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RESEARCH ARTICLE

# Synergistic Antioxidative Potential of Cosmosiin and Vanillic Acid *in vitro* and *ex vivo*

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**Competing Interests:** None

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## Abstract

**Objectives:** To determine the synergistic antioxidative potential of cosmosiin and vanillic acid. **Methods:** The synergistic antioxidative potential of cosmosiin and vanillic acid was evaluated using their ability to scavenge 2, 2′-azino-bis-(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide anions ($O_2^{\bullet -}$), and 1, 1-diphenyl-2-picrylhydrazyl (DPPH). The ability to convert $Fe^{3+}$ into $Fe^{2+}$ was used to evaluate the combined reducing power of cosmosiin and vanillic acid. The synergistic effect of cosmosiin and vanillic acid was also tested in an *ex vivo* setting using mouse erythrocytes and livers, respectively, for their anti-haemolytic activity and inhibitory effects on lipid peroxidation. **Findings:** When compared to the individual compound, the mixture of cosmosiin and vanillic acid exhibited higher scavenging activities for DPPH, ABTS and $O_2^{\bullet -}$ with $IC_{50}$ of 112.0 $\pm$ 0.46 $\mu$M, 126.17 $\pm$ 4.97 $\mu$M, and 5.14 $\pm$ 0.18 $\mu$M, respectively. Consistently, the combination of cosmosiin and vanillic acid possessed higher reducing power than either compound alone. A mixture of cosmosiin and vanillic acid also showed higher inhibitory activities against mice erythrocyte hemolysis and lipid peroxidation in the liver homogenate with an inhibition rate of 51 % and 90% respectively. **Novelty:** Our study corroborates that cosmosiin and vanillic acid have stronger antioxidative effects when used in combination than when used individually.

**Keywords:** Cosmosin; Vanillic acid; Free radical scavenging activity; Antioxidant; *In vitro*

## 1 Introduction

Reactive Oxygen Species (ROS) or free radicals are unstable atoms that can damage cells, causing malfunction and cell death [(1)].

Aerobic organisms naturally generate free radicals during cellular metabolism, which are neutralized by the cell's antioxidant defense mechanism. ROS such as nitric oxide (NO), superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}$OH), and peroxyl radicals ($ROO^{\bullet}$) are byproducts of cellular respiration that are formed in mitochondria. The overproduction of highly reactive free radicals interacts with DNA, proteins, carbohydrates, and lipids, thereby causing pathogenic complications called oxidative stress[2]. Numerous lifestyle-related diseases, such as cancer, diabetes, coronary heart disease, inflammatory diseases, and neurodegenerative illnesses, are reported to be highly correlated with oxidative stress[3]. As a result, exogenous antioxidant supplements can assist biological systems in achieving their demands to scavenge free radicals and decrease oxidative stress[2].

Antioxidants are compounds that, when administered sparingly, can prevent or decrease the oxidation that is produced by an oxidant[4]. Plant-derived bioactive compounds have attracted considerable attention due to their well-established medicinal properties, including anti-inflammatory, anti-apoptotic, antioxidants, anticancer, and antimicrobial properties, as well as a wide range of illnesses, including neurological conditions such as Alzheimer's disease, depression, anxiety, Parkinsonism, and seizures[5,6]. These compounds have antioxidant properties that protect against cellular oxidation and free radical damage[7]. In addition to establishing the foundation for traditional medicine, the identification of bioactive compounds from plant sources has proven to be a vital source of pharmacological agents for modern drug development[6].

Cosmosiin, also known as apigetrin or apigenin-7-O-glucoside, is a glycosyloxyflavone with the molecular formula of $C_{21}H_{20}O_{10}$, that is, apigenin substituted by a beta-D-glucopyranosyl moiety at position 7 via a glycosidic linkage. It is present in several citrus plant species, including *Citrus grandis* (L.) Osbeck (red wendun) and *Citrus aurantium* Linn. of the Rutaceae family[8]. Cosmosiin extracted from the whole plant of *Dracocephalum kotschyi* has been reported to exhibit antioxidant, anti-inflammatory, and antidiabetic activities[9].

Vanillic acid (4-hydroxy-3-methoxybenzoic acid, or VA), a phenolic compound produced by secondary metabolism in plants, is widely utilized in the food industry as a flavour, fragrance, and preservation. It is an oxidized vanillin formed during the conversion of vanillin to ferulic acid, with a solid appearance, a melting point of 211.5 $^{\circ}$C, and a solubility of 1.5 mg/ml at 14$^{\circ}$C. Vanillic acid has also been found in cereals, beers, herbs, fruits, wines, juices, and green tea and is known to have antidepressant, antinociceptive, anticancer, antifungal, antioxidant, hypertensive, hepatoprotective, wound healing, sedative activity, and ulcerative colitis effects[10,11]. Despite the fact that cosmosiin and vanillic acid are both widely used independently for various pharmaceutical applications, no scientific research has been done on their synergistic activities. Therefore, the aim of this study is to investigate the free radical scavenging activity and antioxidative potential of cosmosiin and vanillic acid individually as well as synergistically.

## 2 Methodology

### 2.1 Chemicals

2,2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), disodium hydrogen phosphate, ferric chloride, sodium nitrite, potassium persulfate, glacial acetic acid, aluminium chloride and hydrogen peroxide ($H_2O_2$) were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). Thiobarbituric acid and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals were obtained from Sigma Aldrich Inc (Louis, Germany). Folin–Ciocalteu's reagent; sodium carbonate, sodium hydroxide and ferrous sulfate were purchased from SD fine Chem Ltd. (Mumbai, India). Potassium ferricyanide was purchased from Loba Chem i.e. Pvt., Ltd. (Mumbai, India). Cosmosiin and vanillic acid were purchased from Chem Faces (Wuhan, China).

### 2.2 Determination of free radical scavenging activity (*in vitro*)

#### *2.2.1* ***DPPH radical scavenging activity***

DPPH radical scavenging activity of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid were assessed using the previously described method[12]. Briefly, 1 ml of methanolic solution of DPPH (0.1 M) was added to different concentrations of cosmosiin, vanillic acid and a mixture of cosmosiin and vanillic acid, and incubated in dark for 30 min. The optical density of the solution was measured at 523 nm using UV-Visible spectrophotometer (SW 3.5.1.0. Biospectrometer, EppendorfIndia Ltd., Chennai). The scavenging activities of the compounds were expressed as $IC_{50}$, the concentration ($\mu$M) of the compounds that inhibits the formation of DPPH radicals by 50 %. Each test was performed in triplicate and the scavenging activities of different compounds were compared. Based on the percentage of DPPH radicals scavenged, the scavenging activity was then estimated using the formula:

Scavenging (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

Where $A_{blank}$ is the absorbance of the control (solution containing all the reagents except cosmosiin, vanillic acid and a mixture of cosmosiin and vanillic acid) and $A_{sample}$ is the absorbance of the solution containing cosmosiin, vanillic acid and a mixture of cosmosiin and vanillic acid.

#### *2.2.2* ***ABTS radical scavenging activity***

The scavenging activity of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid against ABTS was determined according to the method described earlier [12]. Briefly, a stock solution containing 5 ml each of 7 mM ABTS and 2.4 mM potassium persulfate was prepared and incubated for 12 h at room temperature in the dark to yield a dark-colored solution that contains $ABTS^{\bullet+}$ radicals. A fresh working solution was prepared by diluting the stock solution with 50% methanol having an initial absorbance of 0.70 (±0.02) at 745 nm before each assay. $ABTS^{\bullet+}$ radicals scavenging activity was then determined by mixing each tested compound with ABTS working solution in a ratio of 1:10. The decrease in absorbance was measured immediately at 745 nm. Each test was performed in triplicate and the scavenging activities of different compounds were compared. The scavenging activity was then calculated using the formula:

Scavenging (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

Where $A_{blank}$ is the absorbance of the control (solution containing all the reagents except tested compound) and $A_{sample}$ is the absorbance of the solution containing tested compounds.

#### *2.2.3* ***Superoxide radical scavenging activity***

Determination of the superoxide scavenging activity of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid was done using the method described earlier [12]. Briefly, the reaction mixture containing 1 mM NBT (dissolved in 100 mM phosphate buffer), 1 mM NADH (dissolved in 100 mM phosphate buffer) and different concentration of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid was prepared. Following the addition of 60 $\mu$M PMS (dissolved in 100 mM phosphate buffer) the samples were then incubated for 15 min under visible light and the absorbance was read at 530 nm. The superoxide radical scavenging activity was then calculated using the following formula:

Scavenging (%) = $(1 - Ae / Ao) \times 100$

Where Ao is absorbance without the sample and Ae is absorbance with the sample.

#### *2.2.4* ***Reducing power***

According to the procedure described earlier [12], the reducing power of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid was estimated with minor modifications. Briefly, a solution containing 0.1% potassium ferricyanide and 0.2 M phosphate buffer (pH-6.6) in a ratio 1:1 was mixed with different concentrations (100-500 $\mu$M) of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid. Following the addition of 10% TCA, the mixture was incubated for 20 min at 50°C and then centrifuged at 3000 rpm for 10 min. The supernatant was taken and an equal volume of distilled $H_2O$ was added followed by the addition of 1% ferric chloride solution. The absorbance of the mixture was measured at 700 nm. The increase in absorbance indicated increasing the reducing power of the tested compounds.

### 2.3 *Ex vivo* antioxidant assay

#### *2.3.1* ***Anti-hemolytic activity***

The anti-hemolytic effect of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid was determined according to the previously described procedure [12]. The animals were sacrificed through an overdose of ketamine (90 mg/kg) and xylazine (10 mg/kg). Blood was collected by heart puncture in a heparinized tube. Erythrocyte hemolysis was induced with hydrogen peroxide ($H_2O_2$) which serves as a free radical initiator. A reaction mixture containing 5% (v/v) suspension of RBC in PBS, different concentrations (1000-5000 $\mu$M) of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid and 1 mol/L $H_2O_2$ was prepared. The mixture was incubated at 37°C for 3 h with constant gentle agitation. Following dilution with PBS, the solution was centrifuged at 2000 rpm for 10 min, and the absorbance of the supernatant was measured at 540 nm. The inhibition rate of erythrocyte hemolysis was then calculated.

Inhibition rate (%) = $[1 — (A1 — A2)/ A_0] \times 100$

Where, $A_0$ is the absorbance of control, A1 is the absorbance of the solution containing tested compounds and A2 is the absorbance without RBC.

#### *2.3.2* ***Inhibition of lipid peroxidation***

The hepatic lipid peroxidation inhibitory action of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid was measured according to the method described earlier[12]. The liver homogenate (1% w/v) prepared in PBS was centrifuged at 3000 rpm at 4°C for 10 min and the supernatant was mixed with different concentrations (1000-5000 $\mu$M) of cosmosiin, vanillic acid and a combination of cosmosiin and vanillic acid. Following the addition of 25 mmol/L $FeCl_2$ and $H_2O_2$, the solution was incubated at 37°C for 1 h and absorbance was measured at 535 nm. The rate of inhibition of lipid peroxidation was calculated using the formula:

Inhibition rate (%) = [1 — (A1 — A2)/ $A_0$] × 100

Where, $A_0$ is the absorbance of control, A1 is the absorbance of the solution containing the tested compounds, and A2 is the absorbance without liver homogenate.

### 2.4 Statistical analysis

The results are presented as the mean ± standard error of the mean. To examine the significant differences in free radical scavenging activity and antioxidative potential of cosmosiin, vanillic acid, and the combination of cosmosiin and vanillic acid, a one-way analysis of variance was performed, followed by Tukey multiple comparisons of means. Using GraphPad Prism software version 6.0, the $IC_{50}$ values were obtained by plotting the % inhibition of free radicals against the log doses. A p-value of less than 0.05 was considered statistically significant.

## 3 Results and discussion

### 3.1. *In vitro* antioxidant assays

*In vitro* antioxidant assays using DPPH, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ revealed the antioxidative potential of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid. The free radical scavenging activities of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid increased in a concentration-dependent manner. Log-doses of tested compounds were plotted against inhibition (%) of DPPH, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ radicals for the calculation of $IC_{50}$ [Figure 1].

The scavenging activities of combined compounds (cosmosiin and vanillic acid) against DPPH ($IC_{50}$: 112.0 ± 0.46 $\mu$M), $ABTS^{\bullet+}$ ($IC_{50}$: 126.17 ± 0.66 $\mu$M) and $O_2^{\bullet-}$ ($IC_{50}$: 5.14 ± 0.18 $\mu$M) were found to be significantly higher than cosmosiin ($IC_{50}$: 441.0 ± 4.97 $\mu$M for DPPH; 781.06 ± 3.79 $\mu$M for $ABTS^{\bullet+}$; 170.27 ± 1.07 $\mu$M for $O_2^{\bullet-}$) and vanillic acid ($IC_{50}$: 195.07 ± 1.01 $\mu$M for DPPH; 510.43 ± 3.56 $\mu$M for $ABTS^{\bullet+}$, 59.6 ± 4.2 $\mu$M for $O_2^{\bullet-}$). Significant variation was observed ($p < 0.001$) among the tested compounds in DPPH, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ scavenging activities [Figure 2 **A, B & C**].

The antioxidant property of natural compounds can be evaluated by assessing their ability to reduce methanolic DPPH solution to the non-radical form DPPH-H. Similarly, the antioxidative activity of the mixture of cosmosiin and vanillic acid was also determined by measuring their ability to convert the blue-colored $ABTS^{\bullet+}$, which is formed by the interaction of ABTS and potassium ferricyanide, to ABTS. The free radical scavenging activities of the tested compounds increased in a concentration-dependent manner as indicated by discoloration of the DPPH and $ABTS^{\bullet+}$. The significant scavenging activities of DPPH and ABTS by the mixture of cosmosiin and vanillic acid clearly indicated the antioxidative potential of the test compounds. The effectiveness of the radical scavenging activities of the compounds depends on a number of characteristics such as an abundance of aromatic rings, types of hydroxyl group substitution, and the molecular weight of phenolic compounds. One of the most significant free radicals in organisms is the superoxide anion radical ($O_2^{\bullet-}$), which is the starting point for reactive oxygen species that may be transformed into other free radicals like $H_2O_2$, ONOO−, etc[13]. The mitochondrial electron transport mechanism generates superoxide anion, a weak free radical. However, it can produce other potent free radicals, increasing the risk of numerous diseases[14]. The present study suggests that $O_2^{\bullet-}$ radical scavenging activity of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid increased with increases in concentration, and the mixture of cosmosiin and vanillic acid showed higher scavenging potential when compared with either compound alone. Previous studies showed that phenolic compounds can transfer a single electron to scavenge free radicals, superoxide, and hydroxyl radicals. There is a general agreement that phenolic compounds have a direct relationship with the antioxidant ability[13]. The capacity of phenolic acids to donate hydrogen atoms has been primarily cited as the source of their antioxidant properties. Esterification, glycosylation, the quantity and location of hydroxyl groups with respect to the carboxyl functional group, and substituents on the aromatic ring appear to have an impact on the antioxidant capacity[15]. Numerous studies have demonstrated the antioxidative potential of flavonoids, and several mechanisms have been reported by which flavonoids exerted antioxidant activity. In general, the antioxidative activity of flavonoids is related to their structure, depending on the number of hydroxyl substituents present in their constitution[16].

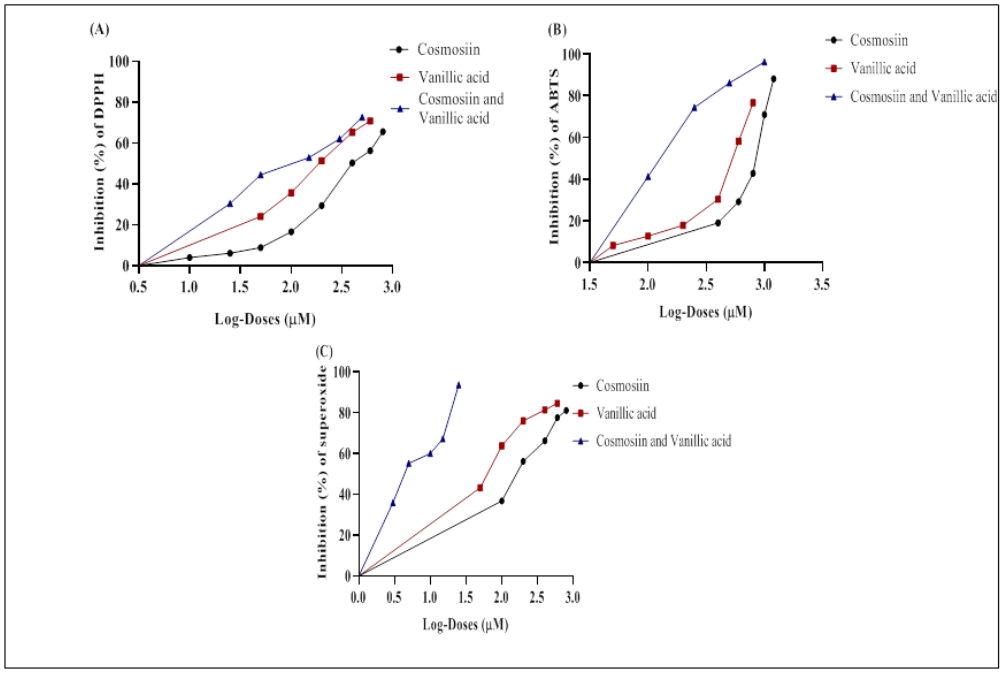


**Fig 1. Plots of log-doses of cosmosiin, vanillic acid and combination of cosmosiin and vanillic acid against DPPH (A), ABTS (B) and $O_2^{\bullet -}$ (C) inhibition (%) for the calculation of $IC_{50}$.**

## 3.2 The reducing power

The reducing power of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid were assessed by measuring their ability to transform ferric ($Fe^{3+}$) into ferrous ($Fe^{2+}$). The reducing activity of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid also increased in a dose-dependent manner [Figure 3]. The total reducing power of the combination of cosmosiin and vanillic acid at 500 $\mu$M (0.559 $\pm$ .001) was found to be significantly higher than cosmosiin (0.110 $\pm$ .004) and vanillic acid (0.280 $\pm$ 0.005) [Figure 3].

It has been shown in several studies that the electron donation capacity indicates the reducing power of physiologically active chemicals in relation to their antioxidant activity. Antioxidants are reducing agents, and the inactivation of oxidants by reductants is a reduction-oxidation (redox) event in which one reactive species is reduced at the expense of the oxidation of the other. Since electron donation is a key mechanism of phenolic antioxidant activity, the reduction of $Fe^{3+}$ is frequently used as a measure of this capacity[17]. One of the most potent classes of natural antioxidants is polyphenols, which include phenolics, flavonoids, and phenolic acids. Their redox characteristics, which enable them to function as reducing agents or hydrogen/electron donors, may be related to their antioxidant activity. As a result, they can neutralize free radicals; put an end to chain processes involving radicals, and chelate transitional metals. ROS-mediated damage to cells can be prevented or repaired by engaging in these activities, which may have positive effects on one's health by lowering the chance of developing cancer or cardiovascular disease. Other studies have also reported that bioactive compound-reducing power is associated with antioxidant activity[18].

## 3.3 *Ex vivo* antioxidant assay

Both the anti-hemolytic activity and lipid peroxidation inhibitory effect of cosmosiin, vanillic acid, and the combination of cosmosiin and vanillic acid occurs in a concentration-dependent manner [Figure 4]. Inhibitory activity of cosmosiin, vanillic acid and the combination of cosmosiin and vanillic acid against mice erythrocyte hemolysis at the dose of 5000 $\mu$M was

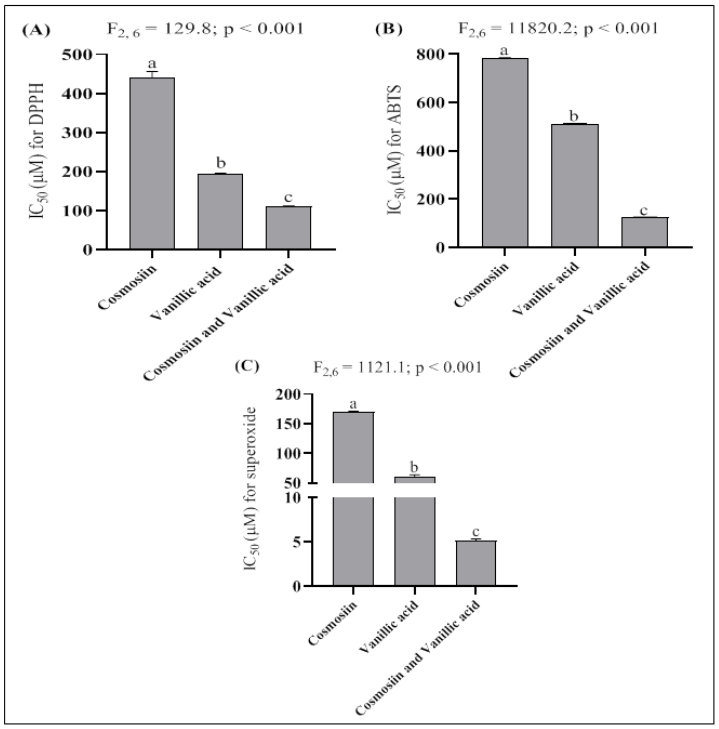


**Fig 2. $IC_{50}$ ($\mu$M) of cosmosiin, vanillic acid and combination of cosmosiin and vanillic acid for DPPH (A), ABTS (B) and $O_2^{\bullet -}$(C). Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.**

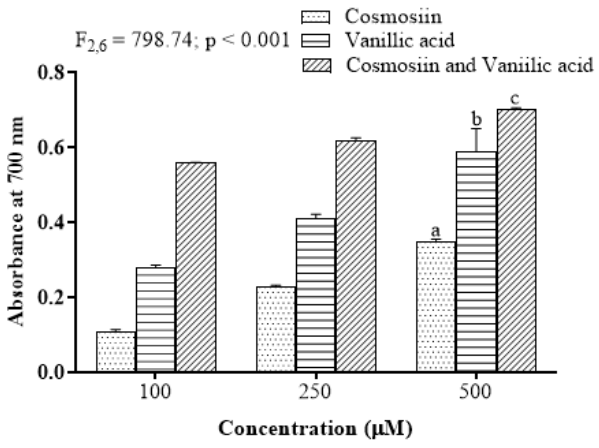


**Fig 3. Reducing power of cosmosiin, vanillic acid, and combination of cosmosiin and vanillic acid at different concentrations. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.**

49%, 26% and 51%, respectively [Figure 4 **A**]. The highest inhibitory effect of cosmosiin, vanillic acid and the combination of cosmosiin, and vanillic acid against lipid peroxidation in mice liver homogenate was recorded at 5000 $\mu$M with an inhibition rate of 38%, 68%, 90% respectively [Figure 4 **B**].

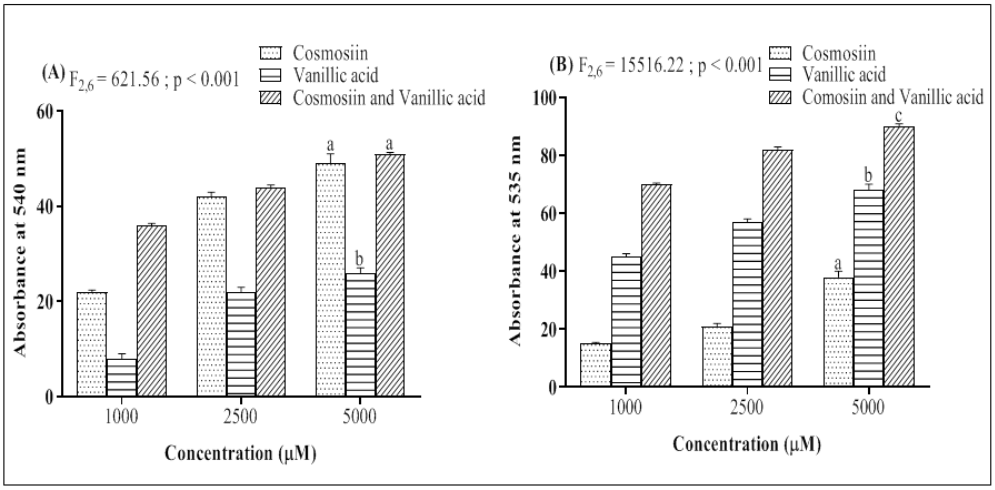


**Fig 4. (A) Anti-hemolytic, and (B) lipid peroxidationinhibitory activities of cosmosiin, vanillic acid and combination of cosmosiin and vanillic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.**

The majority of cells in humans are erythrocytes, which have specific physical and metabolic characteristics necessary for the replication of cells. Erythrocytes are capable of redox-active oxygen transfer, therefore hemoglobins and polyunsaturated fatty acids (PUFA) target these cells. As a result, erythrocyte membrane proteins and lipids are damaged by oxidation during hemolysis. A multitude of circumstances, including radiation, a high concentration of transition metals, hemoglobinopathies, and impairments in erythrocyte antioxidant coordination, are associated with this type of mutilation. Erythrocytes exposed to toxins like hydrogen peroxide experience hemolysis more frequently[19]. Lipid peroxidation is a free radical chain reaction that initiates propagation reactions, leading to damage of the membrane of erythrocytes and consequently to hemolysis. Damage to membranes can result from the peroxidation of lipid moieties, such as polyunsaturated fatty acids, by a cascade of free radicals[3]. Lipid peroxidation was induced in mouse liver homogenate by the application of $FeCl_2$ and $H_2O_2$. Malonaldehyde is produced and used as a marker for oxidative stress and lipid peroxidation. Cosmosiin, vanillic acid, as well as the combination of both compounds showed significant anti-hemolytic and lipid peroxidation inhibitory activities. The OH substitutions found in flavonoid derivatives are connected to their ability to protect erythrocytes, with the quantity of OH groups increasing the antioxidant activity. Together with their known liposolubility, their molecular structure (the C2=C3 link in the C ring enhances antioxidant capacity) raises the anti-hemolytic defence. This enables them to integrate into the membrane and function as antioxidant agents, removing dangerous species before they can damage the membrane. This lowers the quantity of reactive species and therefore stops lysis events[20]. Flavonoids and phenolic acids have recently gained widespread recognition for their antioxidant properties[21]. Phenolic compounds have been demonstrated to scavenge and react with free radicals, effectively ending the free radical reaction chain. As a result, reducing the oxidative modification of erythrocyte lipids using antioxidants is the recommended method of preventing hemolysis[22]. Several researchers claim that membranous phospholipids have the capacity to bind to flavonoids and other polyphenols, preventing the degradation of membranes brought on by fatty acids[23].

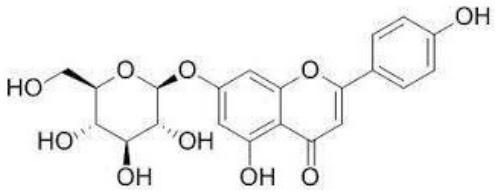


**Fig 5. Molecular structure of cosmosiin**

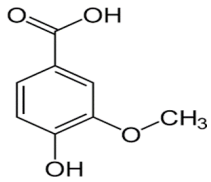


**Fig 6. Molecular structure of vanillic acid**

## 4 Conclusion

The findings of this study demonstrated that the combination of cosmosiin and vanillic acid comparatively exhibits notable antioxidant and anti-haemolytic activities than both compounds individually. The mixture of cosmosiin and vanillic acid exhibited higher scavenging activities for DPPH, $O_2^{\bullet -}$, and ABTS with $IC_{50}$ of 112.0 $\pm$ 0.46 $\mu$M, 126.17 $\pm$ 4.97 $\mu$M, and 5.14 $\pm$ 0.18 $\mu$M, respectively when compared to the individual compound. Consistently, the combination of cosmosiin and vanillic acid possessed higher reducing power than either compound alone. A mixture of cosmosiin and vanillic acid also showed higher inhibitory activities against mice erythrocyte hemolysis and lipid peroxidation in the liver homogenate with an inhibition rate of 51 % and 90 % respectively. According to our study, these two bioactive compounds have the potential to serve as antioxidant sources and are excellent candidates for prospective biomedical applications that will promote human health with few adverse effects.

## 5 Declaration

Presented in 4$^{th}$ Mizoram Science Congress (MSC 2022) during 20$^{th}$ & 21$^{st}$ October 2022, organized by Mizoram Science, Technology and Innovation Council (MISTIC), Directorate of Science and Technology (DST) Mizoram, Govt. of Mizoram in collaboration with science NGOs in Mizoram such as Mizo Academy of Sciences (MAS), Mizoram Science Society (MSS), Science Teachers' Association, Mizoram (STAM), Geological Society of Mizoram (GSM), Mizoram Mathematics Society (MMS), Biodiversity and Nature Conservation Network (BIOCONE) and Mizoram Information & Technology Society (MITS). The Organizers claim the peer review responsibility.

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