitored at 414 nm for 3 min and the initial velocity was recorded. The inhibition rate (I%) was calculated comparatively to a blank (negative control containing Tris-HCl buffer and without extract) using the following Eq. (1):

$$I\ (\%) = [1 - (V_{0\ Sample}/V_{0\ Blanc})] \times 100 \quad (1)$$

$V_{0\ Sample}$ and $V_{0\ Blanc}$ represent the velocities of the sample and the blank.

Acetylcholinesterase inhibition: The inhibitory effect of *Scoparia dulcis* extracts on acetylcholinesterase activity was evaluated using the spectrophotometric method of Ellman (1961). Essays were performed on a CECIL spectrophotometer. Briefly, 100 µL of the extract (1 mg/mL in 50 mM Tris-HCl buffer, pH 8) was mixed with 200 µL of buffer (50 mM Tris-HCl, pH 8 containing 0.1% BSA) and 100 µL of enzyme (AChE) solution (0.22 U/mL in 50 mM Tris-HCl buffer, pH 8 containing 0.1% BSA). The mixture was incubated for 5 min at 30ºC. Then 500 µL of DTNB (3 mM in Tris-HCl buffer, pH 8 containing 0.1 M NaCl and 0.02 M $MgCl_2$) and 100 µL of the substrate ATCI (4 mM in distilled water) were added to initiate the reaction. A blank was also prepared by replacing the enzyme solution with 100 µL of Tris-HCl buffer (50 mM, pH 8, 0.1% BSA). The reaction was monitored for 3 min at 405 nm and velocity (V_0) recorded. Antiacetylcholinesterase activity (I %) was calculated following the equation 1. Galanthamine HBr was used as standard inhibitor for positive control.

Brain protective assay: The assay was performed by adapted method as described by Hsu *et al.* (2007). The brain of a young adult male wistar rat (208 g) was dissected and homogenized with a homogenizer in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1% homogenate. The homogenate was centrifuged at 2000 g for 15 min at 4ºC, and the supernatant was used to perform the assay. The extract solution (0.2 mL, 1.5 mg/mL in Tris-HCl buffer 20 mM, pH 7.4) was mixed with 1.0 mL of the supernatant, 50 µL of $FeCl_2$ (0.5 mM) and 50 µL of H_2O_2 (0.5 mM). The mixture was incubated at 37ºC for 60 min; then the reaction was ended by adding 1 mL of trichloroacetic acid (15%) and 1 mL thiobarbituric acid (0.67%) followed by heating at 100ºC for 15 min. After centrifugation (2000 g for 10 min), the absorbance of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex in the supernatant was measured at 532 nm. Quercetin was used as positive control. The percentage of inhibition of brain oxidation was calculated according to the following equation:

$$\text{Inhibition}\ (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

A_0 is the absorbance of the control (without extract), A_1 is the absorbance of the extract addition and A_2 is the absorbance without brain homogenate.

Protection of rat erythrocytes against haemolysis: The assay was performed as described by Su *et al.* (2009) with slight modifications. The blood sample was collected by heart puncture in heparinized tubes from a male wistar rat (215 g) sacrificed under anesthesia.

Briefly, blood sample collected was centrifuged (1500 g, 10 min) at 4ºC and the erythrocytes were separated from the plasma and buffy coat and then washed three times by centrifugation (1500 g, 5 min) in 10 mL of phosphate buffered saline (PBS, 10 mM, pH 7,4). The supernatant and buffy coats of white cells were carefully removed after each wash. Washed erythrocytes was stored at 4ºC and used within 6 h to prepare 5% erythrocyte suspension in phosphate buffered saline (PBS, 10 mM, pH 7,4).

The reaction mixture was consisted in 0.5 mL of erythrocyte suspension (5%), 0.5 mL extract solution (300 µg/mL in PBS) and 0.05 ml of H_2O_2 (100 mM). After incubation at 37ºC for 60 min, 4.2 mL of distilled water was added and centrifuged at 1000 rpm for 10 min. Then, the absorbance of the supernatant was read at 415 nm. The protective effect was calculated as inhibition percentage of erythrocyte haemolysis in comparison to a blank with complete haemolysis and that did not contain extract solution. The following equation was used:

$$\text{Inhibition (\%)} = [1 - (A_1 - A_2)/A_0] \times 100$$

A_0 is the absorbance of the supernatant without extract, A_1 is the absorbance of the extract addition and A_2 is the absorbance of extract solution.

Statistical analysis: All the reactions were performed in triplicate and data were presented as mean±standard deviation. Data were examined by one-way analysis of variance (ANOVA) followed by Tukey multiple-comparison test using XLSTAT7.1. $p<0.05$ was used as the criterion for statistical significance.

RESULTS

Flavonol content: The yield of each extract was obtained by calculating the ratio of the dried extract by the weight of the initial powder of the plant material. Methanol extract showed the highest yield (15.12%) follow by aqueous-acetone extract (11.23), hexane extract (4.74%) and chloroform extract (2.98%).

Flavonols (Table 1) were quantified from sequential extracts of *Scoparia dulcis* (hexane, chloroform and methanol) and from an aqueous-acetone soaking extract. Flavonols ranged from 0.51±0.10 mg QE/100 mg in hexane extract to 1.37±0.04 mg QE/100 mg in chloroform extract. Hexane extract showed the lowest amount and the chloroform extract exhibited higher content.

Table 1: Flavonol content in *Scoparia dulcis* extracts

Extracts	Flavonol (mg QE/100mg)
Hexane	0.51 ± 0.10^{c}
Chloroform	1.37 ± 0.04^{a}
Methanol	0.74 ± 0.11^{c}
Aqueous-acetone	1.05 ± 0.12^{b}

Flavonol amount was expressed as mg quercetin equivalent (QE)/100 mg of extract; Data are expressed as mean values±standard deviation (n = 3); Values within each column with different superscript letters (a, b, c) are significantly different ($p<0.05$) as determined using ANOVA

Neuroprotective effect: The protective effect of *Scoparia dulcis* extracts on brain was evaluated by inhibition of brain peroxidation, acetylcholinesterase and carboxylesterase (Table 2). In both assay, hexane extract showed the lowest activity.

At 100 µg/mL, all the extracts inhibited acetylcholinesterase (19.29±1.51 to 57.89±2.63%) in a lesser extent than 10 µg/mL of the standard inhibitor Galanthamine HBr (98.32±1.17%). Therefore at the same concentration (100 µg/mL) all the extracts inhibited carboxylesterase (35.62±2.23 to 50.53±4.05%) more than ascorbic acid (34.67±3.42%) as standard.

In brain peroxidation assay, all the extracts protected the brain from oxidation lesser than 50% (14.63±2.88 to 31.01±0.33%) but they were all more effective than the standard quercetin (24.51±1.51%) excepted the hexane extract.

Chloroform extract was the most efficient on acetylcholinesterase inhibition while aqueous-acetone extract was most effective on both carboxylesterase inhibition and brain protection.

Cytoprotective effect: The protective effect of *Scoparia dulcis* extracts on cells was assayed on rat erythrocytes (Table 3). At 300 µg/mL all the extracts prevent more than 50% erythrocytes haemolysis but in a lesser extent than ascorbic acid (96.10±2.32%) used as standard. Hexane extract (83.39±0.83%) and methanol extract (80.96±1.46%) exhibited similar and higher cytoprotective rate than chloroform extract (56.78±1.71%) and aqueous-acetone extract (56.60±2.54%) with also similar inhibitory activities.

DISCUSSION

The protective effect of *Scoparia dulcis* extracts was evaluated as rat brain and erythrocytes protection.

The neuroprotective effect of *Scoparia dulcis* evaluated on brain tissue indicated a rate of inhibition less than 50% at 1.5 mg/mL for all the tested extracts. In this assay, brain oxidation was induced by iron ion (Fe^{2+}) known to be a potent pro-oxidant agent in biological

Table 2: Neuroprotective effect of *Scoparia dulcis*

	Inhibition percentages		
Extracts	Acetylcholinesterase	Carboxylesterase	Brain lipid peroxidation
Hexane	19.29 ± 1,51[d]	35.62±2.23[b]	14.63±2.88[b]
Chloroform	57.89±2.63[b]	45.16±4.26[a]	29.49±1.01[a]
Methanol	33.33±1.51[c]	47.87±4.92[a]	26.55±1.24[a]
Aqueous-acetone	21.05±2.63[d]	50.53±4.05[a]	31.01±0.33[a]
Galanthamine HBr	98.32±1.17[a]	nd	nd
Ascorbic acid	nd	34.67±3.42[b]	nd
Quercetin	nd	nd	24.51±1.51[a]

Acetylcholinesterase and carboxylesterase inhibitions are performed at a final concentration of 100 μg/mL and brain lipid peroxidation at initial concentration of 1.5 mg/mL. Galanthamine HBr (10 μg/mL), Ascorbic acid (50 μg/mL) and Quercetin (1.5 mg/mL) are used as standards. Data are expressed as mean values±standard deviation (n = 3). Values within each column with different superscript letters (a, b, c, d) are significantly different ($p<0.05$) as determined using ANOVA. nd = not determined

Table 3: cytoprotective effect of *S. dulcis*

Extracts	Inhibition of erythrocytes haemolysis (%)
Hexane	83.39±0.83[b]
Chloroform	56.78±1.71[c]
Methanol	80.96±1.46[b]
Aqueous-acetone	56.60±2.54[c]
Ascorbic acid	96.10±2.32[a]

Protective effect of the extracts against erythrocytes haemolysis was performed at initial concentration of 300 μg/mL; Ascorbic acid (300 μg/mL) was used as standard. Data are expressed as mean values±standard deviation (n = 3); Values within the second column with different superscript letters (a, b, c) are significantly different ($p<0.05$) as determined using ANOVA

systems (Oboh, 2009). Iron was pointed to initiate lipid peroxidation in neuronal cells which was further propage by peroxyl and alkoxyl radicals (Oboh *et al.*, 2007). All the extracts showed neuroprotective effect in accordance with the decrease of malondialdehyde (MDA) formation. The percentages of brain protection from Fe^{2+}-induced lipid peroxidation exhibited by the extracts of *Scoparia dulcis* are significant and comparable to those of green and sour teas (Oboh, 2009). Except hexane extract, all the extracts showed a powerful protection than the standard quercetin, known to be a potent anti-lipidperoxidation agent (Terao, 1999). Instead brain was most vulnerable to oxidation due to limited antioxidant system (Vega-Naredo *et al.*, 2005) the extracts still exerted a protective effect that could be explained through different mechanisms. The extracts could prevent the initiation of peroxidation process by chelating or reducing iron ion or by scavenging the free radical produce within the propagation phase, in regard of their known antioxidant potential (Babincová and Sourivong, 2001). According to Rice-Evans (1999), polyphenols inhibit the oxidation by direct scavenging of lipid alkoxyl and peroxyl radicals involved in the propagation phase. In this assay, the inhibition of brain lipid peroxidation by the extract of *S. dulcis* may be related to their flavonol content since each extract with higher flavonol amount was more effective. Such an observation about flavonol impact on brain lipid peroxidation was made *in vivo* by Almaraz-Abarca *et al.* (2007). Moreover, it was reported that anthocyanins reached the brain within minutes from their introduction into the stomach (Passamonti *et al.*, 2005); that could predict the bioavaibility of flavonols and other flavonoid compounds in the sweet broomweed *S. dulcis* to attend and protect the brain in the body. That observation can be supported by the previous *in vivo* studies about protective effect of *S. dulcis* on brain of streptozotocin diabetic wistar rats (Pari and Latha, 2004). Other scrophulariaceae species (*Bacopa monnieri* L. Wettst.) showed such a neuroprotective role in Alzheimer's disease model (Uabundita *et al*., 2010).

Lipid peroxidation in brain has been implicated in various diseases and aging that includes neurodegenerative disorders as Alzheimer's disease (Arlt *et al*., 2002; Praticò *et al.*, 2004; Niki *et al.*, 2005) also associated to inflammatory processes (Stuchbury and Munch, 2005). In this purpose, acetylcholinesterase inhibitors may have a neuroprotective role since they were suggested to be a promising approach for the neurodegenerative Alzheimer's disease therapy (Enz *et al.*, 1993).

All the crude extracts of *S. dulcis* subsequently inhibited acetylcholinesterase from electric eel. The crude chloroform extract exhibited a significant activity but lower than the alkaloid compound galanthamine as standard inhibitor. Hence these extracts could be used for further isolation of potent and safety AChE inhibitors.

Numerous factors were reported to improve the memory impairment including the cerebral blood flow (Tong *et al.*, 2009), the brain oxidative stress status (Nicolakakis *et al*., 2008), the balance function of various neurotransmitters including acetylcholine, serotonin, catecholamine (Reis *et al*., 2009), GABA (Kant *et al*., 1996) and glutamate (Saraf *et al*., 2009). So, by inhibiting AChE, the inibitors in the extracts of *S. dulcis* can help to enhance the level of the neurotransmitter acetylcholine in the neuronal synapse and then preventing the alteration of neuronal function. Free radicals are also reported to target neurotransmitter and neuromodulator systems, altering the chemical synaptic function (Mulkey *et al.*, 2003). Then, in physiological condition, antioxidants may have anticholinesterase properties by directly inhibiting the enzyme in the active site or by preventing free radicals to

target neurotransmitter by their neutralization or moreover by increasing the hippocampal antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) activities. Furthermore, antioxidants were suggested to be useful in the treatment of Alzheimer's disease (Calabrese *et al.*, 2003; Gibson and Huang, 2005). So, the protective effect of *S. dulcis* extracts on brain lipid peroxidation observed in this study and its known antioxidant properties (Babincová and Sourivong, 2001; Ratnasooriya *et al.*, 2005) could be helpful for preventing neurodegenerative disorders.

All the extracts of *Scoparia dulcis* significantly inhibited carboxylesterase as presented in Table 2. Only aqueous-acetone extract reached 50% inhibition. No relationship was observed between carboxylesterase and acetylcholinesterase inhibitions. That suggests the compounds responsible for the inhibition of these two enzymes are not the same and may act by different mechanisms.

Brain is the main target for drug effect and adverse effects because it contain the highest number of receptors (Herz, 1998). Carboxylesterase isoenzymes are known to be serine esterases, which are widely distributed in animal tissue including the brain (Redinbo *et al.*, 2003). They are involved in the detoxification of ester and amide prodrugs including heroin, capecitabine cocaine, meperidine and lidocaine (Redinbo *et al.*, 2003). They are essential enzymes in the catabolism of numerous xenobiotics such as carboxylesters, thioesters and aromatic amides; it also catabolize the angiotensin-converting enzyme inhibitors (temocapril, imidipril, and delapril) known to have a neuroprotective effect by stopping the inhibition of potassium-mediated acetylcholine release in the brain (Barnes *et al.*, 1992; Kehoe, 2003). Furthermore, carboxylesrase was cited to also bind to tacrine (Bencharit *et al.*, 2003), an anti-Alzheimer's drug with neuroprotective capacity. Thus, controlling the activity of these enzymes would be highly useful for management of the biological impact of ester compounds consumed by humans through different ways. Then, it may be important to modulate carboxylesterase activity in order to enhance these drugs effects in neurodegenerative disorders. In this scheme, the inhibitor compounds of carboxylesterase and acetylcholinesterase of *S. dulcis* extracts could be useful for dual protective effect.

The erythrocyte is a cell type, which contains high concentrations of polyunsaturated fatty acids, molecular oxygen and ferrous ions and it might be expected to be highly vulnerable to oxygen radical formation (Clemens and Wailer, 1987). Due to their susceptibility to oxidation, erythrocytes are suitable cellular model for investigation of oxidative damage in biomembranes. Thus, the cytoprotective role of *S. dulcis* extracts was evaluated in wistar rat erythrocytes model. Hydrogen peroxide (H_2O_2) was used to induce membrane oxidation leading to haemolysis, since it causes heme degradation in the presence of hemoglobin with the release of iron ions which catalytically activate lipid peroxidation (Puppo and Halliwell, 1988; Sadrazadeh *et al.*, 1984).

At 300 μg/mL, all the extracts significantly protected the erythrocytes from haemolysis (Table 3). The poorest extracts in flavonol (hexane and aqueous-acetone extracts) exhibited the highest erythrocytes protection. This suggested that the protective role of the extracts is not directly related to their flavonol content. Previous studies related that flavanols isolated from green tea leaves are good antioxidants against free radical initiated lipid peroxidation in solution (Jia *et al.*, 1998) and in human red blood cells (Ma *et al.*, 2000). So, instead of flavonol, other flavonoids may contribute to the protective role of *S. dulcis*.

Despite their significant protective effect, all the extracts of *S. dulcis* exhibited a lesser inhibition of erythrocytes haemolysis than the standard ascorbic acid. Indeed, ascorbic acid (vitamin C) is a known antioxidant compound (Bendich *et al.*, 1986) and was cited to act in a synergistic antioxidative effect with α-tocopherol, a principal liposoluble chain-breaking antioxidant in plasma and erythrocyte (Burton and Ingold, 1986; Liu *et al.*, 1988). This anti-haemolytic capacity of *S. dulcis* was assessed by previous *in vivo* assay in *trypanosoma brucei* infected rabbits (Orhue and Nwanze, 2009) where *S. dulcis* extract prevented these rabbits from anemia. Furthermore, erythrocytes possess an efficient antioxidant system at cytoplasmic level including catalase, superoxide dismutase and low molecular weight antioxidants such as GSH and ascorbate (Meister, 1994) which makes them exceptionally resistant to peroxidation when the radicals are produced within the cell (Clemens and Waller, 1987). Hence, this redundant and overlapping antioxidant system can be supplemented with lipophilic exogenous antioxidants from plants as *S. dulcis*.

CONCLUSION

The different extracts of *Scoparia dulcis* showed a markedly protective role against lipid peroxidation induce in brain and erythrocytes. These neuroprotective and cytoprotective effects might be attributed to its antioxidant components as flavonoidic polyphenols. Thus *S. dulcis* may have a beneficial effect on neurodegenerative disorders as Alzheimer's disease and where lipid peroxidation is strong features.

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AUTHOR'S CONTRIBUTION

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