ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF *NYCTANTHES ARBOR-TRISTIS* LEAF EXTRACTS IN HIGH GLUCOSE-INDUCED SPRAGUE DAWLEY RATS

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ABSTRACT

Objective: To determine the phytochemical constituents and antioxidant activity of *N. arbor-tristis* leaf extracts, and also to investigate the *in vivo* anti-inflammatory effect of *N. arbor-tristis* water extract in high glucose-induced Sprague Dawley rats.

Methods: The ethanolic and water extracts of *N. arbor-tristis*were prepared by soaking oven-dried powdered leaves in ethanol and distilled water, respectively.The antioxidant activity and total antioxidant capacity of the leaf extracts were assessed using DPPH scavenging method and phosphomolybdenum assay, respectively. The changes in the vascular permeability and leukocyte migration were assessed using an Evans blue assay.

Results: The phytochemical studies exposed that water extract of *N. arbor-tristis* containsrelatively high amounts of flavonoids and saponins, compared to the ethanolic extract. *N. arbor-tristis* water extract also showed significantly higher DPPH radical scavenging activity and total antioxidant capacity than the ethanolic extract. *In vivo*results showed that 1000 mg/kg and 2000 mg/kg of *N. arbor-tristis* water extract significantlyattenuated D-glucose-induced increased vascular permeability and leukocyte migration. The results advocate that the antioxidant and anti-inflammatory properties of *N. arbor-tristis*might be related to the high flavonoid and saponin contentsfound in the leaves.

Conclusion: This study demonstrated that *N. arbor-tristis* water extract possesses antioxidant activity and a novel protective role against vascular inflammation *in vivo*. This reveals the rational use of *N. arbor-tristis* leaf extracts in traditional medicinal practice, and the possibility to further explore the therapeutic effect of *N. arbor-tristis*in vascular inflammatory diseases.

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INTRODUCTION

Reactive oxygen species (ROS) are formed by numerous enzymatic sources, including NADPH oxidases, the mitochondria, xanthine oxidase and uncoupled nitric oxide synthases. Under physiological condition, ROS regulatesvascular tone, which is crucial for the homeostatic function of blood vessels. Oxidative stress transpires when there isimbalance betweenROS production and antioxidant defence mechanisms. In vascular diseases, ROShave been reported to cause endothelial dysfunction, inflammation, vasoconstriction, vascular remodelling and fibrosis.[1]

High plasma glucose has beentestified to cause progressive oxidative damage to the vascular endotheliumofseveral organs including eyes, kidneys, the heart, the brain and peripheral nerves.[2]High glucose inducesROS through various mechanisms such as polyol pathway flux, increased advanced glycation end-product formation, activation of protein kinase C and hexosamine pathways.[3, 4]Oxidative stressup regulates the expression of endothelial proteins andelevates theproduction of both pro-inflammatory metabolites and pro-inflammatory cytokines.[4] This pro-inflammatory phenotype promotes the adhesion of leukocytes to the endothelium and causes vascular leakage.[4, 5]

Nowadays, there is a flourishing interest in searching antioxidants from natural resources that are generally shown to be effective, safe, and affordable. Natural antioxidants, especially polyphenols and carotenoids, displayanextensive range ofbiological effects including anti-inflammatory, anti-aging, anti-atherosclerosis and anticancer effects.[6, 7]Antioxidants protectcells against free radicals throughseveralways,including inhibition of ROS generation by sequesteringmetal ions or activating the anti-oxidant enzymatic system, breakage of chain propagation reactions by scavenging ROSand removal of oxidized proteins by activating cytosolic enzymes.[8]

Nyctanthesarbor-tristis L. (Oleaceae),also known as‘night jasmine’,is commonly found in southern Asiacountries such as Thailand, Malaysia, and Indonesia.[9]The plant is characterized by hefty shrub that nurtures up to 10 metres tall with flaky grey bark, and the younger branches are sharp and quadrangular. The plant is consumed by rural communities, mainly tribal people of India, and various parts of the plant have been used in Ayurveda, Siddha and Unani systems of medicine.[10]The flowers of *N. arbor-tristis* are used to provoke menstruation.Decoctions made from the leaves have been used as tonics, laxatives or for the treatment of arthritis, liver disorders, biliary disorders, chronic fever, malarial fever,intestinal worms, and others.[9-11]Furthermore, *N. arbor-tristis* have been known to possess antiviral, antihelmintic, and antimicrobial activities.[11, 12]Researchers demonstrated that aqueous extract of flowers from *N. arbor-tristis*not only reduces fasting blood glucose levels, glucose uptake by intestinal lumen, total cholesterol levels and triglycerides levels, but also increases glucose uptake by diaphragm and HDL-cholesterol levels, which implyhypoglycemic and hypolipidemic activities of *N. arbor-tristis*.[13]*N. arbor-tristis* has also been reported to suppresscarrageenan-induced paw edema in rats, implying its anti-inflammatory activity.[14, 15] Despite a few studies have reported anti-inflammatory activity of *N. arbor-tristis*,anti-oxidant and anti-inflammatory activities of *N. arbor-tristis* leaf water extract in high glucose-inducedrats have not been revealed yet.

MATERIALS AND METHODS

Ammonia (NH_3), ferric chloride ($FeCl_3$), sulphuric acid (H_2SO_4), sodium phosphate (Na_3PO_4) hydrochloric acid (HCl), acetic acid (CH_3COOH), crystal violet ($C_{25}N_3H_{30}C$), and Evan's blue dye were purchased from Merck, Germany. DPPH was purchased from Sigma, United States. D-glucose ($C_6H_{12}O_6$), technical grade ethanol 100 % (C_2H_5OH), and sodium hydroxide (NaOH) were purchased from Thermo Fisher Scientific, United States. Benedict reagent was obtained from Orioner, Malaysia. L-ascorbic acid ($C_6H_8O_6$) was purchased from Scharlab, Spain. Carboxymethyl cellulose was purchased from TCI America, United States. Dexamethasone was purchased from Y.S.P Industries, Malaysia.

Plant Collection and Identification

The fresh *N. arbor-tristis* leaves were obtained from Taman Semangat in Sungai Petani, Kedah, Malaysia. The plant species was identified by School of Biological Sciences, University Sains Malaysia and the voucher specimen was 11800.

Preparation of Nyctanthesarbor-tristis Leaf Extracts

Leaves were washed thoroughly with tap water and then dried at 40 °C using an oven. The weight of the dried sample was monitored daily until a constant weight was obtained. The leaves were powdered with a conventional blender (Waring, United States). The powdered leaves were extracted with 100 % ethanol or distilled water at a ratio of 1:10 (w/v). The mixture was swilled at room temperature and was filtered every 24 h for three consecutive days. The solvents were then removed completely from the collected filtrate using a rotary evaporator (Eyela, United States) and the extracts were stored at 4˚Cfor future use. For *in vivo* assays, *N. arbor-tristis* water extract and dexamethasonewere solubilized in 1 % carboxymethlycellulose. Working solution of *N. arbor-tristis* leaf extracts used in all experiments wasfreshly prepared before use.

Experimental animals

The study was approved by the Animal Ethics Committee of AIMST University, Malaysia. Sprague-Dawley rats weighing 180 to 200 g were acquired from the animal house of School of Biological Science, University Sains Malaysia, Penang. The animals were maintained at standard room temperaturewith 12-hour light/dark cycles andfed with tap water and standard food pellet. They were acclimatized for two weeks prior to the experiment.

Phytochemical Screenings of N.arbor-tristis Leaf Crude Extracts

Qualitative phytochemical analyses were used to detect the presence of flavanoids, reducing sugar, saponin, tannins, and anthraquinone in *N. arbor-tristis* leafextracts. All the chemical tests were carried out as described previously by.[16]

i) Flavonoid Test

1 mL of 1 % NaOH was added into the test tube containing 2 mL of crude extract and the colour change was observed. Then, 1 mL of 1 % HCl was added into each extract sample and the final colour change was recorded. An intense yellow colour appearance in the sample upon addition of 1 % NaOH followed by a colourless appearance upon addition of 1 % HCl indicates the presence of flavonoids.

ii) Benedict Test

The presence of reducing sugar was detected using Benedict test. 2 mL of crude extract was added to1 mL of Benedict solution then the mixture was shaken vigorously and incubated at 45 ºC. After 10 minutes, the colour changes were recorded. A brick red precipitate indicates the presence of reducing sugars.

iii) Frothing Test

The presence of saponins was detected using Frothing test. 2 mL of crude extractwas placed in a tube and shaken vigorously. Then the tube was left to stand for 10 minutes at room temperature. A thick persistent froth indicates the presence of saponins.

iv) Borntrager's Test

To detect the presence of anthraquinone derivatives, 1 mL of 10 % ammonia was addedinto the tube containing 2 mL of crude extract and shaken vigorously. A pink-red colour in the ammonia layer indicates anthraquinone derivatives.

v) Tannins Test

In this test, 2 mL of 15% ferric chloride was added to 2 mL of crude extract, either ethanolic or water extract of *N. arbor-tristis*. A blue or green colour indicates the presence of tannins.

DPPH Radical Scavenging Activity Assay

The assay was carried out as described previously [17]with some modifications. Three mL of each extractwith concentration ranges from 0.5 - 8 mg/mL was mixed with 1 mL of DPPH and the mixture was shaken vigorously.After 30 minutes, the absorbance was measured at a wavelength of 517 nm. Ascorbic acid was used as a reference compound. DPPH radical scavenging activity percentage was calculated.

Total Antioxidant Capacity (TAC)

The TAC of the extracts was determined using the phosphomolybdenum method.[18]The extract at a dose of 0.5 mg/mL was mixed with phosphomolybdenum solution and incubated at 95 °C. After 30 minutes, the absorbance was measured at a wavelength of 695 nm. Ascorbic acid was used as a standard. The results were expressed in ascorbic acid equivalent (AAE)/100 mg dry weight.

In VivoVascular Permeability Assay and Leukocyte Count

The assay was performed as described previously[19] with slight modifications. Rats were pre-treated with different dosesof *N. arbor-tristis* water extract, once daily for four consecutive days through oral gavage. Positive groupwaspre-treated with 10 mg/kg dexamethasone.

On the fifth day, the pre-treated rats were given intravenously 5 mL/kg of 1 % of Evan's Blue dye. Then, 25mM D-glucose (5 mL/kg) was injected intravenously to all the rats, except for the normal control. After 30 minutes, the rats were sacrificed,and the peritoneal exudates were collected. The absorbance of the exudates was measured at 650 nm. By using a standard curve of Evan's blue dye, the amount of dye leaked into the peritoneal cavities was determined.The number of leukocytes in the exudates was counted under a light microscope (Olympus, Japan).

Statistical Analysis

Statistical analysis was performed usingIBM SPSS (Version 23) one-way ANOVA followed byDuncan *post hoc* test.$p<0.05$ was considered to be statistically significant.

RESULTS

Sample Plant and Extraction Yield

Figure 1 shows the *N. arbor-tristis* plant.The plant can grow up to 9 m tall. The leaves are green, hairy, rough, decussate and entire. The flowers are usually white with anorange-red centre. The flowers bloom profusely at night and lose their fragrance and colour as the day approaches and dropping in the morning. The fruit consists of two sections in heart-shaped and each contains a single seed.

As shown in **Table1**, the extraction yields of ethanolic leaf extract and water leaf extract of *N. arbor-tristis*were20.7 % and 32.3 %, respectively. Thus, water gave ahigher yield compared to ethanol.

Qualitative Phytochemical Analysis of N.arbor-tristis Crude Extracts

Table 2 showedthat flavonoid contents in water extract of *N. arbor-tristis*was higher thanthe ethanolic extract. Tannins were found to be in low amount in both of the extracts. Saponins

were highly concentrated in the water extract, whereasthe ethanolic extract did not contain saponins. Reducing sugar and anthraquinone were not found in both the crude extracts.

DPPH Radical Scavenging Activity

Table 3 showed thatcrude ethanolic extract of *N. arbor-tristis,*at doses ranging from 0.5 – 4.0 mg/mL, showed radical scavenging activities which were significantly different from the activity of vitamin C.Notably, 8 mg/mL of *N. arbor-tristis* ethanolic extract possessed a radical scavenging activity of 95.73 ± 0.38 %,which was comparable to vitamin C. The scavenging activities of *N. arbor-tristis* water extract at doses of 2 and 4 mg/mL wascomparable to vitamin C, while 8 mg/mL of *N. arbor-tristis*water extract showed a better scavenging activity than vitamin C.The IC_{50} value of the water extract was 1.29 mg/mL, which was comparatively lower than the ethanolic extract, 1.46 mg/mL. This indicates that water extract of *N. arbor-tristis*possesses greater antioxidant activity than the ethanolic extract.

Total Antioxidant Capacity (TAC)

As shown in **Table 4**,TAC of *N. arbor-tristis*ethanolic and water extracts, at a dose of 0.5 mg/mL,were 0.17 ± 0.006 AAE/100 mg dry weight and 1.14 ± 0.032 AAE/100 mg dry weight, respectively. This indicates that *N. arbor-tristis*water extract possesses higher TAC than the ethanolic extract. As a result, water extract was chosen for *in vivo* permeability assay.

In vivo Vascular Permeability Assay

Figure2 showed that the amount of dye leaked into the peritoneal cavities of normal rats was 0.029± 0.003 mg/mL. In D-glucose-induced group, a significant increase in rat's peritoneal vascular permeability was observed, which was represented by increased dye leakage to 0.383 ± 0.019 mg/mL.Dexamethasone at 10 mg/kg significantly suppressed the D-glucose-induced dye leakage to 0.225 ± 0.019 mg/mL.Pre-treatment of the rats at doses of 1000 mg/kg and 2000 mg/kg significantly inhibited the dye leakage stimulated by D-glucoseto 0.333± 0.011 mg/mL and 0.256 ± 0.002 mg/mL, respectively.

Furthermore, the effect of water extract on high glucose-induced increased leukocyte recruitment was investigated. As shown in **Figure3**, the total leukocyte count in normal control group was 8566.67 ± 327.02 cells/mm^3. D-glucose caused a significant increase in leukocyte migration into the peritoneal cavity,18650.00 ± 1294.86 cells/mm^3.The total leukocyte count in dexamethasone-treated rats was 11116.67 ± 818.91 cells/mm^3.Pre-treatment of the rats at doses of 1000 mg/kg and 2000 mg/kg significantly inhibited the increased leukocyte countto 15408.33 ± 208.73 cells/mm^3 and 13808.33 ± 460.51 cells/mm^3, respectively. Taken together, the findings indicate that *N. arbor-tristis* crude water extract significantly suppresses the increased vascular leakage and leukocyte recruitment in a dose-dependent manner.

DISCUSSION

Alcoholic extracts of*N. arbor-tristis* have previously been reported to exhibit some biological activities such as anti-diabetic, anti-oxidant and anti-inflammatory effects.[15, 20]However, effects of *N. arbor-tristis*leaf water extracton increased free radical production and high-glucose-induced vascular inflammation remain poorly understood.This study showed that leaf water extract of *N. arbor-tristis* contains relatively high amounts of flavonoids and saponins, compared to the leaf ethanolic extract. The *N. arbor-tristis* water extract also showed significantly higher DPPH radical scavenging activity and TAC than ethanolic extract. Importantly, *in vivo*assaydemonstrated that 1000 and 2000 mg/kg of *N. arbor-tristis* water extract significantly attenuated D-glucose-induced increased vascular permeability and leukocyte migration to the peritoneal cavities.

Extraction of the N. arbor-tristis Leaves

In this study, extractions were donewith water and ethanol using maceration technique. This is a cold extraction method whichis able to conserve the thermolabile compounds.[21] Other extraction methods that involve high temperature (Soxhlet extraction) or high pressure (pressurized liquid extraction) can lead to thermal decomposition of the target compounds, orextractions of undesirable impurities.[21]A study has reported that types of solvent affect the extraction yield and biological activity due to the presence of different bioactive compounds in different solvents.Polar and medium polar solvents such as water, ethanol and methanol are widely used to extract hydrosoluble antioxidants such as phenolics, anthocyanins and flavonoids.[7]Ethanol was used as an extraction solvent because it is cheap, reusable, nontoxic and relatively safe for consumption, while water was used in reference to the traditional decoction made from the leaves of *N. arbor-tristis*.[9]

Phytochemical Screening of N. arbor-tristis Leaf Extracts

Preliminary phytochemical studies of *N. arbor-tristis* crude water extract showed that the leavescontain high amounts of flavonoids and saponins, and a low amount of tannins (**Table 2**). Flavonoids, belong to a class of plant secondary metabolites, have been shown to possess antioxidant properties against several diseases such as cardiovascular diseases and atherosclerosis. Flavonoids are used extensively due to their anti-inflammatory, anti-mutagenic and anti-carcinogenic properties.[22]Saponins are glycosidic compounds which exert various biological activities including expectorant, anti-inflammatory, vasoprotective, hypocholesterolemic, immunomodulatory and hypoglycaemic.[23]Tannins are polyphenols with pharmaco-therapeutic potentials such as maintenance of vascular health, anti-inflammatory, management of burns and wound due to their anti-hemorrhagic and antiseptic potentials, antihelmintics, antioxidants, antimicrobial and antivirals.[24-27] Some previous studies have reported that alcoholic leaf extracts of *N. arbor-tristis*containactive compounds such as alkaloids, steroids, tannins, flavonoids, reducing sugars, saponins, glycosides, phenolic and terpenoids.[28, 29]A previous study has also reported that aqueous leaf extract of *N. arbor-tristis* contains anthraquinone.[30] Our findings are only in partial agreement with those studies because reducing sugars and anthraquinone were not detected in both the crude extracts of *N. arbor-tristis*, probably due to different extraction process (**Table 2**).

DPPH Scavenging Activity and Total Antioxidant Capacity (TAC)

A standard DPPH antioxidant activity assessment was performed using *N. arbor-tristis* water and ethanolic leaf extracts.DPPH is a stable nitrogen-centred radical where the nitrogen atom can be reduced by antioxidants.[31] The data of DPPH assay(**Table 3**) demonstrated that *N. arbor-tristis* extracts exerts their antioxidant effects by scavenging free radicals. Forphosphomolybdenummethod, which was used to determine TAC, molybdenum (VI) can be reduced to molybdenum (V) by a reducing agent and forming a green phosphomolybdate (V) complex. [32] As shown in **Table 4**, TAC of *N. arbor-tristis*water extract was higher than the ethanolic extract. Antioxidants derived from plants are classified into phenolic compounds, vitamins and carotenoids.[33]In this study, the free radical scavenging activity of the *N. arbor-tristis*could be contributed by flavonoids in the leaf extracts, in view of high amount of flavonoids present in *N. arbor-tristis* extracts.

In Vivo Vascular Permeability and Total Leukocyte Count

In physiological conditions, the vascular system maintains vascular homeostasis by regulating blood fluidity, maintaining perfusion of different organs, preventing inappropriate activation of leukocytes, and controlling

exchange of molecules between blood and tissues.[34]Elevated plasma glucose has been reported to promote vascular injurythrough oxidative stress and inflammation.[4, 35, 36]Analteration in vascular homeostasis leads to increased vascular permeability, enhanced leukocyte adhesion, and apoptosis of vascular cells, which may participate in the pathogenesis of diabetes-related vascular diseases.[37]As shown in **Figure 2** and **Figure 3**, pre-treatment of the rats with *N. arbor-tristis*crude water extracts significantly suppressed ($p< 0.05$)both the increased vascular permeability and elevated leukocyte migration in the peritoneal cavity in a dose-dependent manner,implying the possible therapeutic usage of the plant forhigh glucose-induced vascular inflammation.

Furthermore, the inhibitory effects of the extracts might be attributedto the presence of flavonoids and saponins in the leaves of *N. arbor-tristis*. Themajor mechanism of action of flavonoids as anti-inflammatory agents isinhibition of key regulatory enzymes and transcription factors.[38, 39]Saponins have been shown to exert anti-inflammatory activity through inhibition of cyclooxygenases-2 and inducible nitric oxide synthase, which results indecreased production of nitric oxide, prostaglandins and tumor necrosis factor-α[40, 41].

CONCLUSION

In this study, wedemonstratedfor the first time that *N. arbor-tristis* crude water extractprotects against vascular inflammation triggered by high glucose*in vivo*. We also showed that *N. arbor-tristis* leaf extracts contain high amounts of phytochemical constituents and exhibit antioxidantactivity. However, further exploration of effects of *N. arbor-tristis*inhyperglycemia-mediatedvascular inflammation are needed to support itsuse as a potential therapeutic agent.

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Figure 1:*N. arbor-tristis* (A: Leaf; B: Flower; C: Seed)

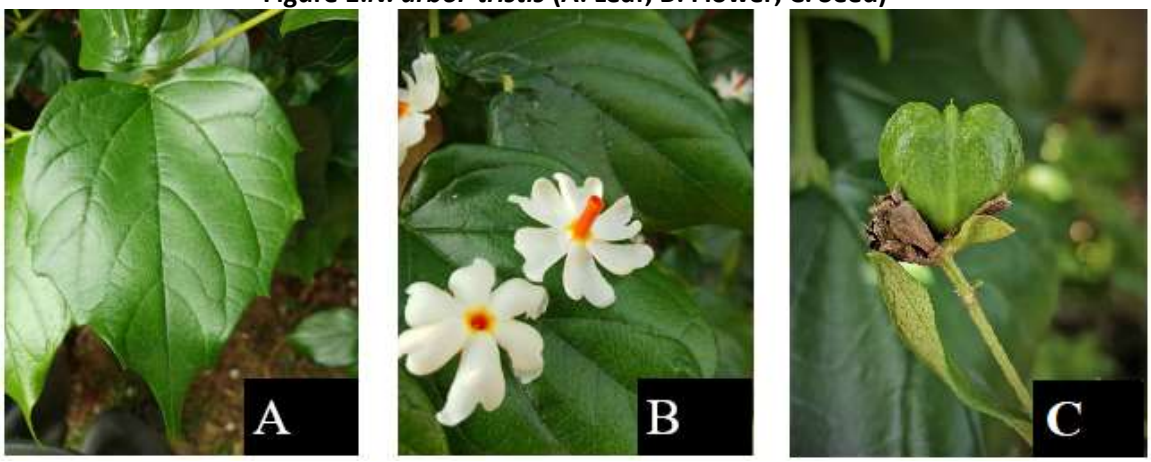

Table 1:Extraction yields of ethanolic and water crude extracts of *N. arbor-tristis*.

Extracts of *N. arbor-tristis*	Weight (grams)		Extraction Yield (%)
	Raw material used	Quantity of extracts	
Crude ethanolic leaf extract	160	33.176	20.7
Crude water leaf extract	160	51.626	32.3

Table 2:Phytochemical analysis of *N. arbor-tristis* crude extracts.

Phytochemicals	Observations	Outcomes	
		Ethanolic extract	Water extract
Flavanoids	Extract change from intense yellow to colourless	++	+++
Tannins	Extract colour changed to blue/green	+	+
Saponins	Formation of frothing	-	+++
Reducing sugar	Extract colour changed to brick red	-	-
Anthraquinone	Formation of pink-red layer	-	-

Descriptions: '+' indicates low amount; '++' indicates moderate amount; '+++' indicates high amount; '-' indicates absent

Table 3:The DPPH radical scavenging activity and IC_{50} of *N. arbor-tristis* crude extracts. Data are expressed as means ± SEM (n=3).
Values with different lowercase letters within the same row are significantly different at $p < 0.05$. NA, not available for positive control.

Concentration (mg/mL)	DPPH radical scavenging activity (%)		
	Ethanolic extract	Water extract	Vitamin C
0.5	34.31 ± 0.46^a	34.27 ± 0.37^a	79.32 ± 1.11^b
1.0	66.43 ± 0.33^a	70.70 ± 0.26^b	81.46 ± 0.20^c
2.0	77.19 ± 0.29^a	82.24 ± 0.60^b	83.31 ± 0.80^b
4.0	84.93 ± 0.12^a	91.19 ± 1.10^b	90.54 ± 0.45^b
8.0	95.73 ± 0.38^a	98.01 ± 0.33^b	95.32 ± 0.36^a
IC_{50} DPPH (mg/mL)	1.46	1.29	NA

Table 4: The total antioxidant capacity of *N. arbor-tristis* crude extracts.
Data are expressed as means ± SEM (n=3).
TAC was expressed as ascorbic acid equivalent (AAE)/100mg dry weight.

Extracts	Absorbance Wavelength = 695 nm	Total antioxidant capacity (AAE/100 mg dry weight)
Ethanolic	0.106 ± 0.001	0.17 ± 0.006
Water	0.014 ± 0.003	1.14 ± 0.032

Figure 2:Effect of *N. arbor-tristis* water extract on high glucose-induced vascular hyperpermeability. Dexamethasone at 10 mg/kg was served as the positive group.
Data are expressed as means ± SEM (n= 6).
[a]*p*< 0.05 compared to normal control group; [b]*p*< 0.05 compared to D-glucose-induced group.

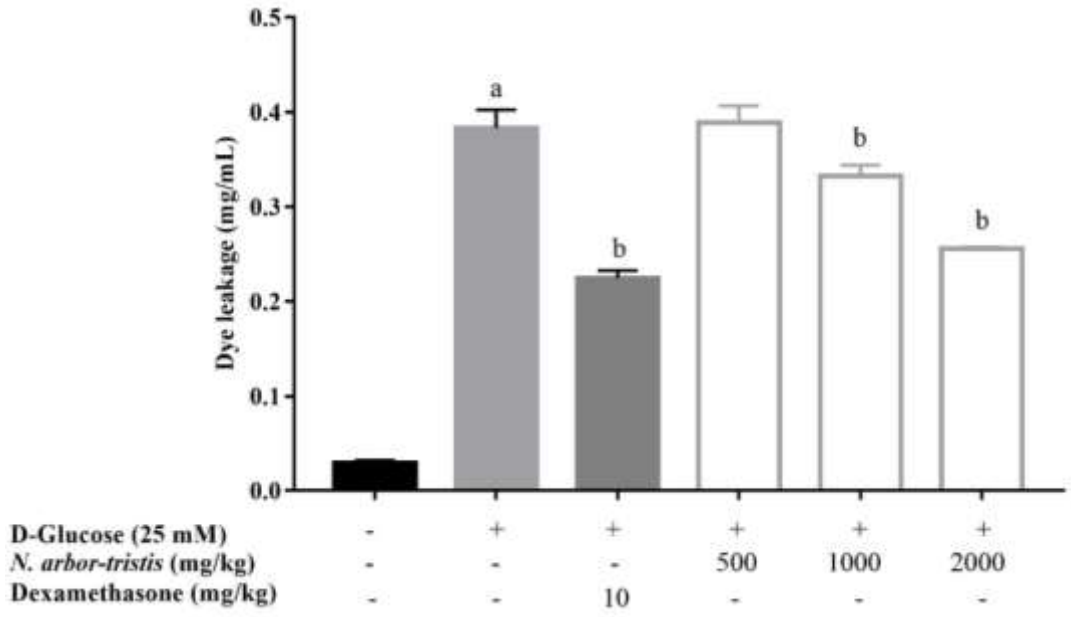

Figure 3:Effect of *N. arbor-tristis* water extract on high glucose-induced leukocyte migration into the peritoneal cavity. Dexamethasone at 10 mg/kg was served as the positive group.
Data are expressed as means ± SEM (n= 6).
[a] *p*< 0.05 compared to normal control group; [b]*p*< 0.05 compared to D-glucose-induced group.