



## Screening of the Phytochemicals, Determination of Antioxidants, Inhibition of Acetylcholinesterase and Lipid Peroxidation by *Spondias mombin* in *Drosophila melanogaster*

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### Abstract

#### Keywords:

Acetylcholinesterase,  
*Drosophila melanogaster*,  
Antioxidant, *Spondias mombin*, flavonoid

For ages, *Spondias mombin* ('Iyeye' in Yoruba) has been in use for medicinal purposes in the treatment of learning impairment even at the time when there was no scientific evidence to substantiate its significant functions. *Drosophila melanogaster* is a fruit fly with very rapid life cycle that is used in the laboratory to test for functional foods and nutraceuticals just like in animals as a way to extend beyond using humans screening. The aqueous extract of *S. mombin* was screened for phytochemical contents, antioxidant activities and in vivo assay of superoxide dismutase (SOD) activity, acetylcholinesterase (AChE) and lipid peroxidation inhibition. The present study however reveals the presence of saponins, tannins, flavonoids and alkaloids. The quantitative values of aqueous extract of *S. mombin* exhibited 1.65 mg/100 g, 1.17 mg/100 g and 0.11 mg/100 g values of total phenol content, total flavonoid content and ferric reducing antioxidant property respectively. Iron (II) sulphate ( $FeSO_4$ ) and aluminium chloride ( $AlCl_3$ ) were used as prooxidants in the induction of neurodegeneration in *Drosophila melanogaster*. However, the aqueous extract of *S. mombin* inhibited the activities of the lipid peroxidation and AChE in *D. melanogaster*. Interestingly, SOD concentration was also observably increased in *D. melanogaster* pretreated with *S. mombin*. These results implied that the aqueous extract of *S. mombin* can serve as an economic source of antioxidants that can be employed in the treatment and management of learning impairment conditions.

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### INTRODUCTION

The research into unique and medicinal plants dated to the ancient time has continued to attract attention irrespective of climatic, phytogeographic and faunal characteristics (Jamshidi-Kia *et al.*, 2018). In Nigeria ethnomedicine, plants are used for the treatment of a variety of diseases, plant parts such as leaves, stem parts and roots are chopped into extractable pieces, and allowed to macerate in water for a period of time. Portions of the extract are then taken orally as soon as the acceptable concentration is achieved (Agbor and Naidoo, 2015).

Neurodegeneration is a process involved in both neuropathological conditions and brain ageing. It is known that brain pathology in the form of cerebrovascular and neurodegenerative disease is classified as the foremost cause of death globally (Gan and Johnson, 2014). Oxidative stress is a condition that is characterized by the imbalance between free radical generation and free radical scavenging abilities which has been implicated as a major factor that contributes to aging (Phaniendra *et al.*, 2015). Apart from these, the term 'oxidative stress' generally describes the interactions between oxygen-containing molecules, hydroxyl radicals, peroxides, nitric oxide radicals, the superoxide anion

radicals, hypochlorite radicals and singlet oxygen and membrane lipids, proteins, enzymes and nucleic acids which impairs their functional capacity (Saliu and Olabiyi, 2017).

### LITERATURE REVIEW

The advent of Alzheimer's disease (AD) as a progressive and complex neurological disease that has been revealed with cases of more than 80% among 65 years old people around the world (Reitz and Mayeux, 2014).

AD is profiled by cognitive dysfunctions which include cerebral cortex, systemic loss of memory, low reasoning and learning ability as a result of neuronal and synaptic depletion in the sub-cortical regions (Vijayan and Reddy, 2016). In spite of various findings, none of the experimental and epidemiological evidences on the pathophysiology of this disease has been able to procure a substantive form of treatment (Bacigaluppi *et al.*, 2010). As regards the cholinergic hypothesis, acetylcholinesterase (AChE) is a critical hydrolytic enzyme that catalyzes the acetylcholine metabolism, which in turn affects the cholinergic dysfunction associated with AD when there is an elevation in its mode of action (Bacigaluppi *et al.*, 2010). An observed lower level of choline in the synaptic gap is an indication of higher concentration of the cholinesterase enzyme.

Earlier research has correlated the cognitive decline in AD patients with the progressive cholinergic degeneration through the cholinesterase enzymes in postmortem brain samples derived from AD patients (Freirra-Vieira *et al.*, 2016). The inhibition of cholinesterase has attracted much attention recently because of its potential use in the treatment of Alzheimer's disease. Synthetic drugs such as rivastigmine, tacrine, donepezil and galanthamine are designed as cholinesterase-I (ChE-I) to restore the level of acetylcholine and slow down neurodegeneration via the inhibition of cholinesterase to subsequently provide further health benefits for AD treatment. However, their side effects and contraindications cause discomfort after a long-time use. Some medicinal plants have earlier been employed traditionally to enhance cognitive function and alleviate other symptoms associated with AD (Sharma, 2019). In addition, the sensitive nature of mammalian brain particularly galvanizes towards oxidative damage, as a result of high oxygen demand and high content of unsaturated lipids in the central nervous system coupled with the reduced access to the antioxidant defense mechanism and the high concentration of redox-active transition metal ions (Salim, 2017).

The existence of oxidative stress in the central nervous system is an underlying factor for the

occurrence of neurodegeneration and neural dysfunction. This has resulted in considerable research efforts being put towards reducing the risks and advent of oxidative stress with the use of antioxidants. The presence of phytochemicals in fruits and vegetables has been reported to reduce the risk of several major diseases such as cardiovascular diseases, cancers as well as neurodegenerative disorders. In folklore medicine, numerous plants have been employed to treat cognitive disorders, including neurodegenerative diseases and other memory-related disorders (Hashimoto and Hossain, 2011). Herbal products contain complicated mixtures of organic chemicals, which may include fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, tannins, terpenes and so forth (Winter *et al.*, 2017).

Therapeutic applications of *S. mombin* leaf has long time been adopted in folklore medicine in the prevention/management of Alzheimer's disease with no or little scientific basis. This study therefore sought to evaluate phytochemicals, antioxidant ability and the *in vivo* inhibitory property of aqueous extract of *S. mombin* leaf on the key enzyme (acetylcholinesterase) linked to neurodegenerative diseases (e.g., Alzheimer's disease) using iron sulphate and aluminium chloride as pro-oxidants to induce oxidative stress in *D. melanogaster*.

### MATERIALS AND METHODS

#### Chemicals and Reagents

All chemicals used in this study were of analytical grade.

#### Sample Collection and Preparation

Fresh leaves of *S. mombin* fruit was collected from Okusa, Akungba-Akoko. The plant was identified as *Spondias mombin* Linn, designated with Voucher number AAUA\_225 at Plant Science Biotechnology, Adekunle Ajasin University, Akungba, Ondo State, Nigeria. The plant was rinsed with distilled water to remove attached soil particles, debris and dirt. The plant was then air-dried at room temperature until fully dried. The sample was chopped into smaller pieces, milled to powder and kept in a covered container.

#### *D. melanogaster* Stock Culture

Wild type *D. melanogaster* (Harwich strain) stock culture was obtained from *Drosophila* research laboratory, Functional Food and Nutraceutical Unit, Department of Biochemistry, Federal University of Technology Akure, Nigeria. The flies were maintained and reared on normal diet made up of corn meal medium containing 1% w/v brewer's yeast and 0.08% v/w nipagin at constant temperature and humidity ( $25 \pm 1$  °C; 60% relative humidity) respectively under 12 h dark/light cycle conditions.

All the experiments were carried out with the same *D. melanogaster* strain.

## Methods

### Aqueous Extract Preparation

The supernatant of the plant material was prepared according to a previously reported method Oboh *et al.* (2010). Briefly, the plant material (blended sample) was soaked in cold distilled water for 24 hours placed in an orbital shaker. The mixture was then filtered through Whatman No. 1 filter paper and the filtrate centrifuged at  $805 \times g$  for 10 minutes. The clear supernatant was labeled and stored in small capped plastic container at  $4^\circ C$  until required.

### In vitro Assays

#### Determination of Total Phenol Content

The total phenol content was determined according to the method of Singleton *et al.* (1999). Appropriate dilution of the extract was oxidized with 2.5 mL 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at  $45^\circ C$  and the absorbance was measured at 765 nm in the spectrophotometer.

#### Determination of Total Flavonoid Content

The total flavonoid content of the herbs was determined using a slightly modified method reported by Meda *et al.* (2005). Briefly, 0.5 mL of appropriately diluted sample extract was mixed with 0.5 mL methanol, 50  $\mu L$  10%  $AlCl_3$ , 50  $\mu L$  1M potassium acetate and 1.4 mL water and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm.

#### Determination of Ferric Reducing Antioxidant Property (FRAP)

The ferric reducing antioxidant property of the extracts was determined by assessing the ability of the extracts to reduce  $FeCl_3$  solution as described by Patel *et al.* (2012). A 2.5 mL aliquot of the extract was mixed with 2.5 mL of 200mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at  $50^\circ C$  for 20 min and then 2.5 mL of 10% trichloroacetic acid was added. This mixture was centrifuged at  $801 \times g$  for 10 min. 5 mL of the supernatant was mixed with an equal volume of water and 1mL of 0.1% ferric chloride. The absorbance was measured at 700 nm.

#### Secondary metabolites Screening of Secondary metabolites

##### Secondary metabolites Analyses

Chemical test was conducted on the aqueous extract of plant sample and also of the powdered form of the plant sample using standard methods by Edeoga *et al.* (2005). This was conducted for the determination of the presence of saponins, tannins, flavonoids and

alkaloids. The aqueous extract of plant was prepared by soaking 10.0 g of powdered samples in 200 ml of distilled water for 12 hours. The extract was then filtered using Whatman No.1 filter paper.

### Test for Tannin

The test for tannin was carried out according to the method of Edeoga *et al.* (2005). Briefly, 0.5 g of powdered sample of the plant was boiled in 20 ml of distilled water in a test tube and filtered. Then 0.1%  $FeCl_3$  was added to the filtrate sample and observed for brownish green or a blue – black coloration, which showed the presence of tannin.

### Test for Saponin

2.0 g of powdered sample of the plant was boiled in 20.0ml of distilled water in a water bath and then filtered. 10.0ml of the filtrate was mixed with 5.0ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins Edeoga *et al.* (2005).

### Test for Flavonoid

A portion of crude powder was heated with 10 ml of ethyl acetate over a steam bath for 3 min. the mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and observed. A dark yellow colour indicated the presence of flavonoids (Edeoga *et al.*, (2005).

### Test for Alkaloid

The plant sample (5.0 g) was prepared in a beaker, 200 ml of 10%  $CH_3COOH$  in  $C_2H_5OH$  was added to the plant sample. The mixture was covered and allowed to stand for 4 hours. The mixture was then filtered and the extract was allowed to become concentrated in a water bath until it reached  $\frac{1}{4}$  of the original volume. Concentrated  $NH_4OH$  was added until the precipitate was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute  $NH_4OH$  and then filtered. The residue is alkaloid (Trease and Evans, 1996).

### Nitric Oxide (NO) Scavenging Activity

Nitric oxide scavenging assay was performed using the method reported by Susanta *et al.* (2006), where in 0.3 mL of sodium nitroprusside (5 mM) was added to 1 mL of concentration of the extract. The test tube was then incubated at  $25^\circ C$  for 150 minutes. After 150 minutes, 0.5 mL of Griess reagent (equal volume of 1% sulfanilamide on 5% orthophosphoric acid and 0.01% naphthylethylenediamine in distilled water, used after 12 hours of preparation) were added. The absorbance was measured at 546 nm.

### In vivo Assays

#### Experimental Design

The *D. melanogaster* Harwich strain (both gender, 5 days old) was divided into six groups for the superoxide dismutase (SOD) activity assay and nine groups for acetylcholinesterase (AChE) activity and lipid peroxidation inhibition assays. Each group comprised of 40 flies which were fed on diet as prescribed in Odubanjo *et al.* (2020) but with slight modifications.

#### Preparation of Tissue Homogenate

The flies were anesthetized in ice and homogenized in 0.1 M phosphate buffer, pH 7.4, using a Teflon homogenizer. The resulting homogenates were centrifuged at 10,000 X g, 4 °C for 10 min in a Kenxin refrigerated centrifuge Model KX3400C (KENXIN Intl. Co., Hong Kong). Subsequently, the low - speed supernatant (S1) was separated from the pellet into Eppendorf tubes and used for lipid peroxidation assay (Belle *et al.*, 2004).

#### Lipid peroxidation assay

The lipid peroxidation assay carried out using the modified method of Ohkawa *et al.* (1979). Briefly, 100 µL of S1 fraction was mixed with a reaction mixture containing 30 µL of 0.1 M (pH 7.4) Tris-HCl buffer, aqueous extract of *S. mombin* (50 µL), and 30 µL of the pro-oxidant (250 µM FeSO<sub>4</sub>), and the volume was made up to 300 µL by water before incubation at 37°C for 1 h. The colour reaction was developed by adding 300 µL of 8.1% sodium dodecyl sulfate to the reaction mixture containing S1. This was subsequently followed by addition of 600 µL of acetic acid/HCl (pH 3.4) buffer and 600 µL of 0.8% thiobarbituric acid. This mixture was incubated at 100°C for 1 h. Thiobarbituric acid-reactive species (TBARS) produced were measured at 532 nm in the spectrophotometer (JENWAY 6305) and the absorbance was compared with that of a standard using malondialdehyde (MDA).

#### Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was determined by the method of Alia *et al.* (2003), in which 0.05 ml of tissue homogenate was treated with 1.0 ml of 50 mmol/L carbonate buffer (pH 10.2) and 0.017 ml of adrenaline (0.06 mg/ml). The absorbance was read at 480 nm in spectrophotometer for 2 min at 15 s intervals. SOD activity was expressed as U/g protein.

#### Enzyme Inhibition Assay

##### Cholinesterase Activity Assay

The Acetylcholinesterase (AChE) inhibitory activity was determined according to the method of Ellman *et al.* (1961). In a reaction mixture containing 200 µL of AChE solution in 0.1M phosphate buffer, pH 8.0, 100 µL of a solution of 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB), 100 µL of a solution of the sample extracts, and 500 µL of phosphate buffer (pH 8.0) were incubation for 20min at 25 °C. Thereafter, 100 µL of the enzyme substrate

(acetylthiocholine iodide [0.05 mM]) solution was added. The AChE activity determined by monitoring changes in absorbance at 412 nm for 3 min. The AChE inhibitory activity was expressed as percentage inhibition as follow:

% Inhibition =  $[(Abs_{Control} - Abs_{Samples}) / Abs_{Control}] \times 100$ , where  $Abs_{control}$  is the absorbance without the extract and  $Abs_{sample}$  is the absorbance with extract.

#### Data Analysis

The result of replicate (n=3) experiments was pooled and expressed as mean ± standard deviation (SD). The means were analyzed using one-way analysis of variance (ANOVA) and the Duncan test was used for the post hoc treatment (Zar, 1984).

## RESULTS AND DISCUSSIONS

The results of the secondary metabolites analyses on aqueous extract of *S. mombin* (leaf) are shown in Table 1. The result revealed the presence of tannins, saponins, flavonoids and alkaloids of the extracts.

**Table 1.** Qualitative Screening of *Spondias mombin* Analysis

Tannin	+
Saponin	+
Flavonoid	+
Alkaloid	+

+: positive

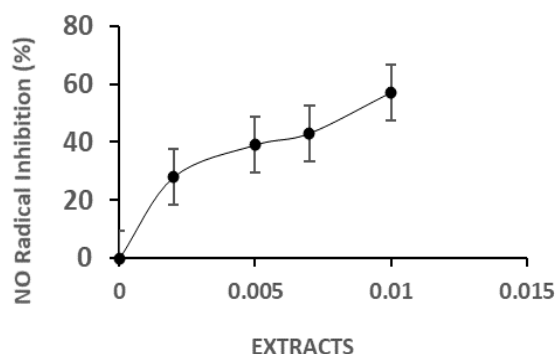
Table 2 shows the results of the total phenol, flavonoid content and ferric reducing antioxidant power of the aqueous extract of *S. mombin* leaf. The extract of *S. mombin* has total phenolic content of 1.65 mg/100g, total flavonoid content of 1.17 mg/100g and ferric reducing antioxidant power value of 0.11 mg/100g.

**Table 2.** Total phenol content, total flavonoid content and ferric reducing antioxidant activity of *Spondias mombin*

Analysis	
Total phenol (mg/100g)	1.65±0.02
Total flavonoid (mg/100g)	1.17±0.03
Ferric reducing antioxidant activity (mg/100g)	0.11±0.02

Values represent mean ± standard deviation (n=3)

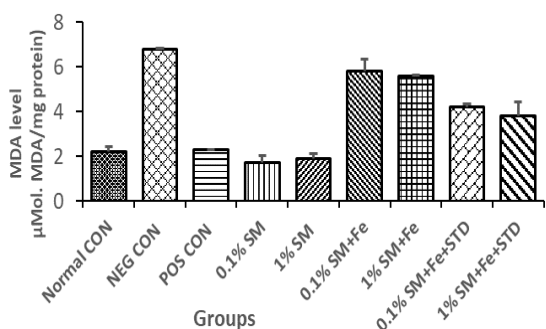
The quantified nitrite level in this present study as a measure of the nitric oxide (NO) concentration is presented in Fig. 1.



**Figure 1.** The inhibitory effect of aqueous extract of *Spondias mombin* on nitrile level in a dose dependent manner.

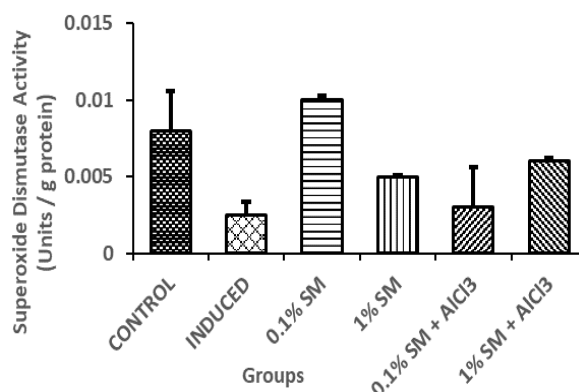
From the graph, the aqueous extract of *S. mombin* leaf scavenged NO radicals in a concentration dependent capacity that ranged from 0.0 - 0.015 µg/mL with IC<sub>50</sub> value of 0.06 µg/mL. The steady increase in the percentage was calculated as evaluation for their NO scavenging ability.

The ability of the aqueous extracts of *S. mombin* leaf to inhibit Fe<sup>2+</sup>- induced lipid peroxidation in fruit flies (*in vitro*) is presented in Fig. 2. The introduction of *S. mombin* extract (0.1% and 1%) caused a significant dose – dependent decrease (P<0.05) in MDA content of the Fe<sup>2+</sup> - stressed fly homogenate. However, incubation of flies' homogenate with 250 mM FeSO<sub>4</sub> (negative control) resulted in a significant (P< 0.05) increase in malondialdehyde (MDA) content. According to the result (Fig. 2) the incorporation of aqueous extract of *S.mombin* leaf inhibited FeSO<sub>4</sub> induced MDA production but with a slight decrease of inhibition in comparison to the negative control.



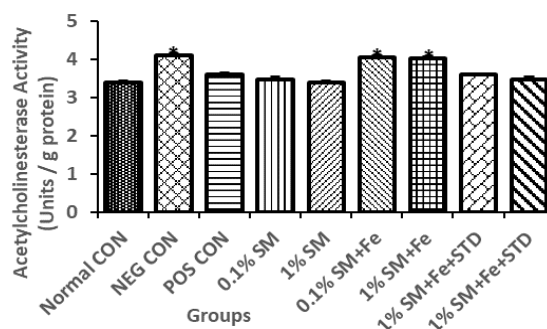
**Figure 2.** Inhibition of FeSO<sub>4</sub> induced lipid peroxidation on malondialdehyde (MDA) in *Drosophila melanogaster* by aqueous extracts of *Spondias mombin*. SM; *Spondias mombin*; NEG; negative, CON; control; POS; positive, Fe; iron, STD; standard.

The result of the renal antioxidant enzyme [superoxide dismutase (SOD)] is shown in Fig. 3.



**Figure 3.** Effect of aqueous extract of *Spondias mombin* on superoxide dismutase (SOD) activity in Aluminium chloride induced *Drosophila melanogaster*. SM; *Spondias mombin*, AlCl<sub>3</sub>; Aluminium chloride

The result revealed a significant (P< 0.05) reduction in the SOD level of the AlCl<sub>3</sub> (as pro-oxidant) induced fly group. However, pretreatment with *S. mombin* extracts (0.1% and 1%) prior to AlCl<sub>3</sub> administration significantly (P< 0.05) increased the SOD level. Following the trend of the observed SOD result, there was significant (P< 0.05) elevation in the brain acetylcholinesterase activity in the FeSO<sub>4</sub> induced - amnesic flies' when compared to the normal control in Fig. 4. However, the reduction in acetylcholinesterase activity observed in *S. mombin* pretreated flies was also in a dose – dependent manner (0.1% and 1%), a significant (P< 0.05).



**Figure 4.** showing the effect of aqueous extract of *Spondias mombin* on Acetylcholinesterase in Fe<sup>2+</sup> induced *Drosophila melanogaster*. CON; control, POS; positive; NEG; negative; SM; *Spondias mombin*; Fe; iron, STD; standard.

## DISCUSSION

Over the years, the efficacy of plant materials in boosting the immune system, healing the allergies, raising and renewing the body vitality (Wang *et al.*, 2010) have been employed in the treatment/management of cognitive dysfunction most especially in African countries. The result of this present investigation revealed the positive response of tannins, saponins, flavonoids and alkaloids in *S. mombin* (leaf) extracts. According to Sharifi-Rad *et al.*, (2020), incorporation of secondary metabolites through correct dietary habits may promote health benefits by protecting individuals against the chronic neurodegenerative disease. An advantage of exposure to these secondary metabolites has been attributed to their various components which act synergistically to exert curative effects.

The present study shows the scavenging effect of the aqueous extract of *S. mombin* leaf on the nitrile level in a dose dependent manner that ranged from 0 - 0.015 µg/mL. In mammalian cells, the production of NO as a free radical is involved in the regulation of various physiological processes such as synaptic activity, memory functions and neural plasticity. Its excess production is however associated with several neuronal disorders (Sivakumar *et al.*, 2012). The expression of the percentage (%) increase in the NO radical scavenging ability of aqueous extract of *S. mombin* leaf follows the trend of the *in vitro* work carried out by Jagetia and Baliga (2014). Also, in line with the earlier behavioural studies (Pitsikas, 2015), this present study indicates that the aqueous extract of *S. mombin* could initiate the attenuation of NO to eventually reverse an impaired learning tendency.

Oxidative stress damages the neuronal cells, impairs communication flow and cognitive function (Nwanna, *et al.*, 2016). However, consumption of phenolic-rich compounds augments the antioxidant status of the body by scavenging free radicals, and chelates metals as a strategic means of mitigating oxidative stress in neurodegenerative diseases (Odubanjo *et al.*, 2017). The MDA reducing effect of the aqueous *S. mombin* leaf extract can be attributed to the phenols, tannins, saponins and flavonoids present in the plant as documented by Nkaa *et al.* (2016). In agreement with a previous study by Shodehinde *et al.* (2016), plants phenolic constituents' act as antioxidants capable of preventing the production of free radicals and/or scavenging radicals produced in the body. Hence, the presence of these phenolic-rich compounds in *S. mombin* leaf extract confers a medicinal property that inhibits the formation of lipid peroxidation and prevent the destruction of cellular membranes.

Superoxide dismutase (SOD) is an important endogenous enzyme that catalyzes the dismutation of superoxide radical into ordinary molecular oxygen and hydrogen peroxide, thereby reducing the likelihood of cellular damage caused by reactive oxygen species (ROS) (Celino *et al.*, 2012). The *in vivo* antioxidant assays as presented in this study showed that *S. mombin* extract increases the activity of superoxide dismutase (SOD). The increased activity of SOD as observed in this study suggests that the extract has an *in vivo* antioxidant activity and is capable of ameliorating the effect of ROS in biologic system (Manonmani *et al.*, 2005; Bakirel *et al.*, 2008). Hence, the positive effect of the extract on *D. melanogaster* SOD activities implies that the antioxidant system of the flies is being optimized to ameliorate oxidative stress.

Inhibition of acetylcholinesterase (AChE) activity has been accepted as an effective treatment/management strategy against AD (Sharma, 2019). The determined acetylcholinesterase (AChE) inhibitory activity in this present research (Fig.4) agrees with some earlier reports where phytochemicals from *Citrus medica*, *G. biloba*, and *S. lavandulaefolia* plant inhibited acetylcholinesterase (AChE) and showed a significant improvement in cognitive performance and memory (Mazza *et al.*, 2006). The presence of flavonoids and phenolics in the extract contributes to its ability to lower cellular oxidative stress and also inhibit Acetylcholinesterase activities (Adefegha *et al.*, 2015). This result also corresponds with the work of Oboh *et al.* (2010) who reported that red and white ginger inhibited Acetylcholinesterase (AChE) activity *in vitro*. Hence, the AChE inhibitory property of *S. mombin* leaf aqueous extract could be suggested to be responsible for its therapeutic use in traditional/ayurvedic medicine.

## CONCLUSION

This present study reveals the potentials of *S. mombin* leaf aqueous extracts as a natural antioxidant with the presence of secondary metabolites that impair the destruction of the components of cellular membranes. The aqueous extracts of *S. mombin* leaf in this research showed a promising effect by reducing lipid peroxide formation (MDA level), NO free radicals formation and reduction in acetylcholinesterase activity. The observed increase in SOD concentration in *D. melanogaster* depicts an improved antioxidant system, which could be attributed to the presence of phenolic rich compounds of the extract. Thus, the result of this study on *S. mombin* could be further explored as an economical therapeutic approach in the management of Alzheimer's disease due to its nutraceuticals potentials as well as its ability to prevent oxidative stress-induced neurodegeneration.

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