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Full length Research Article

Virgin Coconut Oil-Supplemented Diet Reverses Behavioural Phenotypes of Sodium Benzoate-model of Acetylcholinesterase Dysfunction and Cognitive Impairment: Role of Nrf2/NfKb Signaling Pathway

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Summary: The effect of virgin coconut oil (VCO)-supplemented diet on sodium benzoate (SB)-induced neurotoxicity in male Wistar rats was investigated. Twenty (20) male Wistar rats weighing 160-180g were divided into four (4) groups: Control which received 1ml /kg b.w. of normal saline, SB-treated received 200 mg/kg b.w, SB + Low Dose VCO-treated (SB + 5% VCO mixed with 95g of rat chow), and SB + High Dose VCO-treated (SB+ 15% VCO mixed with 85g of rat chow). The brain was removed, homogenised and centrifuged for NRF-2, NF-kB, and acetylcholinesterase (AchE) gene expression levels. Also, the blood sample was collected and centrifuged for assessment of superoxide dismutase (SOD), catalase (CAT), and IL1B levels. One-way ANOVA and Tukey post hoc tests were used to analyse data. SB-treated rats with no intervention showed anxiety-like behaviour and impaired memory as depicted by a significant ($p<0.0001$) increase in anxiety index, brain NF-KB, serum IL1B and AchE gene expression level, decreased in the recognition ratio, spontaneous alternation performance, CAT and SOD levels, and NRF-2 expression level when compared to other groups (especially control and SB + 5% VCO). VCO-supplemented diet (both 5% and 15%) significantly ($p<0.0001$) increased the CAT and SOD levels, increased the NRF-2 gene expression level, and significantly ($p <0.0001$) decreased the IL1-B level. Moreover, 5% VCO significantly ($p<0.0001$) decreased the anxiety index, decreased AchE and NFkB gene expression levels, increased spontaneous alternation performance, and increased recognition ratio compared to 15% VCO. VCO showed a neuroprotective effect in attenuating cognitive impairment and anxiety-like behaviour in SB-induced model by modulating oxidative stress and inflammatory pathways, and also enhancing cholinergic neurotransmission.

Keywords: *Virgin coconut oil; sodium benzoate; acetylcholinesterase; catalase; superoxide dismutase; oxidative stress*

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INTRODUCTION

Sodium benzoate (SB) (C_6H_5COONa) is commonly utilized to preserve food and as an antimicrobial substance in many drinks. Moreover, it is found in a variety of other products, including pharmaceuticals and shampoo (Saad *et al.*, 2005). SB is used to treat acute hyperammonemia in people with urea cycle abnormalities (Brahmachari *et al*., 2009), multiple sclerosis, and acute liver disease (Yadav *et al*., 2016). The FAO/WHO expert council on food additives considers a daily intake of 5 mg/kg body weight as an acceptable quantity of SB (Nair, 2001). However, the safety of food additives has been established, and this substance is generally regarded as safe (Lennerz *et al*., 2015), although excessive consumption of these preservatives may pose a risk to consumers. Because SB is joined with glycine in the liver and removed as hippuric acid in the kidney, investigations have recently revealed that increasing use of SB causes alterations in serum clinical markers, indicating liver damage (Oyewole *et al*., 2012). Furthermore, SB has been shown to cause nephrotoxicity, neurotoxicity, and teratogenicities in zebrafish larvae during early development (Tsay *et al.*, 2007).

In addition, a significant body of knowledge has emerged indicating the potential negative consequences of SB when used to preserve food (Beezhold *et al.*, 2014; Yetuk *et al*., 2014; Khoshnoud *et al*., 2018; El-Shennawy *et al*., 2020). The use of SB has been linked to oxidative stress,

memory loss, anxiety, motor impairment, testicular inflammation, and apoptosis in both in-vivo and in-vitro investigations (Beezhold *et al*., 2014; Yetuk *et al.,* 2014; Khoshnoud *et al*., 2018; El-Shennawy *et al*., 2020).

In a study (Anthony *et al.,* 2021), the effect of SB on increasing oxidative stress was demonstrated in the mouse brain. Increased lipid peroxidation and lower levels of the antioxidant enzyme superoxide dismutase (SOD) were reported after cells were treated with SB. It also caused the activation of apoptosis and an increased inflammation (IL-6 and TNFα) (Anthony *et al*., 2021).

Oxidative stress leads to neurodegeneration through its harmful effect via different pathways (Barnham *et al.,* 2004). Although reactive oxygen species (ROS) may not be the cause of neurodegenerative disorders, but they may increase the chances of disease development through oxidative damage and interactions with mitochondria (Liu *et al.,* 2017). Excessive ROS causes astrocytes and microglia to proliferate and engage in pro-inflammatory activities in response to oxidative stress (Solleiro-Villavicencio and Rivas-Arancibia, 2018). Moreover, ROS abnormally changes all organic molecules in living organisms, leading to protein modification and lipid peroxidation (Li *et al.,* 2013).

There is a plethora of information that oxidative stress is associated with initiation of neurodegenerative disorders (Hu *et al*., 2008; Dean *et al*., 2009). The amelioration of ROS in the brain by reduced glutathione (GSH) is a vital mechanism of neuroprotection against cognitive problem (Abdel-Salam *et al*., 2012). Hence, combating oxidative stress with strong antioxidants could yield good results in offering protection against oxidative stress and cognitive decline.

Virgin coconut oil (VCO) is gotten from mature coconut kernel by either natural method or the use of machine. In natural method, VCO is extracted from the coconut kernel by chilling and centrifugation while for the mechanical method; the kernel is heated and pressed with machine to get the oil (Ghani *et al*., 2018). VCO retains its active ingredients which include phenols and vitamin E (Choi *et al*., 2012; Hayatullina *et al.,* 2012).

In general, saturated fats make up the majority of VCO with unsaturated fats accounting for the remainder. VCO is mostly composed of the following fatty acids: stearic acid, linoleic acid, myristic acid, capric acid, caprylic acid, lauric acid and palmitic acid. It was found to be potent for the treatment of tooth decay, hair and skin problems (Wallace, 2019). Coconut oil has been proven to be good for the skin of newborns (Nangia *et al*., 2015), children (Evangelista *et al.,* 2014), and adults (Agero and Verallo-Rowell, 2004; Verallo-Rowell *et al*., 2008). Coconut oil was reported to prevent loss of protein in hair more effectively than sunflower and mineral oil when used as a pre- and post-wash product (Rele and Mohile, 2003).

VCO consists of high antioxidant flavonoids and phenolic acids, which are responsible for its positive effects in animal models (Famurewa *et al*., 2018a; Narayanankutty *et al.,* 2018). Despite the fact that studies have linked the beneficial effects of VCO to antioxidant phenolics, more research is still needed to find the significance of positive effects of VCO consumption on health. There is paucity of information on the impact of VCO supplementation on memory loss and anxiety. The effect of the oil on SB–induced anxiety like behavior and memory impairment in Wistar rats has not been reported. Hence, this study was designed to investigate the effect of VCO supplemented diet on SB-induced neurotoxicity in Wistar rats.

MATERIALS AND METHODS

Chemicals: Sodium benzoate salt was purchased from May and Baker Ltd. Dagenham, England. Other chemicals were sourced from Sigma Chemical Company, St Louis, USA.

Animals: Twenty mature male Wistar rats weighing between 160-180g were procured from the Ekiti State University, Ado–Ekiti, Ekiti-State, Nigeria. They were housed and kept in plastic cages with good aeration in the Animal Laboratory of the Department of Physiology, Ekiti State University, Ado-Ekiti, Ekiti-State, Nigeria, under standard light, feeding, and temperature conditions. The animals had unlimited access to rat feed and water throughout the experiment, which lasted 30 days. All experimental protocols were in accordance with the guidelines and standards of animal's care approved by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (1985). Ethical approval was given by the Ekiti State University, with protocol number EKSU/P100/2021/08/046.

Experimental Protocol: After acclimatizing the animals to the laboratory environment for the first fourteen days, they were divided randomly into one of the following experimental groups (n = 5 per group) and treated accordingly:

Group 1:- Control received 1 ml /kg b.w. of Normal saline only

Group 2:- Sodium Benzoate (SB) treated (SB-treated; received 200 mg/kg b.w (Oladele *et al.,* 2020).

Group 3:- Sodium Benzoate (SB) + Low Dose Virgin coconut oil-treated (SB + 5% VCO mixed with 95g of rat chow) (Famurewa *et al.,* 2018c).

Group 4:- Sodium Benzoate (SB) + High Dose Virgin coconut oil-treated (SB+ 15% VCO mixed with 85g of rat chow) (Famurewa *et al.,* 2018c).

Virgin Coconut oil-Supplemented Diet Preparation: Virgin coconut oil (VCO) was prepared as reported by Azubuike-Osu *et al.* (2021). Briefly, fresh mature coconut was procured from a market in Ado- Ekiti, Ekiti State, Nigeria. The coconuts were grated, and their tepid water was mixed and strained into a viscous paste until all creamy milk was released. The product was stored at room temperature for 48 h until adequately fermented. The following three layers were created: a creamy mixture on top, VCO in the middle, and water on the bottom. The virgin coconut oil was gently gathered and filtered with cheesecloth into a container. The supplemented diet was subsequently prepared by weighing 5% w/w VCO and 15% w/w VCO from the fresh VCO and separately added to the standard rat feed of 95g and 85g mixed thoroughly respectively (Famurewa *et al.,* 2017)

Behavioural Tests: Tests were performed in Physiology experimental room. Elevated plus-maze was carried out first followed by Y maze, both procedure were spaced

adequately (elevated plus maze between 10:30am-1:30pm and Y-maze between 5:30pm- 7:00pm) 4 hours apart. Both tests were carried out on day 28. While the Novel object recognition test was carried out on the 29th days between 6:00pm-9:00pm.

Elevated plus maze test: The animals were tested for anxiety using an elevated plus-maze on experimental day 28. This was constructed using black plywood. The four arms were elevated 80cm above the plane surface - consisting of two opposing open arms (45x10cm) and two opposing closed arms with similar dimensions (50cm wall height). On test day, each rat was placed on the maze's central platform, facing the open arm, and the rat was permitted to explore the arms for 5 minutes. A blind observer was made to record the time spent in either closed or open arms in seconds or the number of entries into the arms. Percentage time spent in open arms was calculated as total time spent on open arm/300sec x 100. Percentage entry was calculated as (total open arm entry/ total numbers entries into both arms) x 100, also applicable to the close arms. Anxiety Index (AI) was calculated as 1-[(time spent in open arms/ total time spent)+(open arm entries/total entries)]/2. Entry was assumed when the four limbs of the rat is completely inside the particular arm.

Novel Object Recognition test: Cognitive performance was examined using a novel object recognition task experimental approach. This procedure has three separate phases; the habituation phase; when the rodent can freely explore an empty test arena (70x25x50cm) for 10 minutes on experimental day 29. Next is the familiarization phase, where the rat was placed in the test chamber in which two familiar objects (Red toys of similar size and shape) were placed in the two adjacent corners of the chamber. The animals were permitted to explore the objects for 10 minutes and returned to their cages. Moreover, the last phase is performed 24 hours after the concluded phase. Now one new different toy from familiar object in shape, size, and color (Novel objects) was used to replace one of the familiar objects. The animal was permitted to explore freely for 5 minutes. The time spent examining each object was monitored and recorded with a camera placed on the chamber's roof. The recognition ratio (RA) was calculated as time spent exploring the novel object/ total time spent exploring both objects. In between tests, the chamber was cleaned with ethanol solution (20%) soaked cotton wool and then allowed to dry properly before using for the next rodent to eliminate olfactory memory.

Y maze: The short-term working memory of the animals was evaluated using the Y maze apparatus. A smooth plywood structure with three arms (25x10x75cm dimension) alternating at 120^0 between one another.
Each rat was placed in the center of the maze on an experimental day and allowed to explore the three arms for exactly six minutes. A blind observer helped record the pattern of arms entries which were initially designated as A, B, and C respectively. Normally, rats are expected to visit a relatively new arm such that it does not return to the arm it is just coming out of or the recently visited arm. In such order, the rat that has a higher visit sequence of arms A, B, and C consecutively without repetition referred to as Spontaneous alternations (SAP) indicates a better short-term memory performance. Percentage spontaneous alternation (%SAP) was calculated as (number of SAP) / (total number of arm entries – 2) x 100. Entry was assumed when the four limbs of the rat were completely inside the particular arm. The maze was cleaned between tests with ethanol solution (20%) soaked cotton wool and then allowed to dry properly before using for the next rodent.

Preparation of Serum for Biochemical Assay
After the behavioural tests on days 28 and 29, the animals were fasted overnight. On day 30, the rats were anaesthetized using intraperitoneal xylazine/ketamine (10/50 mg/kg) and sacrificed. Blood was collected through a retro-orbital sinus puncture, transferred into non-heparinized tubes, and centrifuged at 3,000 rpm for 15 minutes for biochemical analysis of serum catalase, superoxide dismutase, and interleukin 1B using appropriate kits according to the manufacturer's instructions.

Gene Expression: For each group, the two testes were longitudinally divided into two halves; the first half was used for gene expression using instructions given by the manufacturer. Firstly, total RNA isolation was done using TRIzol Reagent (ThermoFisher Scientific). Secondly, the DNA in the extracted RNA was removed by DNAse I treatment (ThermoFisher Scientific). Next, the ProtoScript® First Strand cDNA Synthesis Kit (NEB) was used to transcribe the DNA-free RNA into cDNA. Finally, polymerase chain reaction amplification was conducted using OneTaq® 2X Master Mix (NEB) using the following forward and reverse primer sets: Nrf2, HMOX-1, CAT, GSR, FSHR, LHR, AR, NFKB, TNF-a, IL-1B, and Caspase-3. The bar charts represent mean ± SEM (n = 5) values of the gene/β-Actin ratio of the gel (1.5% agarose in TAE buffer) image for each sample as computed using (Image-J). The photographs are representative snapshots of the pooled sample. The nonparametric (one-way ANOVA) test was conducted with Graph-pad Prism version 9 for statistical comparison ($p \leq 0.05$).

Gene	Forward Primer	Reverse Primer
NRF2	GTCAGCTACTCCCAGGT TGC	CAGGGCAAGCG ACTGAAATG
NFKB	CCACTGTCAACAGCAGA TGG	TTCTTCTCACTG GAGGCACC
AchE	ACGTGAGCCTGAACCTG AAG	CTCGTCCAGCGT GTCTGTG

Statistical Analysis
The groups were analysed with Graph-pad Prism version 9 for statistical comparison ($p < 0.05$) using one-way analysis of variance (ANOVA) followed by *post hoc* Tukey tests

RESULTS

Low dose virgin coconut oil mitigates anxiety–like behaviour in SB-induced neurotoxicity in a male Wistar rat: The SB animals' AI was significantly higher than the control and the low dose VCO treated groups ($p<0.0001$; <0.0001, respectively). Relative to the low dose treatment group, the high dose VCO rats spent more time in the close

arm and less time in the open arm during this procedure (p=0.0022; 0.0020, respectively). SB-only treated rats showed a statistically evident anxiety-like behavior which was reversed by the low-dose VCO treatment group.

Table 1:
Behavior evaluation of SB induced anxiety-like behavior in male rats using elevated plus maze test.

	Control	SB	SB+5% VCO	SB+15% VCO
%Time (Open)	83.5 ±3.59	5.33 ±1.60**a**	51.0 ±5.53**ab**	29.33 ±2.53**abc**
% Time (Close)	16.5 ±3.6	94.67 ±1.61**a**	49.0 ±5.5**ab**	70.67 ±2.54**abc**
% Open Arm Entry	66.54 ±9.52	10.67 ±6.11**a**	31.67 ±5.86**a**	48.33 ±3.07**b**
% Close Arm Entry	34.17± 9.34	98±6.54 **a**	68.3 ±5.86**a**	55.0 ±2.23**b**
Anxiety Index (AI)	0.26 ±0.06	0.92 ±0.03**a**	0.59 ±0.03**ab**	0.62 ±0.01**ab**

Data expressed are means±SEM, n = 5. Data were analysed by one-way ANOVA followed by Tukey's multiple *post hoc* test. a,b,c p < 0.05 vs Control, SB, and SB+5%VCO respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil); % (Percentage).

Short-term memory loss effect of SB ameliorated by low dose VCO: Table 2 shows that SB-only treated rats spent more time exploring familiar objects (p=<0.0001; 0.027; <0.0001) compared to other groups. Our low dose VCO-treated rats spent significantly more time with the novel object than the high dosage-treated group (p<0.0001). SB-only treated animals demonstrated a low recognition ratio that was reversed by only low VCO dosage treatment (p=<0.0001). Spontaneous alternation performance test, as seen in table 3, shows that SB-only treated and high dosage VCO treated rats visited the same arm (SAR) more statistically compared to control and low dose VCO treated rats (p=<0.0001, <0.0001; < 0.0001, <0.0001 respectively). This short-term memory distortion was best reverted significantly by the 5% VCO treatment option compared to our 15% VCO treatment group (p<0.0001).

Table 2:
Effect of SB on short-term memory in a male rat using novel object recognition method.

	Control	SB	SB+5 %VCO	SB+15%V CO
Ex. Familiar (Sec)	89.33 ±2.87	173.7 ±3.2^{a}	75.2 ±2.0^{ab}	167.3 ±4.5^{ac}
Ex. Novel (Sec)	156.8 ±3.2	76.5 ±4.7^{a}	159.5 ±5.7^{b}	79.8 ±3.6^{ac}
Rec Ratio (RR)	0.64 ±0.007	0.31 ±0.012^{a}	0.68 ±0.007^{b}	0.31 ±0.018^{ac}

Data expressed are means±SEM, n = 5. Data were analysed by one-way ANOVA followed by Tukey's multiple post hoc test. a,b,c p < 0.05 vs Control, SB, and SB+5%VCO respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil); Ex (Exploration time); Rec Ratio (Recognition Ratio).

VCO ameliorates the oxidative effect of SB in male Wistar rats: Orally-ingested SB caused a significant oxidative response which was evident in the rats' serum level of catalase compared to the control rats (p <0.0001). This was attenuated by both dosages of VCO treatment groups, as shown in fig 1a. A similar result was seen in fig 1b; the serum level of SOD revealed statically-raised values in both dosages of our VCO treatment compared to the SB group. Fig 1c shows the brain homogenate NRF-2 gene expression was shut down in the SB group compared to the remaining groups (p <0.0001, p<0.0001, and <0.0001 respectively) while both 5% and 15% up-regulated Nrf2 gene significantly (p <0.0001, p <0.0001).

Table 3:
Spontaneous alternation performance test in SB induce neuro-toxicity

	Control	SB	SB+5% VCO	SB+15% VCO
SAR	1.33 ±0.33	5.16 ±0.30^{a}	1.17 ±0.30^{b}	4.83 ±0.40^{ac}
SAP	13.00 ±0.73	3.17 ±0.47^{a}	10.67 ±0.61^{ab}	6.0 ±0.36^{abc}
SAP%	84.83 ±2.77	24.67 ±3.04^{a}	72.0 ±3.29^{ab}	40.33 ±2.71^{abc}

Data expressed are means±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. a,b,c p < 0.05 vs Control, SB, and SB+5%VCO respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil); SAP (Spontaneous Alternation Performance); SAR (Same Arm Return); SAP% (Spontaneous Alternation Performance ratio)

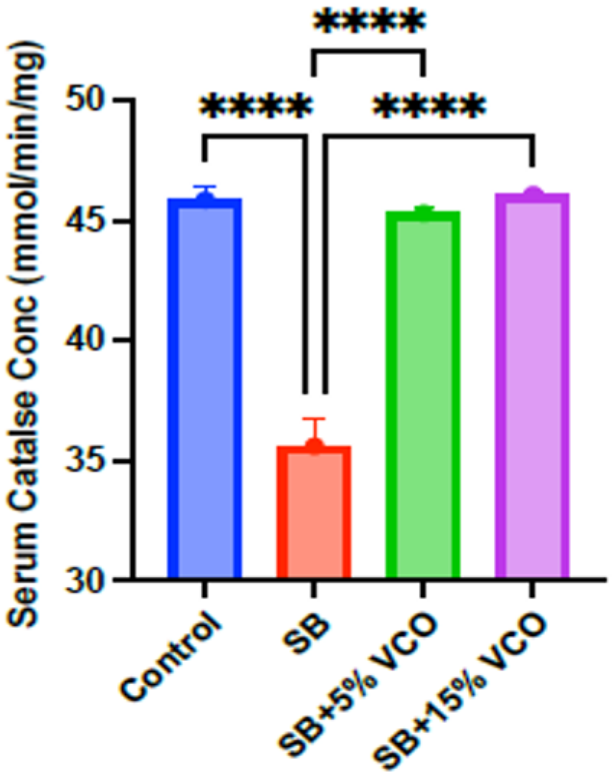

Figure. 1a
Serum concentration of catalase. Expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Tukey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil).

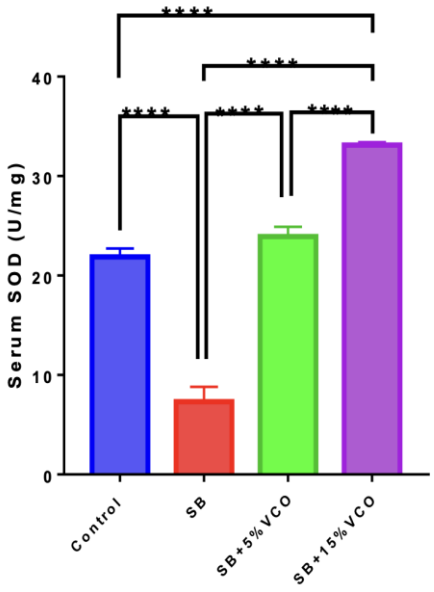

Figure 1b
Serum concentration of SOD. Expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil); SOD (Superoxide demutase)

Anti-inflammatory properties of Low dose VCO in SB-induced neurotoxicity in male Wistar rats compared to the High dose VCO: Our result in fig 2a showed that 5%VCO treated rats had a statistically lower level of serum IL-1B compared to SB and 15% VCO treated groups (p<0.0001; 0.0128 respectively). An up-stream inflammation regulator "NF-KB" mRNA expression was seen in fig.2b. A significantly upgraded expression was noted in SB treated animals compared to the control group. However, it was statistically reversed only in the 5% VCO group.

Fig. 2a shows serum concentration of IL 1B. Expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil)

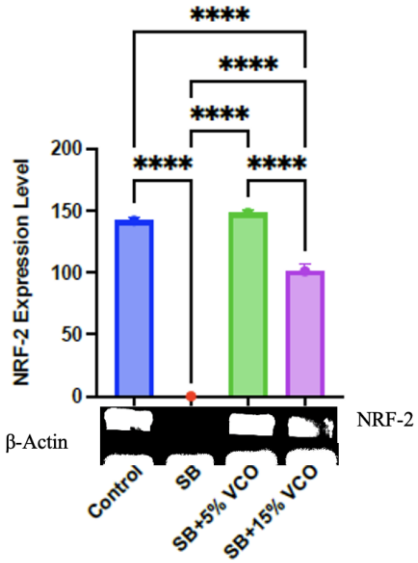

Figure 1c
Frontal brain homogenate anti-oxidative genes expressions in the Control, SB, and treatment groups. Values of quantified band from each sample for specified inflammatory gene across the 4 groups are expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. ****p< 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. The gel image is the representative snapshot of the pooled samples. (Each bar graph represents control normalized relative expression (specific gene/ β-actin). SB (Sodium Benzoate); VCO (Virgin coconut oil); NRF -2 (Nuclear factor-erythroid-related factor 2).

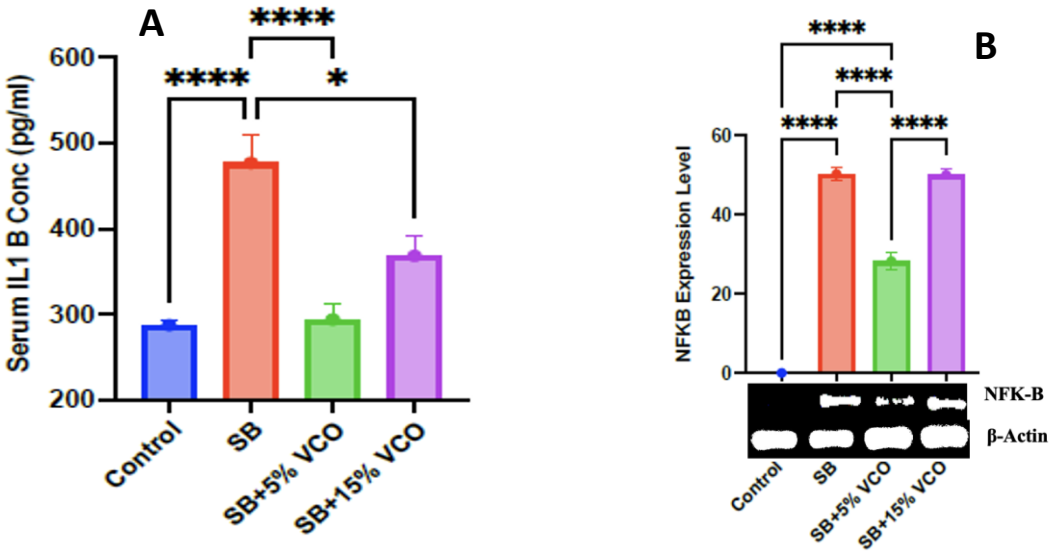

Figure 2
2a shows serum concentration of IL 1B. Expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. SB (Sodium Benzoate); VCO (Virgin coconut oil)
2b shows frontal brain homogenate inflammatory genes expressions in the Control, SB, and treatment groups. Values of quantified band from each sample for specified inflammatory gene across the 4 groups are expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. The gel image is the representative snapshot of the pooled samples. (Each bar graph represents control normalized relative expression (specific gene/ β-actin). SB (Sodium Benzoate); VCO (Virgin coconut oil); NF-KB (Nuclear Factor-kappaB).

Only 5%VCO reverted the raised acetylcholinesterase expression in frontal brain homogenate of SB induced neurotoxicity in male Wistar rats: The result from this finding showed an up-regulated AchE gene expression in SB and high dosage VCO treated groups as seen in fig. 3. This was reverted statistically by low dose VCO treatment

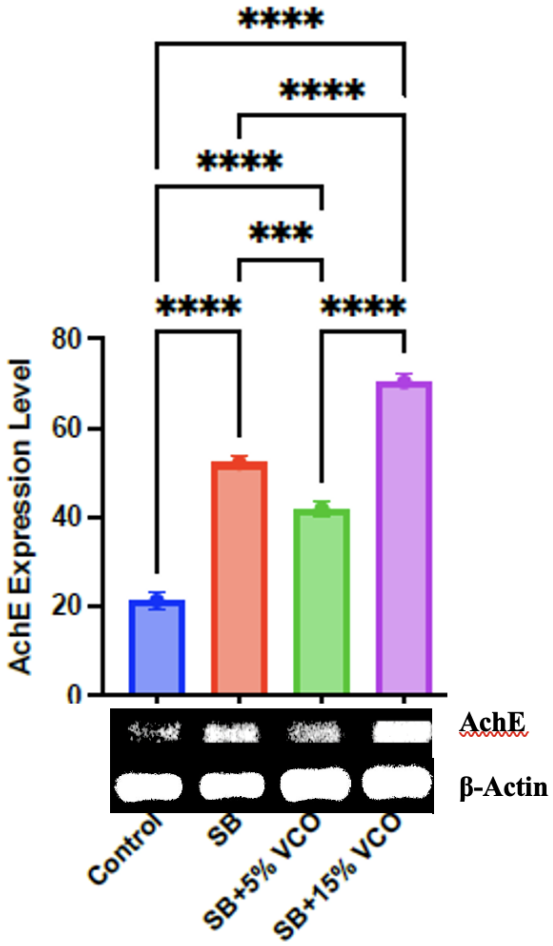

Figure 3
Frontal brain homogenate genes expressions of acetylcholinesterase in the Control, SB, SB+5%VCO and SB+15%VCO groups. Values of quantified band from each sample for specified gene across the 4 groups are expressed as mean±SEM, n = 5. Data were analysed by one-way ANOVA followed by Turkey's multiple *post hoc* test. **** p < 0.0001; ***p< 0.0003; **p< 0.001; *p< 0.05 respectively. The gel image is the representative snapshot of the pooled samples. (Each bar graph represents control normalized relative expression (specific gene/ β-actin). SB (Sodium Benzoate); VCO (Virgin coconut oil); AchE (Acetylcholinesterase).

DISCUSSION

SB, a food preservative, was found to be dangerous to cognitive functions in rats in this study, possibly through a process associated with the initiation of inflammation and oxidative stress. This discovery also revealed VCO's neuroprotective properties.

In the elevated plus-maze test, SB-only treated rats showed anxiogenic-like behaviour. Anxiety was also seen in mice given SB (Noorafshan and Karbalay-Doust, 2014). Treatment with VCO, on the other hand, considerably reduced these anxiogenic indices, demonstrating that VCO had an anxiolytic effect against anxiety induced by SB. This, however, was not dose-dependent.

On the Y maze platform, memory impairment was tested; results indicated that SB dramatically reduced the proportion of correct arm alternation, showing poor spatial memory. However, several human studies have found that taking SB improved cognitive ability in patients with neurodegenerative diseases (Lane *et al*., 2013; Lin *et al*., 2014; Modi *et al.*, 2015). Nevertheless, our finding is in tandem with two earlier rat studies that found decreased learning and memory functions in SB-treated animals (Noorafshan and Karbalay-Doust, 2014; Khoshnoud *et al.*, 2018). Furthermore, the ameliorative impact of VCO on memory impairment induced by SB suggests that VCO has a neuroprotective effect. This might be based on the possibility of VCO interfering with the arachidonic acid cascade, which is involved in memory formation (Kusnandar *et al.*, 2011). In vivo evidence has recently begun to link VCO's memory-enhancing benefits to its antioxidant qualities (Rahim *et al.*, 2017). It is important to state that a significant increase in the percentage of correct alternation between the arms by VCO in this study is not dose-dependent.

Also, in the novel object recognition test, SB-only treated animals demonstrated a low recognition ratio. This may be a result of zinc deficiency. SB can cause alteration in the brain that can make the zinc content in the brain drop significantly, and ultimately lead to changes in the rats' behavior (El-Nouby *et al*., 2009). Also, zinc insufficiency has been linked to motor function and cognitive problems, as well as anxiety and depression (DiGirolamo *et al*., 2010). This corroborates the finding of Khoshnoud *et al.* (2018), who discovered memory impairment after SB was administered orally. On the other hand, this low recognition ratio was reversed statistically ($p<0.0001$) by only low VCO dosage (5%) treatment which suggests the memory-enhancing effect of VCO. This might result from its antioxidant constituents like polyphenol and vitamin E.

SB administration caused a significant reduction in NRF-2 expression, and the activities of catalase and SOD enzymes in this study. However, 5% and 15% VCO administration reversed the pro-oxidant and antioxidant imbalance in the brain of SB-treated rats.

The memory-restoring ability of VCO is indicated by increased levels of NRF-2 gene expression, SOD, and catalase enzymes. This is in tandem with the finding of Famurewa *et al.* (2018b), who found that adding VCO to rats' diet reduced oxidative stress-mediated inflammation and neurotoxicity induced by methotrexate. In-vivo and in-vitro investigations have recently demonstrated VCO's antioxidant capacity (Famurewa *et al*., 2017; Illam *et al*., 2017). Although VCO is a saturated natural oil, its phytochemistry reveals the existence of powerful antioxidants that may be responsible for the improved antioxidant ability and subsequent anxiolytic and memory-enhancing effects shown in this study (Srivastava *et al.*, 2016).

Furthermore, shreds of evidence suggest that oxidative stress might trigger systemic inflammatory responses (Afolabi *et al*., 2012; Kelleni *et al*., 2016) As a result, the current study looked at the role of IL-1B and NF-kB in SB-

induced neurotoxicity. It was observed that SB markedly elevated serum IL-1B and NF-kB.

Oxidative stress has been shown to activate the expression of NF-kB (Hong *et al*., 2019). NFkB controls cytokines and iNOS expressions, which take part in a variety of inflammatory pathways. The translocation of NFkB to the nucleus, where it stimulates inflammatory gene expression, is mediated by its activation. NF-kB regulates the expression of all inflammatory cytokines, and higher levels of inflammatory cytokines can be linked to greater NF-kB expression. The elevation of IL-1B cytokine as a result of SB indicates that this preservative damages brain tissues and activates NF-kB signaling (Raposa, 2016).

Evidence has shown that ROS activates NF-kB (Sanchez-Gonzalez *et al.,* 2011), which causes elevated expression of IL1B, as seen in the current study. Our findings confirmed that NF-kB expression levels were significantly raised in SB-treated rats, as reported above. Our finding shows that VCO significantly reduced ($P<0.0001$) IL-1B in rats given SB and treated with VCO (5% and 15%), indicating that it reduces the pro-inflammatory effects of SB. This show the memory-enhancing and anxiolytic effects of VCO. However, this reduction was not dose-dependent as a low-dose VCO supplemented diet (5%) demonstrates relatively more potent anti-inflammatory properties in SB-induced neurotoxicity in Wistar rats. The capacity of VCO to counteract SB-induced oxidative stress, as seen by increases in SOD and CAT, could be the mechanism behind the anti-inflammatory effect. Furthermore, polyphenols from the diet are modulators of inflammatory pathways, preventing pathogenesis and producing positive health effects (Li *et al*., 2014). Polyphenols trigger an indirect mechanism for the release of IL-10 (anti-inflammatory signaling molecules) and reduction of IL-1B-induced NF-kB, thereby counteracting inflammation (Li *et al*., 2014).

The brains of SB only-treated rats have higher AchE activity, according to our findings. An upsurge in AchE activity in brain areas may affect cholinergic neurotransmission by speeding up the breakdown of acetylcholine. Acetylcholine is a neurotransmitter in the brain that plays important role in behavior, learning, and memory functions (Schmatz *et al*., 2009). Increased acetylcholine breakdown causes acetylcholine receptor downregulation, which has negative consequences on cognitive function (Teodorak *et al.,* 2015). Enhanced AchE activity has been linked to programmed cell death in-vivo and in-vitro (Zhang and Greenberg, 2012). Low dose VCO supplemented diet (5%) in this study reverted the raised acetylcholinesterase expression in frontal brain homogenates of SB-induced neurotoxicity in male Wistar rats. This shows the ability of VCO to improve cognitive functions and enhance memory. This study shows that low dose VCO (5%) demonstrates relatively more potent anti-inflammatory, anxiolytic and cholinergic properties in SB-induced neurotoxicity in male Wistar rats than in high dose (15%). This might result from some heavy metal constituents in VCO, as reported by Amit *et al*. (2022), which is more pronounced in the higher dose used for this study.

In conclusion, neurotoxicity is a catastrophic side effect of SB use, and to combat it with a natural product that could be added to one's daily diet would be appealing. It was discovered from this study that a low dose VCO-supplemented diet (5%) could successfully counteract SB-induced neurotoxicity in Wistar rats by reducing acetylcholine esterase expression, improving antioxidant mechanisms, and downregulating NF-kB cascade signaling pathways. As a result, VCO could be used as a complementary agent to combat the neurotoxic effects of SB

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