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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE PLASMA AND ERYTHROCYTES TREATED *IN VITRO* BY LEAF EXTRACT OBTAINED FROM *FICUS RELIGIOSA* L. (MORACEAE)

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In the present study, we highlight the antioxidant potential of aqueous extract of *Ficus religiosa* L. leaves in equine plasma and erythrocyte suspension. In this study, we have focused on the antioxidant effect of leaf extract obtained from *F. religiosa* on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification (OMP), total antioxidant capacity (TAC)] using the equine erythrocytes model. Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w). The equine erythrocyte aliquots and plasma were used in the study. A volume of 0.1 ml of the *F. religiosa* extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (blank), phosphate buffer was used. Treatment by extract reduced the erythrocytes TBARS level by 25.3% (p = 0.009), while plasma TBARS level was increased by 75.6% (p = 0.000), as compared to untreated erythrocytes. When plasma was incubated with extract, the ketonic derivatives level was significantly increased by 22.8% (p = 0.000), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed. Treatment by *F. religiosa* extract caused the increase of TAC in plasma and erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant. All these data suggest that *F. religiosa* could be explored for its antioxidant potential in equine erythrocyte suspension.

Keywords: *Ficus religiosa*, leaf extract, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

Introduction

Increase in prevalence of disease-related oxidative stress disorders has been on the rise in the entire world since the past decades. Oxidative stress has been implicated in numerous chronic degenerative diseases such as cardiovascular diseases, cancers, type 2 diabetes,

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neurodegenerative diseases, obesity, and hypertension. However, reactive oxygen species (ROS) may have dual roles in many pathologies (Paur et al., 2011). Oxidative stress, characterized by an imbalance between oxidants and antioxidants in favor of oxidants, leads to disruption of redox signaling and physiological function (Sies, 1986, 2015).

It is clear that a beneficial effect of a large intake of one single antioxidant (such as high-dose vitamin C, vitamin E, or β-carotene supplement) would not be expected. An alternative and much more likely strategy would be to test the potential beneficial effects of antioxidant-rich foods, since such foods typically contain a large combination of different antioxidants, which are selected through plant evolution to protect every part of the plant cells against oxidative damage (Blomhoff, 2005; Paur et al., 2011). Significant positive effects with few antioxidant properties in the modern drugs pave for the alternative medicines in managing the disease (Lee et al., 2014).

Ficus religiosa L. a large deciduous tree up to 35 m in height known by more than 150 names, is known to possess high antidiabetic, anticonvulsant, antiamnesic, wound healing, anti-inflammatory and antibacterial property (Singh et al., 2011). It is native of the sub-Himalayan tract, Bengal, and central India. *F. religiosa* tree begins its life epiphytically and then strangle the host by its far-growing roots that extend to the ground, establishing it as an independent tree. The therapeutic utilities of *F. religiosa* have been indicated in traditional systems of medicine like Ayurveda, Unani, etc. It has been used to cure the disorders of the central nervous system (epilepsy, migraine, etc.), endocrine system (diabetes, etc.), gastrointestinal tract (vomiting, ulcers, stomatitis, constipation, liver diseases, etc.), reproductive system (menstrual irregularities, etc.), respiratory system (asthma, cough, etc.) and infectious diseases (chickenpox, elephantiasis, leprosy, tuberculosis, gonorrhea, scabies, etc.) (Singh et al., 2011).

Fresh plant materials, crude extracts and isolated components of *F. religiosa* showed a wide spectrum of *in vitro* and *in vivo* pharmacological activities, i.e. antidiabetic, cognitive enhancer, wound healing, anticonvulsant, anti-inflammatory, analgesic, antimicrobial, antiviral, hypolipidemic, antioxidant, immunomodulatory, antiasthmatic, parasympathetic modulatory, estrogenic, antitumor, antiulcer, antianxiety, antihelmintic, endothelin-receptor antagonistic, apoptosis inducer and hypotensive activity (Singh et al., 2011). Moreover, different extracts of *F. religiosa* showed high antimicrobial activity (Tkachenko et al., 2016). Medicinal importance of this plant encouraged us to carry out the antimicrobial investigation of the ethanolic extract of *F. religiosa* leaves against Gram-negative bacteria *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922), as well as Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 49619). We noted in preliminary experiments, that ethanolic extract from *F. religiosa* leaves showed potent antibacterial activity against *S. aureus* (diameter of growth of inhibition zones was 20.5 mm), *E. coli* (16.5 mm), and *P. aeruginosa* (14.0 mm), while antibacterial activity against *K. pneumonia* and *S. pneumoniae* was less profound (Tkachenko et al., 2016).

An increasing number of studies are published on markers of oxidative stress in a whole range of human diseases (Frijhoff et al., 2015). The World Health Organization has defined

a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predicts the incidence of outcome or disease (WHO, 2001). The oxidant and antioxidant equilibrium are known to play an important role in equine medicine and equine exercise physiology. Moreover, interest in the role of oxidative stress (OS) status in equine medicine and exercise physiology has increased the need for the development of reliable methods to assess the biomarkers related to OS (Kusano et al., 2016). Nevertheless, not many studies have been conducted to quantify the antioxidant effect of leaf extract various plants on oxidative stress biomarkers in horses (Tkachenko et al., 2016, 2017).

Consequently, in this study, we have focused on the antioxidant effect of leaf extract obtained from *F. religiosa* on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] using the equine plasma and erythrocytes model. Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). The erythrocytes represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure and cytotoxicity assay (Pagano and Faggio, 2015). Exposure of erythrocytes to oxidative stress lead to lipid peroxidation that could alter the membranes of RBCs inducing membrane protein conformation and protein cross-linking by decreasing membrane protein content and consequently lead to abnormal cell morphology and hemolysis that could disturb the microcirculation (Asha Devi et al., 2005; Farag and Alagawany, 2018). Thus, equine erythrocytes were proved to be a good tool for analyzing the oxidative stress biomarkers as a mechanism of antioxidant action of *F. religiosa* leaf extract.

Material and methodology

Collection of plant materials

The leaves of *F. religiosa* were collected in M.M. Gryshko National Botanic Garden (NBG), Kyiv, Ukraine (Figure 1). The whole collection of tropical and subtropical plants at NBG (including *Ficus* spp. plants) has the status of a National Heritage Collection of Ukraine. Plant samples were thoroughly washed to remove all attached material and used to prepare extracts.

Preparation of plant extracts

Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

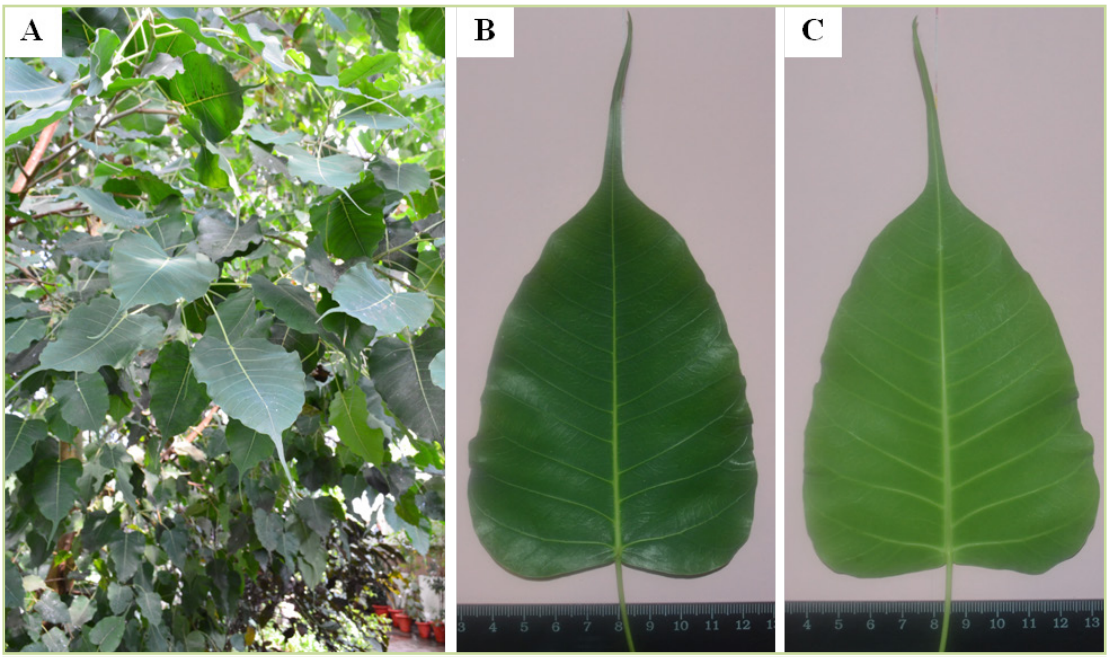

Figure 1 Leaf morphology of *F. religiosa*: A – general view; B – adaxial leaf surface; C – abaxial leaf surface

Horses

Eighteen clinically healthy adult horses from central Pomeranian region in Poland (village Strzelinko, N 54º 30' 48.0" E 16º 57' 44.9"), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. Before sampling, all horses were thoroughly examined clinically by a veterinarian and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma Blood was stored into The pellet of blood was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for

determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of sample (blood, plasma, and erythrocytes' suspension) was added to 2 mL of distilled water, 1 mL of 20% TCA and 1 mL of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3,000 g for 10 minutes. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1 M DNPH (dissolved in 2M HCl) was added to 0.1 mL of the sample after denaturation of proteins by 20% trichloroacetic acid (TCA). After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h at 37 °C. The tubes were spun in a centrifuge for 20 min at 3,000 g. After centrifugation, the supernatant was decanted and 1 mL of ethanol-ethylacetate solution was added to each tube. Following the mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then spun again (20 min at 3,000 g). The supernatant was decanted and the pellet washed thrice with ethanol-ethylacetate. After the final wash, the protein was solubilized in 2.5 mL of 8M urea solution. To speed up the solubilization process, the samples were incubated in a 90 °C water bath for 10–15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient 22,000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37ºC. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The

level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney U test (Zar, 1999). In addition, the relationships between oxidative stress biomarkers were evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Recently, a vast number of methods have been developed and used to measure the extent and nature of oxidative stress, ranging from the oxidation of DNA to proteins, lipids, and free amino acids (Frijhoff et al., 2015). As we know, lipid peroxidation is one of the consequences of oxidative damage, and it is one of the chief mechanism for cell injury and death (Çimen Burak, 2008). It is well documented that lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids and its occurrence in biological membranes causes impaired membrane function, impaired structural integrity (Halliwell and Gutteridge, 1985), decreased fluidity, and inactivation of a number of membrane-bound enzymes.

Malondialdehyde (MDA), the well-characterized product of the lipid peroxidation of erythrocytes, is a highly reactive bifunctional molecule, that could impair various membrane functions by cross-linking the erythrocytes' proteins and phospholipids leading to diminished survival and induce hemolysis (Farag and Alagawany, 2018). Moreover, lipid peroxidation of erythrocytes may be implicated in cell aging and variable pathological conditions. The determination of MDA level provides a good measure of lipid peroxidation. The most common method used to assess the MDA level is the 2-thiobarbituric acid reactive substances (TBARS) assay. The TBARS content as a biomarker of lipid peroxidation in the equine erythrocytes suspension after *in vitro* incubation with *F. religiosa* leaf extract and shown in Figure 2A. As shown in Figure 2A, treatment by extract reduced the erythrocytes TBARS level by 25.3% (p = 0.009), while plasma TBARS level was increased by 75.6% (p = 0.000) when compared to untreated erythrocytes.

Nevertheless, it was noted that despite their widespread use, all methods that detect both MDA and TBARS have their pitfalls (Spickett et al., 2010). Specifically, Moore and Roberts (1998) have found that in the TBARS assay, up to 98% of the measured MDA can be formed by the high-temperature conditions during the procedure itself.

Oxidative damage of proteins can occur directly by the interaction of the protein with ROS or indirectly by the interaction of the protein with a secondary product (resulting from the interaction of radical with lipid or sugar molecule). Modification of a protein under oxidative stress can occur *via* peptide backbone cleavage, cross-linking, and/or modification of the side

chain of virtually every amino acid (Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009). Moreover, most protein damage is irreparable and oxidative modification of the protein structure can lead to loss of enzymatic, contractile, or structural function in the affected proteins, thus making them increasingly susceptible to proteolytic degradation (Levine and Stadtman, 2001; Fisher-Wellman and Bloomer, 2009). Albumin is the main (48–76% of total proteins) and the most osmotically active protein fraction of a horse serum (Winnicka, 2011) whereas a globulin fraction is a heterogeneous group of blood proteins including carrier proteins, enzymes, immunoglobulins and other inflammatory molecules (Abeni et al., 2013). ROS induced oxidation of arginine, lysine, threonine, or proline amino acid residues generates reactive carbonyl derivatives (RCD), which can be readily measured by reaction with 2,4-dinitrophenylhydrazine (Radák et al., 2000, 2002). Protein RCD is used very often as a marker of oxidative modification of proteins (Radák et al., 2000, 2002, 2008). When equine plasma was incubated with extract, the ketonic derivatives level was significantly increased by 22.8% (p = 0.000) (Fig. 1B), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed (by 1.6 and 8.9%, p >0.05).

The total antioxidant capacity (TAC) includes an enzymatic antioxidant such as superoxide dismutase, catalase, glutathione peroxidase, as well as some macromolecules (albumin, ceruloplasmin, and ferritin), and its assessment may contain more information than a single review of its constituent parts (Gad et al., 2011). As shown in Figure 2C, treatment by *F. religiosa* extract caused the increase of TAC in plasma and erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant.

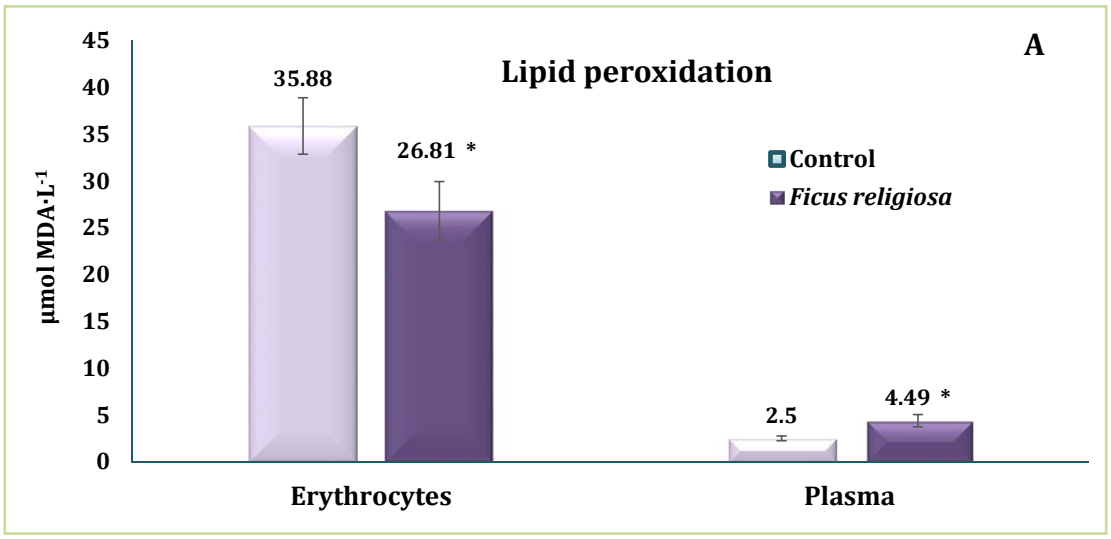

Figure 2A The TBARS content as a biomarker of lipid peroxidation (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B), and total antioxidant capacity (C) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract (M ±m, n = 18)

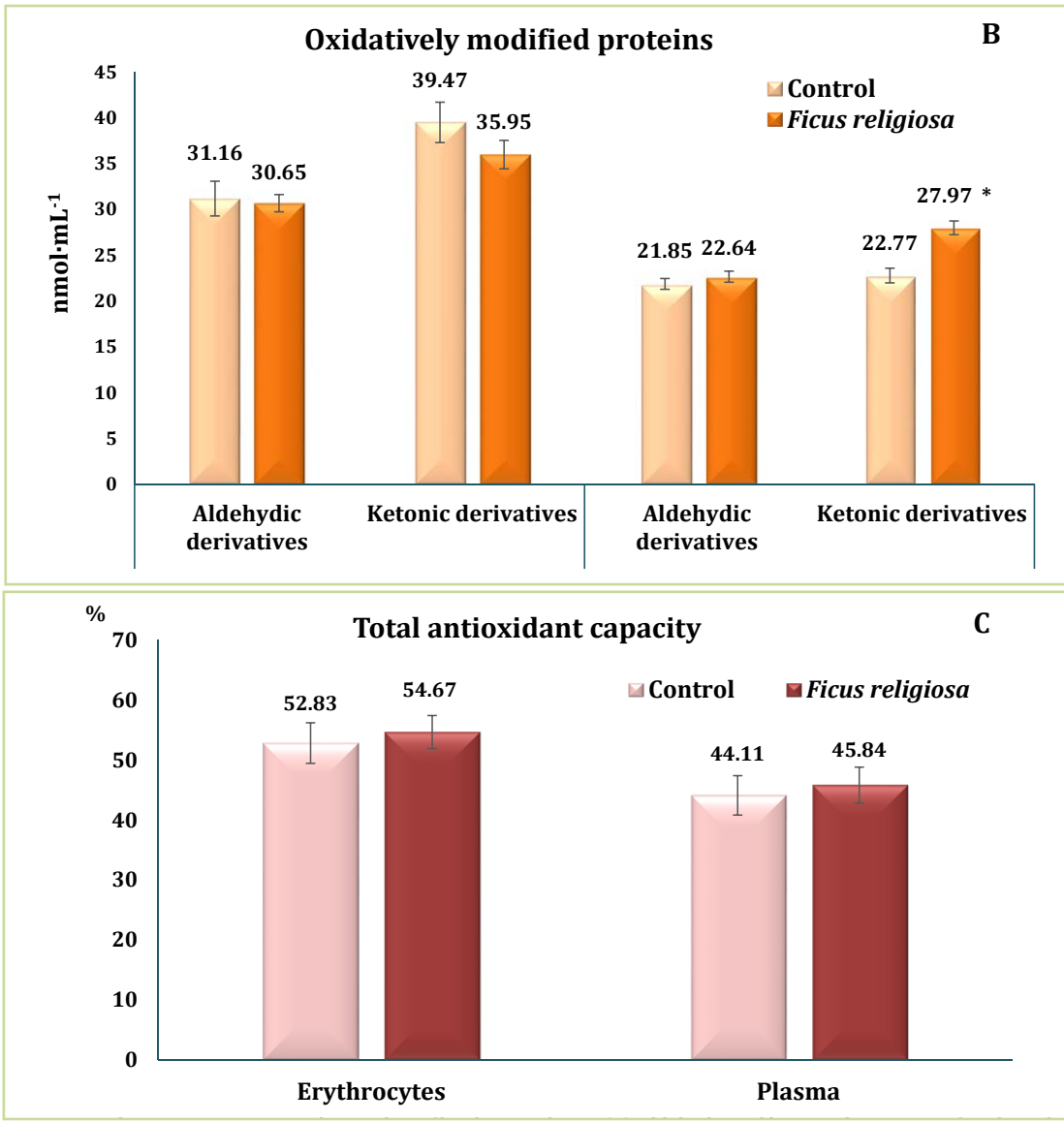

Figure 2B–C The TBARS content as a biomarker of lipid peroxidation (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B), and total antioxidant capacity (C) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract (M ±m, n = 18)

Several correlations between checked parameters were found (Figure 3). Erythrocyte TBARS level correlated inversely with plasma TBARS level (r = -0.532, p = 0.023) and plasma TAC level (r = -0.525, p = 0.025) (Figure 3A). Decreased erythrocyte TBARS level induced the increase of plasma TBARS level and TAC level. The erythrocyte TAC level correlated inversely with plasma TBARS level (r = -0.742, p = 0.000) and correlated positively with aldehydic derivatives of OMP in plasma (r = 0.645, p = 0.004). High level of TAC in erythrocyte suspension after

incubation with leaf extract obtained from *F. religiosa* induced reduce plasma TBARS level, while aldehydic derivatives of OMP positively correlated with plasma TAC level (Figure 3).

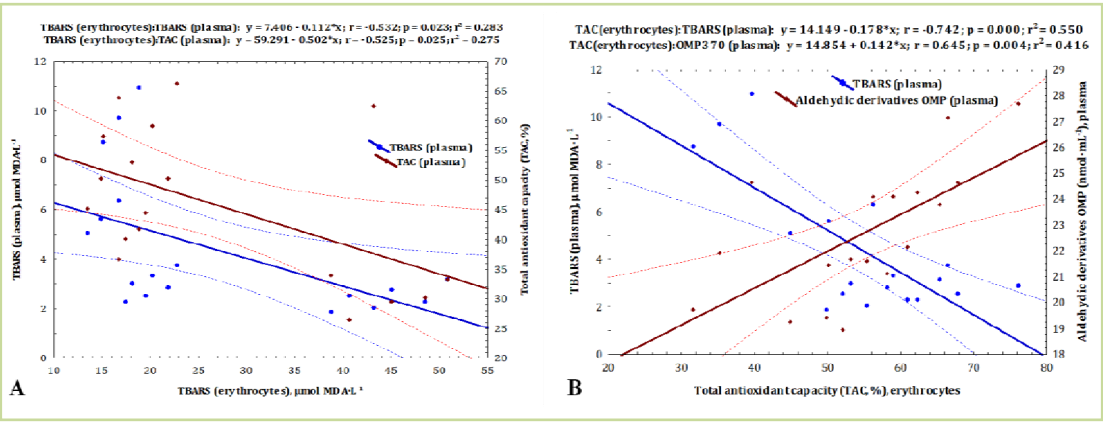

Figure 3 Correlations between oxidative stress biomarkers – plasma and erythrocyte TBARS, plasma TAC level (A), erythrocyte TAC level, plasma TBARS level and aldehydic derivatives of OMP (B) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract

Many results also clearly suggest that treatment by herbal extracts *in vivo* and *in vitro* study prevents organ damage by a decrease of lipid peroxidation and protection of the antioxidant defense system. Several methods have been developed to measure the free radical scavenging capacity, regardless of the individual compounds, which contribute towards the total capacity of a plant product in scavenging free radicals (Lo Shu-Fung et al., 2004). For instance, the *in vitro* antioxidant effect of the ethyl acetate root extract of *F. religiosa* using diphenyl picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, reducing capacity and hydrogen peroxide scavenging assay was investigated by Sharma and Gupta (2007). The extract showed reducing potential, scavenged DPPH radical (87.61%) at 250 $\mu g.mL^{-1}$ and hydrogen peroxide (70.25%) at 1000 $\mu g.mL^{-1}$. The investigators suggested the role of polyphenolic components (determined by the Folin-Ciocalteu's phenolic reagent method) for the observed antioxidant effect (Sharma and Gupta, 2007).

The effects of four extracting solvents [absolute ethanol, absolute methanol, aqueous ethanol (ethanol: water, 80 : 20 v/v) and aqueous methanol (methanol: water, 80 : 20 v/v)] and two extraction techniques (shaking and reflux) on the antioxidant activity of extracts of barks of *Azadirachta indica* A. Juss. (Meliaceae), *Acacia nilotica* (L.) Delile (Leguminosae), *Syzygium cumini* (L.) Skeels (syn. *Eugenia jambolana* Lam.) (Myrtaceae), *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn. (Combretaceae), leaves and roots of *Moringa oleifera* Lam. (Moringaceae), the fruit of *F. religiosa*, and leaves of *Aloe vera* (L.) Burm. f. (syn. *Aloe barbadensis* Mill.) (Asparagaceae) were investigated by Sultana and co-workers (2009). The fruit powder of *F. religiosa* was subjected to shaking and refluxing with absolute ethanol, absolute methanol, 80% hydro-ethanol and 80% hydro-methanol to get various extracts. Total phenolic components and flavonoids were quantified in all the extracts. The extracts were subjected

to DPPH scavenging and percent inhibition of linoleic acid assay. The investigators found that the 80% hydro-methanolic extract obtained by refluxing contained the highest amount of antioxidant components (phenols and flavonoids) and showed maximum antioxidant effect. The study suggested that the extracting solvent and extraction technique affects the antioxidant activity of the plant extracts (Sultana et al., 2009). Apart from the bark, roots, and fruits, the antioxidant effect of the aqueous, methanolic and ethanolic leaf extracts at 35–36 μg.100 mg $^{-1}$ concentration, in similar *in vitro* DPPH assay has also been reported (Preethi et al., 2010).

Anandjiwala et al. (2008) have also reported the free radical scavenging activity of an Ayurvedic preparation *Panchvalkala* and its individual components (stem bark of *Ficus benghalensis*, *F. racemosa* L. (syn. *F. glomerata* Roxb.), *F. religiosa*, *F. virens* Aiton and *Thespesia populnea* (L.) Sol. ex Corrêa (Malvaceae). Being stem barks, these samples contained phenolics (ranging from 3.5 to 10.8% w/w) and tannins (1.6 to 7.0% w/w). This prompted researchers to study the free radical scavenging activity of *Panchvalkala* and its components which were evaluated in three *in vitro* models viz. 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, superoxide radical scavenging activity and reducing power assay. *Panchvalkala* and its individual components showed significant antiradical activity by bleaching 1,1-diphenyl-2-picrylhydrazyl radical (EC_{50} ranging from 7.27 to 12.08 μg) which was comparable to pyrogallol (EC_{50} 4.85 μg). Thin layer chromatography of the methanol extracts when sprayed with 0.2% 1,1-diphenyl-2-picrylhydrazyl in methanol revealed several bands with antiradical activity as seen by bleaching of 1,1-diphenyl-2-picrylhydrazyl. All the samples showed good superoxide scavenging potential (EC_{50} ranging from 41.55 to 73.56 μg) comparable to ascorbic acid (EC_{50} 45.39 μg) in a dose-dependent manner. The reduction ability, Fe^{3+} to Fe^{2+} transformation was found to increase with increasing concentrations of all the sample extracts. *Panchvalkala* and its components showed good free radical scavenging activity which can be attributed to tannins and phenolics along with other compounds. Free radical scavenging activity could be one of the mechanisms of action of *Panchvalkala*, including its anti-inflammatory activity (Anandjiwala et al., 2008).

Since oxidative stress as a consequence of free radicals generated during body‘s cellular respiration has been implicated in the pathogenesis of many human diseases, the use of antioxidants has been suggested as a common treatment approach for these disorders. The antioxidant potential of different parts of *F. religiosa* can, therefore, be further explored in ameliorating the oxidative stress-related disorders (Singh et al., 2011). *F. religiosa* bark possesses also significant antidiabetic activity. The antidiabetic effect of aqueous extract of *F. religiosa* bark in normal, glucose-loaded hyperglycemic and streptozotocin-induced diabetic rats was investigated in a study by Pandit et al. (2010). The aqueous extract of *F. religiosa* bark also showed a significant increase in serum insulin, body weight and glycogen content in liver and skeletal muscle of streptozotocin-induced diabetic rats while there was a significant reduction in the levels of serum triglyceride and total cholesterol. The aqueous extract of *F. religiosa* bark also showed a significant anti-lipid peroxidative effect in the pancreas of streptozotocin-induced diabetic rats (Pandit et al., 2010).

Kapoor et al. (2011) have demonstrated the phytopharmacological potential and anti-asthmatic activity of *F. religiosa*. Histamine and acetylcholine were used to guinea pigs to establish bronchospasm model. In *in vivo* study, the aqueous extract of *F. religiosa* leaves at doses of 150 and 300 mg.kg^{-1} was administrated to guinea pigs, and the broncho-protective activity of the aqueous extract of *F. religiosa* leaves was compared with aminophylline at 25 mg.kg^{-1}. While in *in vitro* study, and 10, 20, 30 g.mL^{-1} of the aqueous extract of *F. religiosa* leaves was administrated to guinea pigs, respectively, and mast cell stabilizing activity of the aqueous extract of *F. religiosa* leaves was compared with ketotifen at 10 g.mL^{-1}. Administration of the aqueous extract of *F. religiosa* leaves (150 and 300 mg.kg^{-1}, ip.) produced a significant effect on latency to develop histamine and acetylcholine-induced pre-convulsive dyspnea. In the mast cell stabilizing model, the aqueous extract of *F. religiosa* leaves at 10, 20 and 30 μg.mL^{-1} could significantly increase the number of intact cells. It can be concluded that the aqueous extract of *F. religiosa* leaves is effective on histamine and acetylcholine-induced bronchospasm in guinea pigs (Kapoor et al., 2011).

F. religiosa latex and constituents have excellent nephroprotective and curative activities and thus have great potential as a source for natural health products. The possible nephroprotective and curative effects of *F. religiosa* latex methanol extract against cisplatin-induced acute renal failure was determined by Yadav and Srivastava (2013). The anti-ulcer activity and acute toxicity of *F. religiosa* leaf ethanolic extract in animal models were evaluated by Gregory and co-workers (2013). Anti-ulcer activity of *F. religiosa* ethanolic extract (250 and 500 mg.kg^{-1} body weight) was studied on stress-induced ulcer animal models. Results showed that the extract treatments prevented ulcer area and gastric secretion in a dose-dependent manner. Administration of 2,000 mg.kg^{-1} extract did not show any acute toxicity in albino mice. The preliminary phytochemical analysis identified the presence of flavonoids in the ethanolic extract of *F. religiosa*. The anti-ulcer activity is probably due to the presence of flavonoids (Gregory et al., 2013).

An orally administered aqueous root extract of *F. religiosa* has dose-dependent and potent anticonvulsant activities against strychnine- and pentylenetetrazole-induced seizures in mice, as described in the study of Patil et al. (2011). The anticonvulsant activity of the extract (25, 50 and 100 mg.kg^{-1}, p.o.) was investigated in strychnine-, pentylenetetrazole-, picrotoxin- and isoniazid-induced seizures in mice. Rat ileum and fundus strip preparations were used to study the effect of the extract on acetylcholine (Ach)- and serotonin (5-HT)-induced contractions, respectively (Patil et al., 2011). The hydroethanolic extract of adventitious roots of *F. religiosa* has anticonvulsant activity. Retention of anticonvulsant effect in the saponins-rich fraction-treated animals indicated the role of saponins for the activity (Singh et al., 2012).

The petroleum ether extract of *F. religiosa* plant showed to be an antioxidant and showed a promising effect in animals with Parkinson‘s disease, significantly attenuating the motor defects and also protecting the brain from oxidative stress. In study of Bhangale and Acharya (2016), effects of *F. religiosa* (100, 200, and 400 mg.kg^{-1}, p.o.) were evaluated using *in vivo* behavioral parameters like catalepsy, muscle rigidity, and locomotor activity and its effects on neurochemical parameters (malonic dialdehyde, catalase, superoxide dismutase, and glutathione) in rats. The experiment was designed by giving haloperidol to induce catalepsy

and 6-hydroxydopamine to induce Parkinson's disease-like symptoms. The increased cataleptic scores (induced by haloperidol) were significantly (p <0.001) found to be reduced, with the petroleum ether extract of *F. religiosa* at a dose of 200 and 400 $mg.kg^{-1}$ (p.o.). 6-Hydroxydopamine significantly induced motor dysfunction (muscle rigidity and hypolocomotion). The 6-Hydroxydopamine administration showed a significant increase in lipid peroxidation level and depleted superoxide dismutase, catalase, and reduced glutathione level. Daily administration of petroleum ether extract of *F. religiosa* (400 $mg.kg^{-1}$) significantly improved motor performance and also significantly attenuated oxidative damage (Bhangale and Acharya, 2016). The effect of flavonoid-rich ethyl acetate fraction of the crude fig extract of *F. religiosa* in combination with phenytoin on seizure severity, depressive behavior, and cognitive deficit in pentylenetetrazol (PTZ)-kindled mice was investigated in a study of Singh et al. (2014). The flavonoid-rich ethyl acetate fraction of the crude fig extract was found to show significant antioxidant potential in various *in vitro* free radical scavenging assays. Biochemical investigations of the brain tissue showed amelioration of TBARS, reduced glutathione (GSH) levels, and reduced catalase and acetylcholinesterase activities, thereby indicating suppression of oxidative stress (Singh et al., 2014).

The antiviral activity of *F. religiosa* extracts against herpes simplex virus type 2 (HSV-2), the main causative agent of genital ulcers and sores was investigated in a study of Ghosh et al. (2016). Water and chloroform bark extracts were the most active against HSV-2, and also against an acyclovir-resistant strain. The water extract has a direct virus-inactivating activity. By contrast, the chloroform extract inhibits viral attachment and entry and limits the production of viral progeny. The chloroform extract of *F. religiosa* did not inactivate extracellular virus particles but targeted early steps of the viral replicative cycle such as virus attachment and/or entry. Moreover, a significant reduction in the number of viral plaques was also observed when the extract was added to the methylcellulose medium after infection. This finding suggests that the virus was blocked in some of the initial infections (Ghosh et al., 2016). The antiviral activity of *F. religiosa* extracts against respiratory viruses such as a human respiratory syncytial virus (RSV) and human rhinovirus (HRV) was demonstrated by Cagno et al. (2015). The antiviral activity of *F. religiosa* was tested *in vitro* by plaque reduction and virus yield assays and the major mechanism of action was investigated by virus inactivation and time-of-addition assays. *F. religiosa* methanol bark extract was the most active against HRV with an EC_{50} of 5.52 $\mu g.mL^{-1}$. This extract likely inhibited the late steps of the replicative cycle. Water bark extract was the most active against RSV with an EC_{50} between 2.23 and 4.37 $\mu g.mL^{-1}$. Partial virus inactivation and interference with virus attachment were both found to contribute to the anti-RSV activity. Replication of both viruses was inhibited in viral yield reduction assays (Cagno et al., 2015).

F. religiosa has been shown to exert diverse biological activities including apoptosis in breast cancer cell lines and it could be explored for its chemopreventive potential in cervical cancer. The anti-neoplastic potential of aqueous extract of *F. religiosa* bark in human cervical cancer cell lines, SiHa and HeLa were demonstrated by Choudhari et al. (2013). The aqueous extract of *F. religiosa* bark altered the growth kinetics of SiHa (HPV-16 positive) and HeLa (HPV-18 positive) cells in a dose-dependent manner. It blocked the cell cycle progression at a G1/S

phase in SiHa that was characterized by an increase in the expression of p53, p21 and pRb proteins with a simultaneous decrease in the expression of phospho Rb (ppRb) protein. On the other hand, in HeLa, FRaq induced apoptosis through an increase in intracellular Ca^{2+} leading to loss of mitochondrial membrane potential, the release of cytochrome-c and an increase in the expression of caspase-3. Moreover, aqueous extract of *F. religiosa* bark reduced the migration as well as invasion capability of both the cervical cancer cell lines accompanied with downregulation of MMP-2 and Her-2 expression. Interestingly, aqueous extract of *F. religiosa* bark reduced the expression of viral oncoproteins E6 and E7 in both the cervical cancer cell lines (Choudhari et al., 2013).

The antioxidant role of *F. religiosa* after *in vitro* incubation with equine erythrocytes suspension might be due to its chemical constituents like flavonoids and phenolic compounds. Furthermore, flavonoids traditionally expose antioxidant activity. Chemical analysis conducted by Suryawanshi et al. (2011) found that leaves of *F. religiosa* contained appreciable amounts of campestrol, stigmasterol, isofucosterol, α-amyrin, lupeol, tannic acid, arginine, serine, aspartic acid, glycine, threonine, alanine, proline, tryptophan, tyrosine, methionine, valine, isoleucine, leucine, nonacosane, *n*-hentricontanen, hexacosanol, and *n*-octacosan. On the other hand, the findings of Taskeen and coworkers (2009) showed, that quercetin was the most abundant among flavonols. Phytosterols (2.8%) like campesterol, stigmasterol, sitosterol and 28-isofucosterol, and triterpene alcohols (28.5%) like α-amyrin, β-amyrin, and lupeol have been isolated from the non-saponifiable fraction of light petroleum leaf extract of *F. religiosa* (Singh et al., 2011). Along with phytosterols and triterpene, 7.1% of long-chain hydrocarbons [*n*-nonacosane and *n*-hentriacontane] and 7.9% of aliphatic alcohols [*n*-hexacosanol and *n*-octacosanol] have also been isolated from the same fraction (Behari et al., 1984; Williamson and Hooper, 2002). The leaves of *F. religiosa* contain a high amount of l-cystine, l-lysine, l-arginine, dl-serine, dl-aspartic acid, glycine, dl-threonine, dl-∞-alanine, l-proline, tryptophan, l-tyrosine, dl-methionine, dl-valine, dl-isoleucine and l-leucine (Verma and Bhatia, 1986). The leaves contain around 1.5% of total tannin content, which comprises a tannic acid and condensed tannins (Singh, 1977; Niranjan et al., 2007; Singh et al., 2011). The leaves are rich in minerals like calcium, phosphorous, iron, copper, manganese, magnesium, zinc, potassium and sodium (Williamson and Hooper, 2002; Singh et al., 2011).

Conclusions

In conclusion, the results obtained from the present studies revealed that leaf extract of *Ficus religiosa* exhibited antioxidant activity after *in vitro* cultivation with equine erythrocytes. These findings suggest that the extensive use of this herbal in treating various types of disorders might, therefore, be justified by its antioxidant activities. The results also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results.

Nevertheless, in order to avoid any misinterpretation of the results obtained in this study, another alternative marker for evaluating lipid peroxidation level in equine erythrocyte suspension and assessing free radical scavenging potency of leaf extracts of plant extracts screened could be employed.

Additionally, further studies aimed at the isolation and identification of active substances from the extract obtained from *Ficus religiosa* leaves could also disclose compounds with better therapeutic value. It is believed that screening of all the investigated plants for other biological activities including anti-inflammatory, wound healing and antioxidant activities are essential in medicine and veterinary.

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