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Full length Research Article

Brain Antioxidant Status and Gene Expressions of Nicotinic and Dopamine Receptors are Improved by Black Seed (*Nigella Sativa*) Oil Administration in Cigarette Smoke or Nicotine Vapour-Exposed Rats

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Summary: Smoking is associated with dysregulation of the antioxidant system and addiction. This study sought to ascertain the effect of *Nigella sativa* (NS) oil on the antioxidant system, nicotine/tobacco addiction as well as the expressions of α4β2 nicotinic (nAChR) and dopamine type-2 (DRD2) receptors in selected brain regions of the rat. Thirty (30) male Sprague-Dawley rats were divided into 6 groups (n=5/group) comprising of vehicle-treated control, NS oil only, Smoke only, Smoke + NS oil, Nicotine only and Nicotine + NS oil. Animals were passively exposed to cigarette smoke or nicotine vapour for 12 weeks (whole body exposure, 60min/session/day), however, NS oil treatment commenced from 9th-12th week of the experimental duration. Conditioned place preference test was carried out at the start and end of the experiment. Cotinine and antioxidants levels were assessed in the plasma. Quantitative RT-PCT was used to assess gene expressions of nicotine subtypes and dopamine receptor in the brain homogenates. Nicotine vapour and cigarette smoke-induced increase in cotinine level were significantly reduced by NS treatment. Cigarette smoke or nicotine vapour exposure significantly ($p<0.05$) decreased the level of antioxidant enzymes while increasing malondialdehyde level in the brain homogenates of the rats. Administration of NS oil significantly ($p<0.05$) reversed the reduced antioxidant level. Cigarette-smoke also significantly increased α4-nAChR expression in the frontal cortex and olfactory bulb of exposed rats compared to control. Nicotine vapour significantly increased DRD2 expression only in the olfactory cortex. NS oil administration reduced both the cigarette-smoke-induced increase in α4-nAChR and nicotine vapour-induced increase in DRD2 gene expression only in the olfactory cortex. Findings from this study suggest that NS oil improves brain antioxidant status while ameliorating nicotine vapour and cigarette smoke addiction through down-regulation of α4-nAChR and DRD2 gene expressions in discrete brain regions of Sprague-Dawley rats.

Keywords*: Antioxidants, cigarette smoke; dopamine receptor; nicotinic receptor; Nigella sativa oil*

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INTRODUCTION

Tobacco addiction is the leading cause of preventable death worldwide (CDCP 2012). According to the World Health Organization (WHO 2013), smoking causes about 6 million deaths yearly. Based on previous studies, the main psychoactive component responsible for the positive reinforcing effects of tobacco use is nicotine (Hoffman and Evan 2013). Several studies have proven beyond any reasonable doubt that α4β2 nicotinic receptors (Kamens *et al*. 2013; Esterlis *et al*. 2016; Melroy-Greif *et al*. 2016) are the main receptors responsible for addiction due to cigarette smoke (Zoli *et al*. 1998; Pons *et al*. 2008) as these receptors are up-regulated during chronic exposure to nicotine and cigarette smoke (Brees *et al*. 1997; Perry *et al*. 1999).

Another gene that plays a very prominent role in addiction is the dopamine type 2 receptors (DRD2). These are G-protein coupled receptors (GPCRs) linked to the G-alpha-inhibitory (Gαi) arm of the receptor to bring about their effects on the reward pathways and addiction (Seamans and Yang 2004; Bronson and Konradi 2010). DRD2 receptors also act by inhibiting adenylate cyclase and activating beta arrestins and protein phosphatase 2A (PP2A) which inhibit protein kinase B (Akt), leading to the dephosphorylation and activation of glycogen synthase kinase 3 beta (GSK3β), a kinase involved in Wnt signaling (Cross *et al*. 1995; Beaulieu *et al*. 2009). Previous study had

earlier linked nicotine exposure to increase dopamine release in mesolimbic terminal fields to cause dependence (DiChiara 2000).

While there are several animal models of addiction, the two commonly used models are the conditioned place preference (CPP) and self-administration (SA) tests (Torres *et al*. 2008; Shram and Le 2010). Conditioned place preference, has been used to study rewarding properties of addictive substances in rodents (Le Foll and Goldberg 2005; Liu and Le Foll 2008; O'Dell and Khroyan 2009). Many smokers are on quit smoking therapies, but the success rates of these regimens remain disappointingly low. In an attempt to quit smoking, some smokers have resulted to the use of Electronic Nicotine Delivery Systems (ENDS) also called e-cigarettes (Singh *et al*. 2016). The primary aim of using the e-cigarettes is to aid quitting process in smokers (Bold *et al*. 2017; Chaffee *et al*. 2018; Leventhal *et al*. 2015; Weaver *et al*. 2015) but may also even escalate addiction to nicotine (Fong *et al*. 2019; Mathur and Dempsey 2018). There is thus, an urgent need for an intervention that would aid voluntary quitting in these individuals.

Nigella sativa L. (Ranunculaceae) oil also known as black seed or black cumin oil has been reported to possess immuno-stimulatory, anti-inflammatory, hypoglycemic, antihypertensive, anti-asthmatic, antimicrobial, anti-parasitic, antioxidant and anticancer properties (Randhawa and Alghamdi 2002; Ali and Blunden 2003; Salem 2005). Interestingly, black seed oil had been reported to be an effective non-opiate treatment for opioid dependence (Sangi *et al*. 2008). Abdel-Zaher *et al*. (2011) also, reported the inhibition of tramadol tolerance and dependence in rats following the use of *Nigella sativa* seed oil. Phytochemical analysis revealed the main active constituent of *Nigella sativa* seed oil to be thymoquinone which had been reported to be safe when used orally (Mansour 2001; Al-Ali *et al*. 2008). This study provides an insight into the mechanism(s) by which oral administration of *Nigella sativa* oil could modulate the mRNA expressions of nicotinic acetylcholine receptor (nAChR) and dopamine type 2 (DRD2) receptors in nicotine and cigarette smoke-induced addiction. The study demonstrates the potential use of black seed oil as an effective adjunct therapy to aid quitting and increase the chance of cessation in smokers.

MATERIALS AND METHODS

Experimental animals: Thirty (30) male Sprague-Dawley rats (80-100g, 4-5 weeks old) were obtained from the animal facility of the Faculty of Basic Medical Sciences, College of Medicine of the University of Lagos. Rats were fed normal rat chow ad libitum. They were housed five per cage under a 12-hour dark-12-hour light cycle in room temperature (30 ± 2ºC) and humidity (55 ± 5%) controlled animal room. Before the commencement of the study, the rats were acclimatized in the new room for a week. Ethical approval was obtained from the College of Medicine of the University of Lagos Animal Care and Use Research Ethics Committee (CMUL-ACUREC) with registration number: CMUL/HREC/08/19/568. All animal experiments complied with, and were carried out following the National Institutes of Health guide for the care and use of Laboratory animals (NIH 1996).

Study design: The chart below illustrates the selection process carried out in the choice of rats used for the study. Using computer generated numbers, Sprague-Dawley rats were randomly assigned into 6 groups consisting of: Vehicle control (Ctrl; 10 ml/kg): rats received only water ad-libitum without any cigarette smoke or nicotine vapour exposure.

Positive control (NS): NS oil (10ml/kg, p.o.) was administered for 4 weeks (9th to 12th week)

Cigarette-Smoke-exposure (SMK): passive whole-body exposure to cigarette smoke for 12 weeks, 60 min/session/day.

Nicotine vapour-exposure (NCV): passive whole-body exposure to nicotine vapour for 12 weeks, 60 min/session/day.

Nicotine vapour–exposure + NS (NCV+NS) passive whole-body exposure to nicotine vapour for 12 weeks, 60 min/session/day, then NS oil was administered for 4 weeks (9th to 12th week).

Cigarette-Smoke-exposure + NS (SMK+NS): passive whole-body exposure to cigarette smoke for 12 weeks, 60 min/session/day, then NS oil was administered for 4 weeks (9th to 12th week).

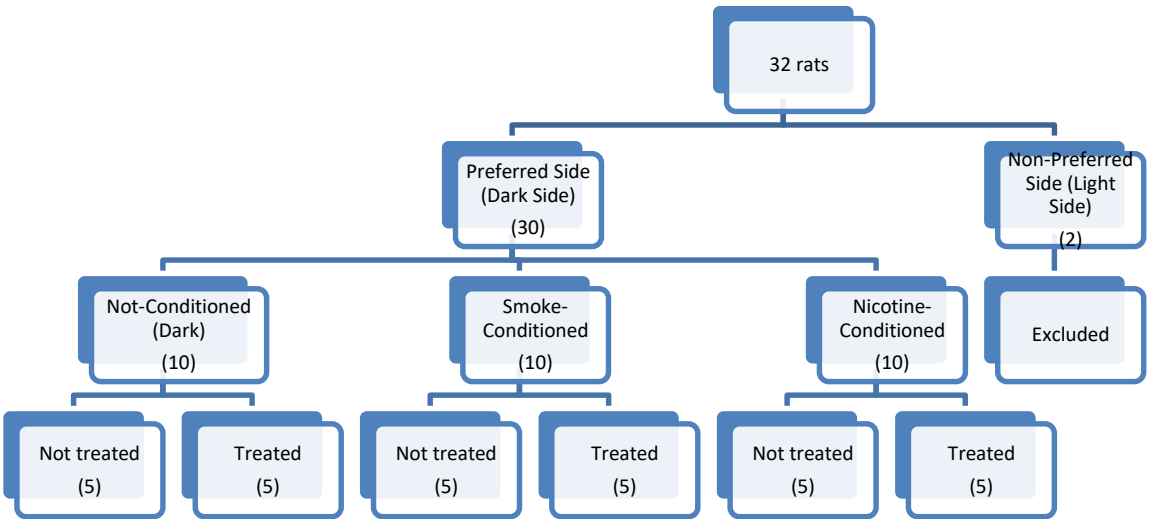

Figure 1:
Chart showing rat selection process.

Rats in groups III and IV were exposed to smoke from 3 sticks (Rothmans®) cigarette (Omotoso *et al*. 2012) daily over a period of 60 minutes for 12 weeks. The Rothmans® cigarette contained 0.738 g of tobacco and 1 mg of nicotine36 and was used because of its wide acceptability and use among Nigerian smokers.

Rats in groups II, IV and VI were administered with *Nigella sativa* (NS) oil (10 ml/kg, p.o) once daily (Kanter *et al*. 2005) during the last 4 weeks (9th to 12th week) of the experimental period.

Experimental procedures

Exposure Chamber: The conditioning chamber consisted of two Plexiglas chambers (l=40cm × b=25cm × w=30cm) each with a door (10cm × 10cm) in dimensions. The two chambers are connected by a central connecting chamber (10cm × 40cm × 10cm) which also has a (10cm × 10cm) door through which the animals were introduced into either side of the chamber. The first side was designated to be the non-preferred side and consisted of white-colored walls with a rough, black floor. On the wall of the first side is an extractor, with power rating of 1200 rpm to prevent excess accumulation of the vapour in the chamber. Also present in the chamber is a Multigas MSA detector (ALTAIR5X) containing a gas analyzer sensor which is used to ensure carbon monoxide level in the chamber is kept between acceptable 150 to 250 ppm during the exposures. The Multigas MSA detector also helped ensure the other gases like sulphur IV oxide and methane were maintained at zero while oxygen was maintained at 20.8%. The other side of the chamber was the dark side designed and designated as the preferred side. The side has a smooth floor and a small vent through which the side is aerated. For smoke exposure, the system consists of a pump (air pump) that has a direct connection to a syringe-system that blows air across the already lit cigarette. The air flow moves backward such that only filtered smoke gets into the chamber which directly mimics the kind of smoke inhaled by smokers in a passive whole-body exposure system.

For the nicotine vapour exposure, nicotine vapour was generated by bubbling air generated by an aerator (air pump) operating at a flow rate of 30 liters per minute through an Airistech vaporizer containing a solution of vaporized nicotine concentrations (80 mg/ml (FEELLiFE Vanilla Orange)) for 12 weeks (60 min/session/day) into the chamber. Nicotine vapour was produced by heating the nicotine solution to a temperature of 200ºC (392ºF) by the vaporizer. While there were other concentrations, it was observed that smokers preferred the 80 mg/ml concentration and that informed the choice of the concentration to mimic the likely effects observed in most smokers. Pure cotton wool was used to soak about 2mls of the pure nicotine and inserted into the vaporizer during each session that lasted for an hour per day for the two concerned groups of rats. Nicotine concentrations were selected putting the following factors into consideration: (1) the nicotine concentration (0-30 mg/mL) usually found in commercial e-cigarette liquids (Goniewicz *et al*. 2013), (2) companies' recommendation of e-cigarette liquids up to 60-100 mg/ml nicotine level; and (3) reported rapid metabolism of nicotine in rats compared to humans smokers (Matta *et al*. 2007). Identical chambers with controlled untreated air were used as the Control group. These chambers were either used for nicotine vapour exposure with the door closed during exposure or with the doors free to open during conditioned place preference assessment.

Conditioned Place Preference test: Conditioned Place Preference test was carried out at the start and end of the experiment using the methods of Okhuarobo *et al*. (2019) with little modifications. The preferred (dark side) and non-preferred side (light side) were first determined by the time spent in each side of the chamber by the rats and recorded as initial values. Rats that showed preference for the light side were removed from the experiment and replaced. The rats that showed preference for the dark side were divided into the different test groups accordingly. Smoke or nicotine vapour exposure was separately used to condition the rats to the initially non-preferred side (light side) and the reversal potential of NS oil tested accordingly. After the smoke or nicotine exposure that lasted for 12 weeks and the eventual application of the NS oil for 4 weeks (9th to 12th week) in the appropriate groups, the rats were placed at the central connecting chamber for free access to either of the main sides of the chamber. The rats were then left for 30 minutes, and the time spent on each sides of the chamber at the end of the 30 minutes was recorded.

Determination of Plasma Cotinine Concentration: Collection of blood samples for cotinine measurement in all groups of rats was performed on the last day of exposure. Plasma (150µL) was separated by centrifugation at 3000 rpm for 15 min and then stored at −80 °C until time for analysis. The concentration of cotinine (nicotine metabolite) was determined with Cotinine Direct ELISA (MBS580061), MyBioSource Inc., San Diego, CA 92195) according to the manufacturer's instructions.

Oxidative Stress Studies: The levels of reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and malondialdehyde (MDA) were assessed in the brain homogenates of the rats using previously described standard methods (Morakinyo *et al*., 2011). Absorbance was recorded using Shimadzu recording spectrophotometer (UV 160) in all measurements. The reduced glutathione (GSH) content of the homogenates was determined using the method described by VanDooran *et al*. (1978). The GSH determination method is based on the reaction of Ellman's reagent 5,5' dithiobis (2-nitrobenzoic acid) DNTB) with the thiol group of GSH at pH 8.0 to produce 5-thiol-2-nitrobenzoate which is yellow at 412nm. The activity of the SOD enzyme was determined according to the method described by Sun and Zigman (1978). The reaction was carried out in 0.05M sodium carbonate buffer pH 10.3 and was initiated by the addition of epinephrine in 0.005N HCl. Catalase (CAT) activity was determined by measuring the exponential disappearance of H2O2 at 240nm and expressed in units/mg of protein as described by Aebi (1984). Absorbance was recorded using Shimadzu recording spectrophotometer (UV 160) in all measurement. For the malondialdehyde (MDA), it was estimated with the method of Uchiyama and Mihara (1978) which is based on its interaction with thiobarbituric acid (TBA) to form a pink complex with absorption maximum at 535nm. Absorbance

was recorded using Shimadzu recording spectrophotometer (UV 160) in all measurements.

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR): At the end of the experiment, rats were sacrificed by cervical dislocation. The brains were quickly removed from the skull, immediately snap frozen in liquid nitrogen (-178ºC) and stored at -80ºC.

RNA extraction: Total RNA was extracted from the brain tissue with RNA extraction kit (ZYMO Quick-RNATM MiniPrep, (Cat. No: R1054, Lot No: ZRC203837)) following the manufacturer's instructions. Total RNA was quantified by measuring the absorbance at 260 nm (U-1100 spectrophotometer, Yokohama, Japan). All RNA samples were treated with amplification grade DNase 1 according to the manufacturer's instructions (ZYMO Quick-RNATM, CA) to eliminate residual DNA.

Reverse-transcription- qPCR (RT-qPCR): Complementary DNA (cDNA) synthesis and qPCR was performed in a single tube using gene-specific primers and total RNA by Protoscript First Strand cDNA Synthesis Kit (NEW ENGLAND BioLabs II E6300S/L) and LunaR Universal Quantitative PCR Master Mix (M3003) following the manufacturer's instruction. For the conversion of total RNA to cDNA, a 50-µl single-tube reaction mixture was prepared from a master mix containing 25µl of 2× reaction mix, 0.5 µg of template RNA. Then, 10 µM of each gene-specific primer pair was added to the tubes. Primer sequences were selected from the unique cytoplasmic domain region of each nicotinic acetylcholine or dopamine receptor as indicated below:

For α4:
Forward: 5'-GTCAAAGACAACTGCCGGAGACTT-3'
300 bp, 57ºC (Anneal temp.)
Reverse:5'TGATGAGCATTGGAGCCCCACTGC-3'

For β2:
Forward: 5'-ACGGTGTTCCTGCTGCTCATC-3'
507 bp, 57ºC (Anneal temp.)
Reverse: 5'-CACACTCTGGTCATCATCCTC-3'

For DRD2:
Forward: 5'-GCAGTCGAGCTTTCAGAGCC-3'
507 bp, 57ºC (Anneal temp.)
Reverse: 5'-TCTGCGGCTCATCGTCTTAAG-3'

For GAPDH:
Forward: 5'-ATGACAATGAATATGGCTACAG-3'
507 bp, 57ºC (Anneal temp.)
Reverse: 5'-CTCTTGCTCTCAGTATCCTT-3'

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to verify the quality of each RNA sample and its subsequent qRT-PCR analysis. The qRT-PCR cycling profiles using a Thermal Cycler (GeneAmp PCR System 9600; BIORAD CFX ConnectTM Real-Time System, USA) was as follows: 1 cycle at 50ºC for 30 min, 1 cycle at 94ºC for 2 min, 35 cycles at 94ºC for 1 min (46-57ºC), 72ºC for 1 min, and a final cycle at 72ºC for 7 min.

Statistical analyses: Intergroup comparisons were carried out using ANOVA to determine the level of significance. Values quantified from the expressions were represented as Mean ± SEM and Tukey post-hoc test was carried out with the significance level set at $p< 0.05$. STATA statistical software (version 13) was used for the analyses.

RESULTS

Impact of NS on rat conditioned place preference (CPP) for cigarette smoke or nicotine vapour: Figure 2 shows the time spent in the cigarette smoke or nicotine vapour-paired side of the chamber at the end of the experiment. The time spent in the drug-conditioned side was not statistically different ($p>0.05$) between the cigarette smoke-exposed group (SMK) and the control (Ctrl). Also, NS oil administration did not significantly change ($p>0.05$) the time spent in the drug-conditioned chamber between the SMK+NS group and SMK group. However, rats exposed to nicotine vapour stayed longer ($p<0.001$) in the nicotine vapour-paired side compared to the control group. The period of stay was equally longer in the NCV group when compared with the SMK group ($p<0.01$). Black seed oil administration caused a significant reduction ($p<0.05$) in the period of stay in the NCV+NS group compared to the NCV group.

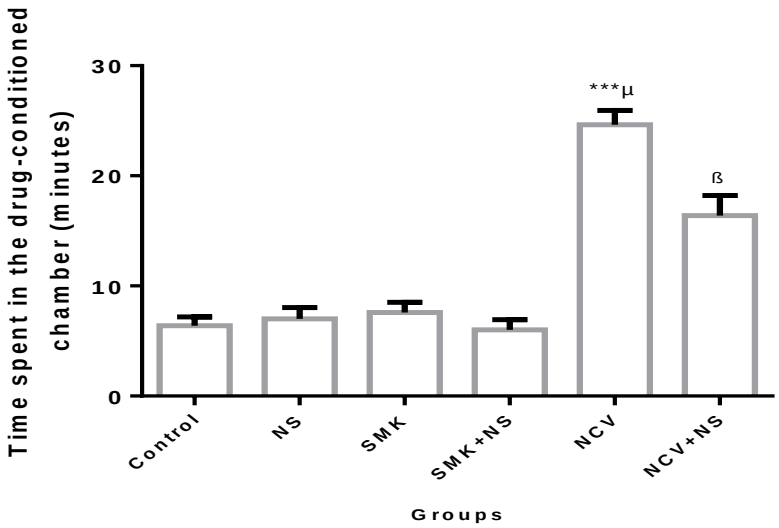

Figure 2:
Effect of treatments on conditioned place preference tests. Values are expressed as mean ± SEM (*n*=5 for each group). $^{***}p<0.001$ versus vehicle control, $^{µ}p<0.01$ versus SMK group, $^{ß}p<0.05$ versus NCV group. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

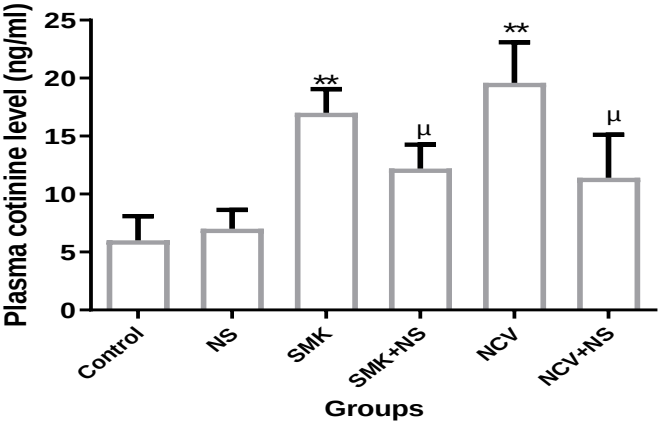

Figure 3:
Cotinine level across the group at the end of the experiment. Values are expressed as Mean ± SEM (*n*=5 for each group). **p<0.001 versus Control group, $^{\mu}$p<0.01 versus corresponding SMK and NCV groups. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

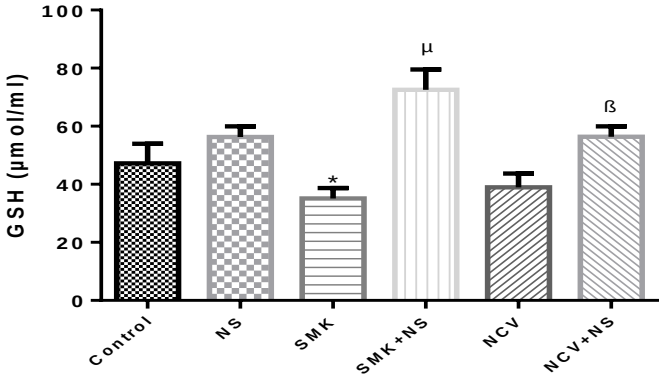

Figure 4:
Brain GSH level across the groups. Values are expressed as Mean ± SEM (*n*=5 for each group). *vs Control (*p<0.05), μ=vs SMK (p<0.0001), β=vs NCV (p<0.01). Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

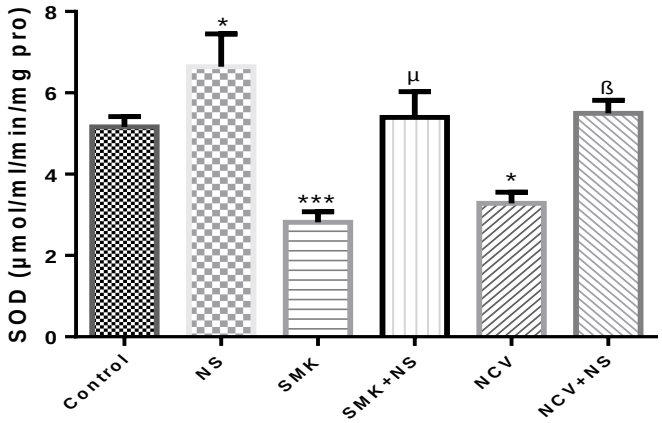

Figure 5:
Brain SOD level across the groups. Bars represent Mean ± SEM (*n*=5 for each group). *VS Ctrl (*p<0.05, ***p<0.001), μ=vs SMK (p<0.001), β=vs NCV (p<0.01). Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

Effect of treatments on cotinine level: Figure 3 illustrates the plasma cotinine level at the end of the experiment. Both cigarette smoke and nicotine vapour exposure caused a statistically significant increase (p<0.01) in the level of cotinine in the SMK and NCV groups when compared with the control group. Black seed oil administration in both the cigarette smoke and nicotine vapour-exposed groups caused a statistically significant (p<0.01) reduction in cotinine level in the SMK+NS and NCV+NS groups compared to the SMK and NCV groups respectively.

Effect of smoke exposure, nicotine vapour exposure and oil administration on GSH level: In the brain tissue, GSH level was significantly lower (p<0.05) in the SMK group (35.12±3.58 μmol/ml) compared to the Control (47.23±6.73 μmol/ml). GSH level was however significantly higher (p<0.0001) in SMK+NS group (72.55±7.02 μmol/ml) compared to the SMK group. While there was no significant difference in the GSH level between the NCV group (38.94±4.81 μmol/ml) and Control, the level was significantly higher (p<0.01) in the NCV+NS group (56.34±3.64 μmol/ml) compared to the NCV group (Figure 4).

Effect of smoke exposure, nicotine vapour exposure and oil administration on SOD level: In the brain as well, SOD level was significantly lower (p<0.001) in the SMK group (2.81±0.26 μmol/ml/mg pro) compared to the Control (5.16±0.26 μmol/ml/mg pro). SOD level was however significantly higher (p<0.0001) in SMK+NS group (5.39±0.63 μmol/ml/mg pro) compared to the SMK group. The SOD level was also significantly lower (p<0.05) in the NCV group (3.29±0.29 μmol/ml/mg pro) when compared with the Control. However, the SOD level was significantly higher (p<0.01) in the NCV+NS group (5.50±0.32 μmol/ml/mg pro) compared to the NCV group (Figure 5).

Effect of smoke exposure, nicotine vapour exposure and oil administration on Catalase level: Catalase level was significantly lower (p<0.001) in the SMK group

(12.71±3.09 µmol/ml/mg pro) compared to the Control (31.45±1.79 µmol/m µmol/ml/mg pro). Catalase level was however significantly higher (p<0.001) in SMK+NS group (29.10±4.41 µmol/ml/mg pro) compared to the SMK group. The Catalase level was also significantly lower (p<0.001) in the NCV group (15.47±2.29 µmol/ml/mg pro) when compared with the Control. However, there was no significant difference (p>0.05) in the Catalase level between the NCV+NS group (19.02±1.51 µmol/ml/mg pro) compared to the NCV group (Figure 6).

Effect of smoke exposure, nicotine vapour exposure and oil administration on GST level: GST level was significantly lower (p<0.05) in the SMK group (0.68±0.13 µmol/ml/mg pro) compared to the Control (1.52±0.04 µmol/ml/mg pro). GST level was however significantly higher (p<0.001) in SMK+NS group (3.46±0.40 µmol/ml/mg pro) compared to the SMK group. The GST level was also significantly lower (p<0.05) in the NCV group (0.79±0.06 µmol/ml/mg pro) when compared with the Control. However, the GST level was significantly higher (p<0.001) in the NCV+NS group (2.56±0.39 µmol/ml/mg pro) compared to the NCV group (Figure 7).

Effect of smoke exposure, nicotine vapour exposure and oil administration on MDA level: In the brain as well, MDA level was significantly higher (p<0.05) in the SMK group (5.70±1.03 µmol/ml) compared to the Control (2.30±0.28 µmol/ml). MDA level was however significantly lower (p<0.05) in SMK+NS group (3.86±0.24 µmol/ml) compared to the SMK group. The MDA level was also significantly higher (p<0.001) in the NCV group (6.27±1.35 µmol/ml) when compared with the Control. However, the MDA level was significantly lower (p<0.001) in the NCV+NS group (3.23±0.46 µmol/ml) compared to the NCV group (Figure 8).

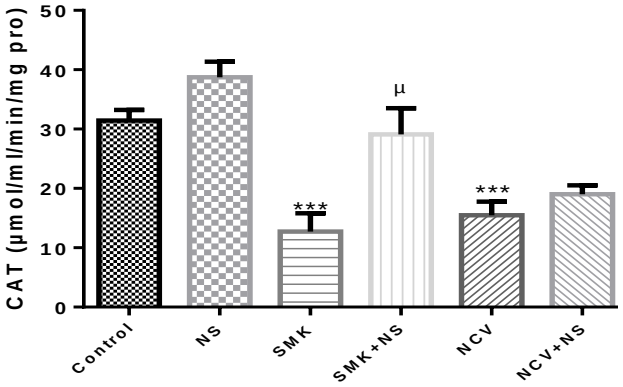

Figure 6:
Brain CAT level across the groups. Bars represent Mean ± SEM (*n*=5 for each group). *vs Control (***p<0.001), µ=vs SMK (p<0.001). Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

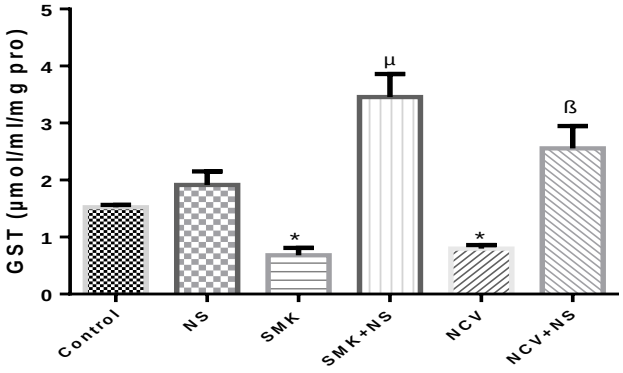

Figure 7:
Brain GST level across the groups. Values are expressed as Mean ± SEM (*n*=5 for each group). *vs Control (*p<0.05), µ=vs SMK (p<0.001), β=vs NCV (p<0.001). Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

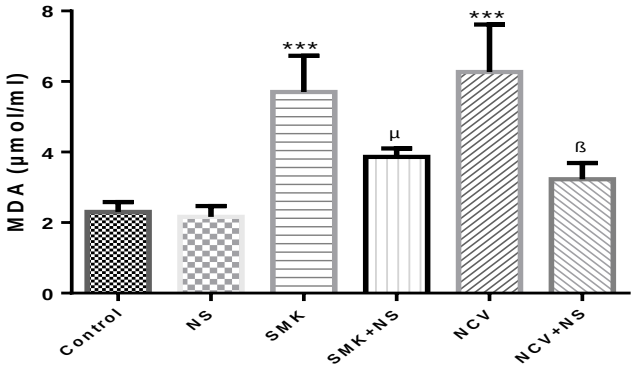

Figure 8:
Brain MDA level across the groups. Bars represent Mean ± SEM (*n*=5 for each group). *vs Control (***p<0.001), µ=vs SMK (p<0.001), β=vs NCV (p<0.001). Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil

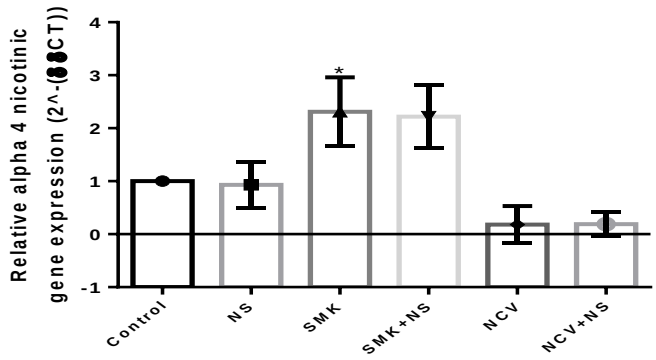

Figure 9:
Alpha-4 nicotinic gene expression in the frontal cortex. Values are expressed as Mean ± SEM (*n*=5). *p<0.05 versus Control group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil

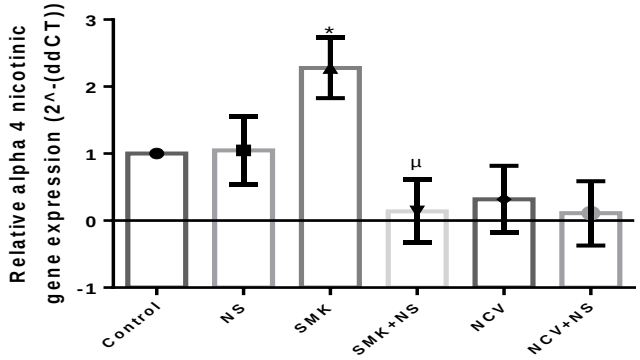

Figure 10:
α4 nicotinic gene expression in the olfactory cortex. Values are expressed as Mean ± SEM (*n*=5 for each group). *p<0.05 versus Control group; μp<0.05 versus SMK group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil

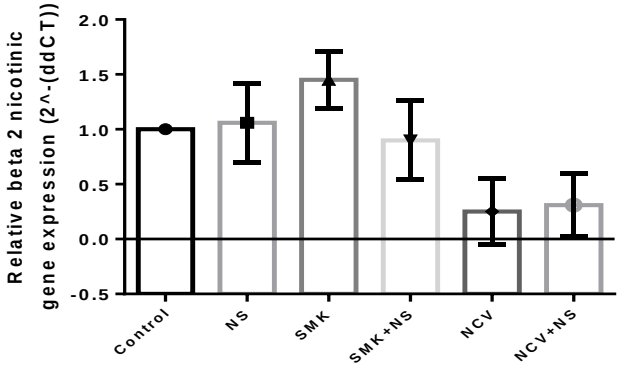

Figure 11:
β_2 nicotinic gene expression in the frontal cortex. Values are expressed as Mean ± SEM (*n*=5). Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil

Alpha 4 subunit nicotinic gene expression in the frontal cortex: As illustrated in Figure 9, cigarette smoke exposure significantly (p<0.05) increased the expression of α4 subunit of nicotinic acetylcholine receptor in the SMK group when compared with the control group. There was no statistically significant (p>0.05) difference in the expression of the subunit in the SMK+NS group when compared with the SMK group. In essence, black seed oil administration failed to alter the expression of this subunit in rats exposed to cigarette smoke. In a similar manner, nicotine vapour caused no significant change (p>0.05) in the NCV group when compared with the control. Oil administration in the NCV+NS group did not significantly alter the level of the subunit expression when compared with the NCV group.

Alpha 4 subunit nicotinic gene expression in the olfactory cortex: Cigarette smoke exposure caused the expression of α4 subunit of nicotinic acetylcholine receptor to be significantly higher (p<0.05) in SMK group when compared with the control group. Its worthy of note that the expression of the subunit was significantly lower in the SMK+NS group compared to the SMK group indicating significant suppression effect on the expression of the subunit in the olfactory cortex. Exposure to nicotine vapour in the NCV group caused no statistically significant difference (p>0.05) in the level of expression of the subunit in the NCV group when compared to the control group. Black seed oil administration also failed to cause a statistically significant change in the level of expression of the subunit in the NCV+NS group compared to the NCV group (Figure 10).

β2 subunit nicotinic gene expression in the frontal cortex: Cigarette smoke exposure caused no statistically significant change (p>0.05) in the gene expression of β2 subunit of nicotinic acetylcholine receptor in the SMK group compared to the control (Figure 11). Black seed oil administration did not significantly change the expression of the subunit (SMKNS versus SMK, p>0.05). Nicotine vapour exposure caused no statistically significant (p>0.05) change in the expression level of the subunit in the NCV group compared to the control group. Black seed oil administration also did not cause a significant change in the expression of the subunit in the NCV+NS group when compared with the NCV group.

β2 subunit nicotinic gene expression in the olfactory cortex: Cigarette smoke exposure significantly increased ($p<0.05$) the expression of β2 subunit in the SMK group compared to the control group. There was no statistically significant ($p>0.05$) difference in the expression of the subunit in the SMK+NS group compared to the SMK group even though, there appears to be a slight reduction in the gene expression of the subunit following oil administration. In essence, black seed oil administration failed to alter the expression of this subunit in rats exposed to cigarette smoke. Nicotine vapour exposure caused no significant ($p>0.05$) change in the subunit expression in the NCV group compared to the control group. Black seed oil administration caused no significant change ($p>0.05$) in the subunit expression in the NCV+NS group compared to the NCV only group.

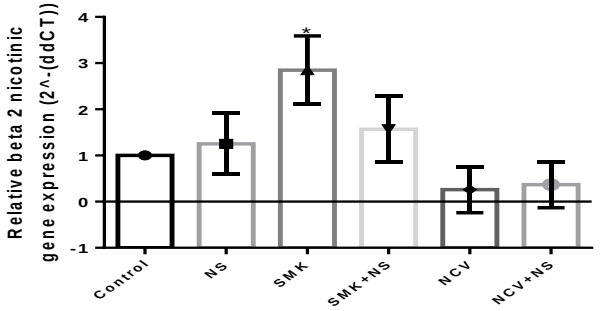

Figure 12:
Beta 2 nicotinic gene expression in the olfactory cortex. Values are expressed as Mean ± SEM (n=5 for each group). *p<0.05 versus Ctrl group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil

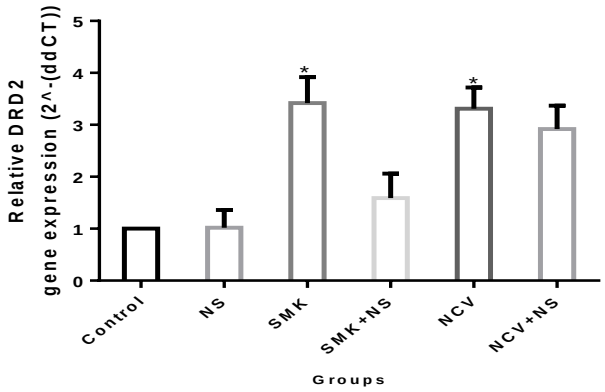

Figure 13:
Dopamine receptor type 2 gene expression in the frontal cortex. Values are expressed as Mean ± SEM (n=5 for each group). *p<0.05 versus Control group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

Dopamine receptor type 2 (DRD2) gene expression in the frontal cortex: Cigarette smoke exposure caused the DRD2 gene expression to be higher ($p<0.05$) in the SMK group compared to the control group. Black seed oil administration did not cause a significant change ($p>0.05$) in the level of expression of the subunit. Nicotine vapour exposure caused significant increase in DRD2 gene expression ($p<0.05$) in the NCV group compared to the control group. In contrast, black seed oil failed to attenuate DRD2 gene expression in the NCV+NS group when compared with the NCV group (Figure 13).

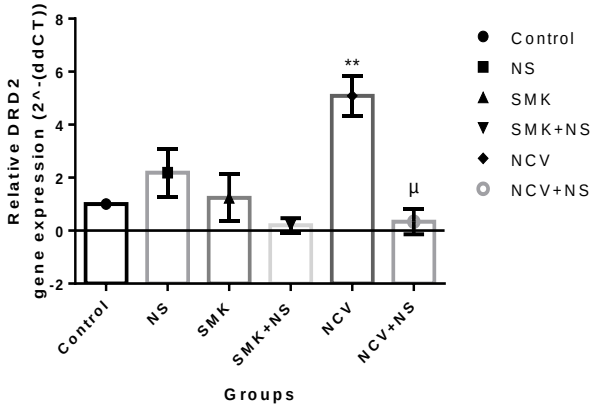

Figure 14:
Dopamine receptor type 2 gene expression in the olfactory cortex. Values are expressed as Mean ± SEM (n=5). *p<0.01 versus control group; μp<0.01 versus NCV-control group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test. Control= Control group, NS= No exposure + black seed oil, SMK= Cigarette smoke exposed group, SMK+NS= Cigarette smoke exposed + black seed oil, NCV = Nicotine vapour exposed group, NCV+NS= Nicotine vapour exposed + black seed oil.

Dopamine receptor type 2 (DRD2) gene expression in the olfactory cortex: Cigarette smoke exposure failed to cause a significant ($p>0.05$) change in the expression of the DRD2 gene in the SMK group compared to the control group. Black seed oil caused no significant change in the gene expression in the SMK+NS group compared to the SMK group. Exposure to nicotine vapour caused a marked statistically significant ($p<0.01$) increase in DRD2 gene expression in the NCV group compared to the control group. Black seed oil administration also caused a statistically significant reduction ($p<0.01$) in the level of DRD2 gene expression in the NCV+NS group when compared with the NCV group (Figure 14).

DISCUSSION

Our results did not only demonstrate the cigarette smoke and /or nicotine vapour induced-damage to the brain antioxidant systems and the gene expressions of the α4β2 nicotinic acetylcholine receptors and dopamine receptor type 2 (DRD2), it further showed the possible ameliorative effects of Black seed oil on the deranged brain antioxidant system and addictive genes expression. In this study, cigarette smoke exposure resulted in a general marked reduction in the brain antioxidant system of the animals as assessed from the brain homogenates. Our results further corroborate some earlier findings (Benowitz, 2014; Nielsen *et al*., 2000; Maritz, 2009). According to Nielsen *et al*. (2000), the degree of lipid peroxidation in the brain of the rats was observed to be high as well. In another study, Maritz (2009) reported marked reduction in the antioxidant capacity of other tissues exposed to cigarette smoke. Benowitz (2014) opined that these observations may be due to the presence of

carcinogens and heavy metals widely known to always lower the concentrations and activity of antioxidant micronutrients or the activation of vagocytic cells that generate potent oxidant species that worsen the degree of lipid peroxidation (Valavanidis *et al*., 2009).

In a similar manner, the brain antioxidant system was suppressed following exposure to nicotine vapour. The damaging effects appeared to be less intense however compared to that of exposure to cigarette smoke. This points to the fact that vaping can never be a better alternative to smoking based on its damaging effects on the antioxidant system. In support of these observations, Lerner *et al*. (2015) reported the ability of nicotine vapours to cause release of vapours that potentiate the oxidant system while suppressing the antioxidant system. Sussan *et al*. (2015) made similar observations. Beyond causing oxidative stress (Rubenstein *et al*., 2015; Ji *et al*., 2016), these vapours could therefore be responsible for the reduced brain antioxidant levels observed in this study. Consistent with these postulations, nicotine vapour and cigarette smoke had been reported to significantly increase cellular reactive oxygen species and decrease total cellular antioxidant capacity in other studies (Ganapathy *et al*., 2017; Biodi-Zoccai *et al*., 2019).

The fact that the success rates of the available quit-smoking therapies are disappointingly low makes it imperative to look for alternative drugs with smoking-quitting properties. However, the mechanisms underlying the therapeutic properties of such agents need to be unraveled. This study sought to provide the molecular basis for the possible therapeutic use of black seed oil as a smoking cessation regimen or as an adjunct therapy to aid the quitting process via modulation of α4β2 nicotinic acetylcholine receptors and dopamine receptor type 2 (DRD2) beyond its potent antioxidant effects. Our results showed that black seed oil administration could modulate the expressions of the selected genes and lower the levels of the major metabolite of cigarette smoke- cotinine. Moreso, young-adult rats were used in this study as they appeared to be more sensitive to the reinforcing effects of nicotine or cigarette smoke-induced addiction (Kota *et al*., 2007; Brielmaier *et al*., 2008).

To first ascertain addiction, we conducted a conditioned place preference (CPP) test which is one of the commonly used methods to assess the rewarding and cue-craving effects of drugs (Prokhorov, 1996; Tzschentke, 1998). The method had been shown to involve Pavlovian learning (Liu and Le Foll, 2008; Le Foll and Goldberg, 2005). What informed the choice of this method was the fact that the test is comparatively easy to perform unlike the self-administration test that requires a lot of technicalities among which is training the animals before the commencement of the study (Le Foll and Goldberg, 2005). In this study, all the rats initially preferred the dark side of the testing apparatus. This observation agrees with earlier studies (Biala and Budzynska, 2006; Brielmaier *et al*., 2008; Le Foll and Goldberg, 2009). After exposure to either cigarette smoke or nicotine vapour, their preference shifted to the light side which hitherto was non-preferred side in the nicotine groups to indicate their attachment to the drug-paired side (Picciotto, 2003) while there was a weakened preference to this drug-paired side in the cigarette smoke-exposed groups. An assessment of the period of stay for thirty minutes (Picciotto, 2003) in the drug-paired side at the start and end of the experiment also indicated the strong attachment of the groups exposed to nicotine vapour while there appeared to be aversion in the groups exposed to cigarette smoke. Earlier studies had shown the ability of nicotine to induce conditioned place preference (Fudala and Teoh, 1985; Horan and Smith, 1997; Le Foll and Goldberg, 2005). Our conditioned place preference result is in line with these earlier results. The "biased" place preference procedure used, whereby the drug of interest is paired with the initially non-preferred side made it easy to achieve the observed place preference (Le Foll and Goldberg, 2005). The observed place aversion after the long period of exposure in the cigarette smoke-exposed group may be due to many reasons among which is the presence of numerous other compounds apart from nicotine whose potential aversive or addictive actions have not been thoroughly investigated (Carter *et al*., 2009).

In the assessments, black seed oil administration significantly reduced the period of stay in the drug-paired side. To buttress this finding, both the cigarette smoke and nicotine vapour exposed groups had higher cotinine levels while black seed oil administration caused a significant reduction in the level of this bye-product of nicotine metabolism. In essence, it's either the oil reduced nicotine metabolism, reduced nicotine reuptake or enhanced the reuptake of cotinine into other tissues or probably caused degradation of cotinine into other metabolites that we could not assay for. Another possibility is the fact that the reduction in the cotinine level could be due to the reduced exposure to the source of nicotine which would consequently reduce the amount of nicotine available for metabolism and definitely its metabolite – cotinine. Sangi *et al*. (2008) had earlier reported the potential of black seed oil to ameliorate dependence caused by opioids. The ability of the oil to improve tramadol-induced tolerance and dependence had equally been established (Abdel-Zaher *et al*., 2011). Our observations for the ameliorative effects of the oil are thus in agreement with these earlier findings. It is worthy of note that the level of cotinine which happens to be the main bye-product of nicotine metabolism was found to be reduced in the test groups exposed to cigarette smoke or nicotine vapour. The cotinine level reported in this study could be extrapolated to that of human smokers. To the best of our knowledge, this report is among the first to indicate the direct effect of the black seed oil administration on this end-product of nicotine metabolism. Considering the other therapeutic benefits of black seed oil, our results indicate the potential use of the oil to improve the cessation rate. This explains the need to account for the molecular basis of such therapeutic potential on selected genes known to be associated with addiction.

The α4β2 receptors are a group of nicotinic acetylcholine receptors mainly responsible for nicotine addiction (Zoli *et al*., 1998; Gotti *et al*., 2006). They are found in large numbers in regions of the brain concerned with addiction and their subunits have been shown to play prominent roles in the modulation of addiction (Pons *et al*., 2008; Picciotto *et al*., 2008). These subunits are usually up-regulated whenever there is exposure to nicotine from cigarette smoke (Breese et a., 1997; Perry *et al*., 1999). Our results on these subunits indicated a significant increase in the gene expression of α4 subunit in the frontal and olfactory cortices

of smoke-exposed rats. Cigarette smoke exposure also increased the β2 subunit gene expression in the olfactory cortex without significantly changing the expression in the frontal cortex of the brain. Black seed oil administration only significantly reduced the α4 subunit expression in the olfactory cortex with minimal modulatory effects in the other regions. There were wide variations in the expressions of both the α4 and β2 subunits expression in the brain areas following exposure to nicotine vapour. Therefore, the likely modulatory effects of black seed oil administration on the expression of these subunits in these areas were minimal. In line with our results, previous studies had reported increased gene expressions of the α4β2 receptors following exposure to either cigarette smoke or nicotine vapour (Marks *et al*., 1983; Schwartz and Kellar, 1983; Flores *et al*., 1992; Marks *et al*., 2011). This was the same observation made in human tobacco users and cultured cells expressing the genes after transfection with nicotine (Lomazzo *et al*., 2011; Zambrano *et al*., 2012). The observed slight but not statistically significant reduction in the expression of the β2 subunit in the smoke-exposed group following black seed oil administration is an indicator of the potential therapeutic effect of reducing addiction caused by cigarette smoke as earlier reports indicated that deletion of some of the subunits of the nicotinic receptors are associated with reduced responsiveness to nicotine (Tapper *et al*., 2007) and nicotine self-administration in mice (Pons *et al*., 2008; Picciotto *et al*., 2008). In this study, the whole oil was used, and the period of oil administration was quite short. There is every possibility that this beneficial effect of the oil could be enhanced if the active component of the oil, which is thymoquinone was used and the period of administration prolonged. However, this study remains an eye-opener to the potential of the oil to cause reduction in the expression of the genes responsible for addiction and the potential use in smoking cessation and aiding the quitting process in smokers.

Another gene of interest is the dopamine receptor type 2 (DRD2). This is a G-protein coupled receptor (GPCR) with G-alpha inhibitory properties (Seamans and Yang, 2004). The dopamine system has been implicated in addiction and reward pathways (Kauer and Malenka, 2007). The established mechanism of action of DRD2 partly involves inhibition of adenylate cyclase (Enjalbert and Bockaert, 1983) and activation of beta arrestins and protein phosphatase 2A (PP2A) which inhibit protein kinase B (Akt), leading to the activation of glycogen synthase kinase 3 beta (GSK3β) involved in Wnt signaling (Beaulieu *et al*., 2009). Cigarette smoke and nicotine vapour exposure increased the mRNA expression of the DRD2 receptor in the frontal and olfactory cortices respectively. Again, black seed oil administration significantly reduced the expression of the gene in the olfactory cortex. In line with our observations, earlier reports had indicated attenuation of nicotine self-administration following disruption of dopamine in the ventral tegmental area of the brain (Corrigall and Coen, 1991; Huston-Lyons *et al*., 1993; Koob and LeMoal, 2008). Dopamine antagonists had earlier been reported to block nicotine-induced conditioned place preference (Acquas *et al*., 1989; Vizi *et al*., 2004). The marked reduction in DRD2 gene expression in group administered with black seed oil indicates the potential use of the oil in aiding the quitting process in smokers. However, further studies would be needed to ascertain this effect. The less attraction of the rats administered with the NS oil could further explain the observed marked reduction in the mRNA expression of the DRD2 receptor.

It is necessary to add that the source of nicotine vapour used in this study is the Electronic Nicotine Delivery Systems (ENDS) which is also known as e-cigarettes. These e-cigarettes were ordinarily intended to aid smoking cessation but the results from this study signal the possibility of the vapour worsening addiction instead of ameliorating it. Other studies had reported e-cigarettes not to be effective in smoking cessation (Leventhal *et al*., 2015; Weaver *et al*., 2015; Bold *et al*., 2017; Chaffee *et al*., 2018) while others even claim it facilitates smoking and dependence (Jensen *et al*., 2015; Sifat *et al*., 2018; Kulik *et al*., 2018; El-Hellani *et al*., 2019).

In conclusion, both smoke exposure from conventional cigarette and nicotine vapour from e-cigarettes have the potential to induce addiction through mechanisms that may involve up-regulation of the gene expressions of the α4β2 subunits of nicotinic acetylcholine receptor and dopamine receptor type 2 (DRD2) in the frontal and olfactory cortices. However, nicotine vapour appears to be more addictive than cigarette smoke. Black seed oil has the therapeutic potential to reduce the expression of these above-mentioned genes especially in the olfactory cortex of the brain. Black seed oil administration is a potential novel regimen that could be further investigated in the search for agents that could be used for smoking cessation. The study demonstrates the potential use of black seed oil as an effective adjunct therapy to aid quitting and increase the chance of cessation in smokers while boosting the antioxidant system. There would be a need to further investigate these potential ameliorative effects of Black seed oil on the antioxidant system and addiction in humans.

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