Jobelyn® Supplement Lowered Neuronal Degeneration: Significance of Altered p53 and α-Enolase Protein Expressions in Prefrontal Cortex of Rat Exposed to Ethanol

Oyinbo A. Charlesa, b  Igbigbi S. Patrickb  Avwioro O. Godwinb

a Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, and b Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, Delta State University, Abraka, Delta State, Nigeria

Key Words
Alcohol use disorder · Neurodegeneration · Nutraceuticals · Neuroprotection

Abstract
Background: Alcohol-induced neurodegeneration, a consequence of chronic ethanol exposure, is a neuroadaptation that drives the progression of alcohol use disorder (AUD). Unfortunately, conventional drugs for AUDs do not prevent neurodegeneration as part of their pharmacological repertoire. Multimodal neuroprotective therapeutic agents are hypothesized to have high therapeutic utility in the treatment of central nervous system. Interestingly, nutraceuticals by nature are multimodal in mechanisms of action. Purpose: This study examined the neuroprotective potential of Jobelyn in prefrontal cortex (PFC) of a binge-alcohol rat model of AUD. Methods: Three groups of rats were fed thrice daily through an orogastric tube with 5 g/kg ethanol (25% w/v), 5 g/kg ethanol (25% w/v) plus Jobelyn (4 mg/kg body weight), and 5 g/kg of a nutritionally complete diet (50% v/v), respectively. Cytoarchitectural study of the PFC was done in slides stained with haematoxylin and eosin. Immunohistochemical analyses were performed with mice monoclonal anti-p53 and anti-neuron specific enolase (NSE) antibodies to detect the degree of apoptosis and necrosis in the PFC. In addition, the degree of tissue damage and the level of lipid peroxidation were evaluated. Results: Jobelyn supplementation significantly lowered the levels of histologic and biochemical indices of neurodegeneration, and caused an increased expression of p53 protein and a decreased expression of NSE immunoreactivity (NSE-IR). Conclusions: Jobelyn supplementation ameliorates neurodegeneration in the PFC of AUD rats by reducing the oxidative stress, reducing the NSE-IR, and by increasing the expression of cellular tumor antigen p53 in the cortical neurons.

Introduction
Excessive alcohol ingestion, characteristic of alcohol use disorders (AUDs), results in neurodegeneration, which is responsible for the cognitive and behavioral impairment that drives the transition to alcohol addiction [1]. Globally, about 80 million people have diagnos-
able AUDs, making it a major global health concern [2]. To date, renowned medications for the treatment of AUDs: acamprosate, disulfiram, and naltrexone, have been clinically unsatisfactory [2]. They targeted the psychoactive properties of alcohol; while the neurodegenerative effect of alcohol that drives alcohol-induced neurological dysfunction was not managed by these specific remedies [1]. The prefrontal cortex (PFC) is highly susceptible to alcohol-induced damage [3]. PFC deficiency is characterized by executive dysfunction such as deficits in working memory, impulse control, and decision making. They are linked with the inability to abstain from alcohol [4]. Executive dysfunction often occurs before general mental status challenges. Studies showed that chronic alcohol exposure is related to the induction of oxidative stress and neuroinflammatory mediators, which lead to neurodegeneration [5, 6]. Consistent with this hypothesis, antioxidants have been effective in reducing binge alcohol-induced neurodegeneration [6]. Neuroprotection mediated by antioxidant treatment is associated with the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells–DNA binding, reduction of cyclooxygenase-2 (COX-2) expressions, and microglial activation [6]; which support the hypothesis that neuroinflammatory signalling and oxidative stress contribute to alcohol-induced neurodegeneration [5]. Jobelyn (Health Forever Products, Lagos, Nigeria), a commercially available nutraceutical, has shown outstanding anti-inflammatory and anti-oxidative properties. Interestingly, it is on record that Jobelyn’s oxygen radical absorbance capacity value (37,622 μmol/TE/g) is the highest thus far recorded in any known plant [7, 8]. Jobelyn’s strong anti-oxidative and anti-inflammatory properties have been utilized in the management and treatment of myriad diseases ranging from cancer, sickle cell, diabetes, arthritis, infertility, and many other diseases [9]. Jobelyn demonstrated a selective COX-2 inhibition property, thus providing an effective reduction in inflammation without side effects of common prescription medications [9]. Although, multimodal neuroprotective agents are considered to have a high therapeutic utility in the treatment of neurodegenerative diseases, there is yet the dilemma of how to safely combine individuals’ conventional agents into a single agent with multimodal mechanisms of neuroprotection [10, 11]. Nutraceuticals by nature are multimodal in mechanism; they produce a vast array of diverse chemical substances in a natural form in an entity, and any search of these resources for unusual or enhanced biological properties can expect some degree of success. Hence, this study was aimed at assessing the neuroprotective potentials of Jobelyn in the PFC of rats with AUD. We evaluated PFC histology with haematoxylin and eosin (H&E) stained slides. Also, cellular tumor antigen p53 and neuron-specific enolase (NSE) immunohistochemistry were performed to detect the extent of apoptosis and necrosis in the PFC.

Methods

Jobelyn supplement capsules were acquired from the manufacturer, Health Forever Products Ltd., Lagos, Nigeria. Thirty adult male Wistar rats (240–310 g) obtained from the animal house of the College of Health Sciences, Niger Delta University, were randomly assigned to 3 groups of 10 animals per group, namely, the control group (A), the alcohol group (B), and the Jobelyn group (C). Rats were maintained on a 12 h light–dark cycle and had ad libitum access to rodent chow and water, except on the night before the start of the experiment, when they were deprived of chow. The protocols for this study (PG/06/07/9/4/94289) were in accordance with the Guide for the Care and Use of Laboratory Animals of the Animal Care and Use Committee of Delta State University.

Induction of AUD

The binge-ethanol rat model of AUD was induced as described earlier [12]. Briefly, 5 g/kg ethanol in a solution of 25% (w/v) ethanol in a diluted nutritionally complete diet (Vita Milk Ghana, 50% v/v) [13] was administered through an orogastric tube to group B and C rats. Additionally, group C rats received 4 mg/kg Jobelyn supplement (i.e., 4 mg of Jobelyn per ml of distilled water) immediately after ethanol administration. Control animals received 5 g/kg of Vita Milk (50% v/v) made isocaloric with glucose. The procedure was managed every 8 h for 4 successive days at 6 a.m., 2 p.m., and 10 p.m. Apart from the first 5 g/kg dose, subsequent doses were determined using a 6-point behavioral intoxication scale [14]. Briefly, the rats were scored according to these behavioral patterns: 0 – normal rat, 1 – hypoactive, 2 – ataxia, 3 – ataxia with dragging abdomen and/or delayed righting reflex, 4 – loss of righting reflex, and 5 – loss of righting reflex and loss of eye-blink reflex. Each of these scores is associated with a particular alcohol dose of 0–5 g/kg in reverse order. Rats in all groups were deprived of rodent chow throughout the duration of the experiment, but still had free access to water.

Brain Isolation

At the end of the 4th day, rats were sacrificed under anaesthesia. Briefly, rats for histological and immunohistochemical studies were anaesthetized with a mixture of ketamine (75 mg/kg) and diazepam (2.5 mg/kg i.p.), and were transcardially perfused with 10% phosphate buffer formal saline (0.1 M, pH 7.4) solution. After complete perfusion, rats were decapitated and their heads with the brains in-situ in respective cranial cavities were post-fixed by immersion in 10% phosphate buffer formal saline for 48 h. Additionally, the rats for biochemical analyses were anaesthetized with dichloromethane, and while fully anaesthetized, the brains were carefully and quickly excised and the prefrontal cortices isolated for biochemical investigation.
Degeneration

The prefrontal cortices were excised from post-fixed brains and transferred to increasing grades of alcohol for dehydration, cleared with xylene. They were embedded in paraffin wax overnight, serially sectioned at 5 μm thickness with a rotary microtome, mounted on a glass slide and stained routinely with H&E [15].

Histopathological Study

Randomly selected paraffin-embedded tissue slices from various groups were stained with H&E and assessed using a light microscope. The representative sections (n = 5 per group) were evaluated and injured or degenerating neurons were identified by any of these 3 criteria: intensely eosinophilic cytoplasm, loss of Nissl substance, and pyknotic nuclei/cell body shrinkage [16]. Using the method of Paterniti et al. [17], damaged neurons were counted and histopathologic change in the gray matter of the PFC was scored on a 6-point scale. No lesion observed = 0, gray matter contained 1–5, damaged/degenerating neurons = 1, gray matter contained 5–10 damaged/degenerating neurons = 2, gray matter contained more than 10 damaged/degenerating neurons = 3, small infarction (less than one-third of the gray matter area) = 4, moderate infarction (one-third to one-half of the gray matter area) = 5 and, large infarction (more than half of the gray matter area) = 6. However, as a modification, a score of 4 was given to the section that met the criterion for the 3-point score and also showed vacuolation in the neuropil. The score of all the sections from each group was averaged to give a final score for the particular group.

Cell Count

Counting was done at 400× magnification. Only cells that were clearly positive for the particular IHC labelling were counted. The counting area was 2 square mm. The score of all the sections from each group was averaged to give a final score for the particular group. Values were expressed as mean ± SEM and were subjected to statistical analysis.

Immunohistochemistry

The Avidin-Biotin Complex technique for paraffin-embedded tissue sections was used in this study [18]. Sections, 2–3 μm thick, were obtained from paraffin-embedded tissues and mounted on a glass slide for the antigen retrieval immunohistochemistry. Sections were deparaffinized and hydrated in increasing grades of ethanol. Endogenous peroxidase activity was blocked with 30% hydrogen peroxidase in methanol (20–40 min). Sections were then boiled for 15 min in citrate buffer (0.01 M). Non-specific binding was blocked with 1% bovine serum albumin TRIS solution. Subsequently, sections were incubated (2–6°C) for 12 h overnight with primary antibodies: mouse monoclonal anti-NSE and anti-p53 (Novocastra, Leica Biosystems Newcastle, UK, 1:100). Thereafter, sections were rinsed in PBS solution and incubated with the biotinylated secondary antibody (Novocastra, Leica Biosystems Newcastle, UK) and horseradish peroxidase-conjugated streptavidin (Novocastra, Leica Biosystems Newcastle, UK) for 1 h. The antigen-antibody sites were developed with 3,3′-diaminobenzidine tetrahydrochloride (Novocastra, Leica Biosystems Newcastle, UK). Sections were finally counterstained with hematoxylin, rinsed with tap water, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX.

Light microscopy examination of H&E stained representative sections of various groups showed that 6 layers of the cortex were distinguishable by characteristic neu-

Estimation of Malondialdehyde

Biochemical evaluation of cortical degeneration was determined by measuring the malondialdehyde (MDA) level in the tissue. Briefly, about 0.5 g sample of the PFC was homogenized in 9 volumes of 0.1 M ice-cold Tris buffer, pH 7.4. The homogenate was then centrifuged at 3,000 g for 15–20 min to remove debris. The supernatant was carefully decanted into a plane sterile tube and stored temporarily at −15°C until when needed. Tissue MDA level was determined in the homogenate as previously described [20]. Briefly, 0.6 ml of the supernatant was added to 3 ml of glacial acetic acid in a test tube followed by the addition of 3 ml of 1% thio-barbituric acid in 0.2% NaOH. The test tube was immersed in a boiling water bath for 15 min and then allowed to cool. The absorbance of the red colored product formed was read in a spectrophotometer at 532 nm against a reagent blank to which was added 0.6 ml of distilled water instead of tissue extract. The concentration of MDA in the sample was estimated and expressed as mmol/g wet tissue.

Statistical Analysis

The one-way analysis of variance (ANOVA) and the Tukey’s post-hoc test were used to assess the intergroup differences (GraphPad Prism 5, San Diego, USA). Values were expressed as mean ± SEM. A value of p < 0.05 was considered statistically significant.

Results

Qualitative Histological Evaluation of Cerebral Changes

Light microscopy examination of H&E stained representative sections of various groups showed that 6 layers of the cortex were distinguishable by characteristic neu-
rons. The PFC of control rats showed characteristic neurons peculiar to each layer. From layer I to layer VI in order: few scattered round-shaped neurons and spiny stellate neurons, small pyramidal neurons and numerous stellate neurons, small and medium-sized pyramidal neurons, and non-pyramidal neurons, different types of stellate and pyramidal neurons, large pyramidal neurons, and a few large pyramidal neurons and many small spindle-like pyramidal and multiform neurons. There was no neurodegeneration in the control group (fig. 1a). The PFC of the alcohol-fed rats showed degenerating neurons (red arrows) and sparse vacuolation in some areas (fig. 1b), and aggregation of inflammatory cells in some portion of the cortex (delineated; fig. 1b1). Degenerative neurons were more evident in the external granular and external pyramidal layers; neurons were generally round instead of pyramidal (fig. 1b and b1). The administration of Jobelyn attenuated these degenerative changes. The level of degenerative changes was not only mild, but the infiltration of inflammatory cells observed in the alcohol-fed rats was also not evident in the Jobelyn group (fig. 1c).

Semi-Quantitative Histological Evaluation of Cerebral Changes

The degree of neurodegenerative changes in the PFC was assessed as previously described [17]. The highest mean neurodegenerative index (NDI) value was recorded in the alcohol group and the lowest in the control group (table 1). The control group was significantly different from the alcohol group (p < 0.001) and Jobelyn group (p < 0.05). However, the extent of neurodegeneration was significantly reduced in the Jobelyn group (p < 0.05) compared to the alcohol group, which suggests that Jobelyn supplementation attenuated alcohol-induced neurodegeneration in the PFC (fig. 2).

Table 1. Degenerative indices and change in body weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>HDI*</th>
<th>BDI**</th>
<th>PBW</th>
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<tr>
<td>Control (A)</td>
<td>0.40±0.24</td>
<td>50±4.6</td>
<td>14±1.7</td>
</tr>
<tr>
<td>EtOH (B)</td>
<td>2.8±0.37</td>
<td>106±6.4</td>
<td>16±1.5</td>
</tr>
<tr>
<td>Jobelyn (C)</td>
<td>1.6±0.24</td>
<td>73±6.0</td>
<td>13±1.3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 6.

HDI = Histological degenerative index; BDI = biochemical degenerative index (lipid peroxidation); PBW = % change in body weight.

* A significant difference from B (p < 0.001); and C (p < 0.05).

** A significant difference from B (p < 0.05).

C significant difference from B (p < 0.01).
Quantitative Biochemical Evaluation of Cerebral Damage

To assess the level of cerebral damage associated with or without Jobelyn supplement, the levels of lipid peroxidation (LP) in these tissues were estimated by their respective tissue MDA concentrations. The mean level of MDA was highest in the alcohol group and lowest in the control group (table 1). Tissue MDA level is directly proportional to the extent or degree of tissue damage. The administration of Jobelyn to alcohol-fed rats lowered the mean cortical level of MDA compared to that of the alcohol group. The mean MDA level in the respective groups was analyzed for intergroup differences. The mean MDA level of the control rats was significantly different from the alcohol group (p < 0.001) and Jobelyn group (p < 0.05). However, there was also a significant difference between the Jobelyn group and the alcohol group (p < 0.05). This suggests that although Jobelyn provided neuroprotection by a reduction in the level of LP, it was, however, unable to completely inhibit the sensitivity of neurons to free radical insult.

Body Weight

The percentage change in body weight was calculated by subtracting the initial body weight from the final body weight, and expressing the change as a percentage of the initial weight. A one-way ANOVA analysis showed no significant differences in body weight among the 3 groups (table 1).

Intoxication Behavioral Assessment during Binge Alcohol Exposure

Intoxication parameters across all ethanol-treated animals were similar, with an overall mean intoxication score of 1.8 ± 0.4 on the 6-point Majchrowicz scale, which indicates that all animals were, on an average, 'hypoactive to ataxic' immediately before dosing. This level of intoxication resulted in an overall mean dose of 10 ± 0.3 g/kg/day of ethanol for all animals used. A one-way ANOVA showed no significant difference (p > 0.05) in the intoxication parameter between the ethanol exposed groups (fig. 3).

Immunohistochemistry

Cellular Tumor Antigen p53

The result of p53-IHC (fig. 4) suggests that Jobelyn suppressed cell apoptosis in the PFC of alcohol-fed rats by inducing increased p53 protein expression in the cortical neurons. We developed a 5-point semi-quantitative arbitrary scale to assess the proportion of p53 positive neurons in representative images of various groups. p53 positive neurons were counted on a 160 × 160 pixels area on the Digimizer® platform and scored: <20% =
The increased expression of NSE proteins in the alcohol-fed rats (fig. 5b) was suppressed in rats that received Jobelyn supplement (fig. 5c). Digimizer® image analyses showed that the NSE-IR in rats treated with Jobelyn does not differ significantly from that of control rats (fig. 5c). However, the NSE-IR of the alcohol-fed rats was significantly different from other groups (p < 0.05).

**Discussion**

This study demonstrates a considerable neuroprotective effect of Jobelyn in a proven rat model of AUD that produces substantial neurodegeneration in the cortico-limbic pathway. Animals treated with Jobelyn exhibited reductions in tissue destruction, oxidative stress, neuronal necrosis, and neuronal apoptosis. However, intoxication behavioral assessment with the 6-point Majchrowicz scale [14] showed that Jobelyn may not significantly alter the level of alcohol intoxication in the 4-day binge rat model.

**Neuroprotective Significance of Jobelyn's Reduction of ε-Enolase Activity**

Our results show that Jobelyn attenuates alcohol-induced neurodegeneration and prevents depletion of ε-enolase proteins in the cerebral cortex of rats after exposure to alcohol.

The dynamic relationship between morphological change and functional deficiency in the central nervous system (CNS) is well known. The loss of cortical cytoarchitecture and neurons are implicated as contributing factors in the decline of cerebral functions following an insult in the CNS [21]. In this study, we observed a significant distortion in the cytoarchitecture organization of the PFC in the alcohol group; especially in the molecular, outer granular, and outer pyramidal layers. Furthermore, most neuronal perikarya show a rounded profile as against the characteristic polygonal profile consistent with normal neurons [22]. The neuropil of rats that received alcohol without Jobelyn supplement showed mild vacuolation and aggregation of inflammatory cells, which are classic indicators of cellular stress [23]. NSE (ε-enolase) is a biochemical marker for pathogenic processes and ongoing neuronal degeneration [24]. NSE overexpression, as seen in the alcohol group of this study, has been previously correlated with an overall histological evidence of neuronal damage [22, 25, 26]. Furthermore, neurons or cells with sustained...
**Fig. 4.** Jobelyn supplement increased p53 protein expression. Representative photomicrographs of the effect of Jobelyn treatment on binge EtOH-exposed rat. p53 labeled (positive) neurons were brownish: it was negligibly expressed in control group (a), but evidently expressed in the EtOH group (b). Observe degenerating or dead neurons (white arrow) in rats that received alcohol without supplement (b). The p53 protein was overexpressed in Jobelyn-treated rats (c). The proportion of p53 positive neurons in the Jobelyn group was significantly different from those of the control and EtOH groups (p < 0.001). The proportion of p53 positive neurons in the EtOH and control groups were not significantly different (p > 0.05), although they were more p53 positive neurons in the EtOH group (p53 labeling ×400). Scale bar = 100 μm. *** p < 0.001.

**Fig. 5.** Jobelyn supplement reduced γ-enolase protein expression. NSE positive neurons displayed a brownish colouration. Observe the normal expression of NSE in control (a). Note the increased expression of NSE protein in the EtOH group (b). The number of NSE positive neurons in the EtOH group was significantly different (d, p < 0.05) from the control and Jobelyn groups. Jobelyn supplement reduced the number of NSE positive neurons (c). There was no significant difference between control and Jobelyn groups (NSE labeling ×400). Scale bar = 100 μm. * p < 0.05.
overexpression of NSE proteins are apt to lysis, which leads to cell death. Additionally, the release of NSE into the neuropil potentiates the severity of injury [27]. Hence, the death of neurons leads to the death of more neurons. Jobelyn supplementation to alcohol-exposed rats largely inhibited this destructive cascade. Though these findings are laudable in that it provided proof that Jobelyn attenuates the severity of alcohol-induced damage in AUD, nevertheless, our result also demonstrated that its neuroprotective mechanisms involve the modulations of cellular tumor antigen p53 and a reduction of oxidative stress.

**Neuroprotective Significance of Jobelyn’s Activation of p53 Protein**

This study suggests that Jobelyn inhibits cell apoptosis signaling in the neurons of the PFC through an increased expression of p53 protein. We know that overexpression (up-regulation) and reduced expression (down-regulation) of p53 protein can be either protective or exacerbating to stressed neurons [28, 29]. The dual tendency of p53 – to destroy or repair damaged DNA – in protecting the genome may be partly responsible for this observation. Cellular tumor antigen p53 safeguarded cells by the activation of DNA repair proteins in damaged or compromised DNA by suspending the cell cycle at the G1/S regulation point on DNA, thus arresting growth and allowing time for repair before continuation [30, 31]. Nevertheless, it would trigger apoptosis if DNA damage becomes irreparable [32, 33]. Though the loss of p53 function is implicated in CNS tumors, p53 overexpression or excessive function is involved in neural tube defects, neuronal degeneration, and embryonic lethality [29, 34]. Consequently, p53 activity or function is tightly controlled or regulated. Therefore, understanding the full repertoire of p53 function in the CNS development and maintenance could offer novel points of therapeutic intervention for human CNS diseases. A critical question that emerged from this study was to determine if the increased expression of p53 protein seen in the neurons of the PFC of the Jobelyn-treated rats confers protection against cell apoptosis. Our results suggest that Jobelyn supplement plausibly confers some sort of neuroprotection. First, we observed that the level of degenerating neurons was significantly less in rats treated with Jobelyn, as demonstrated by NSE labeling and NDI. Furthermore, almost all the neurons in the Jobelyn-treated group displayed an increased expression of p53 protein (fig. 3; table 2); uncharacteristic of p53 labeled apoptotic neurons in the CNS, where selective labeling is the rule for apoptotic neurons. Interestingly, Luo et al. [35] reported that pine pollen suppressed cell apoptosis in the cerebral cortex of arsenic-poisoned mice by reducing Bax, Bcl-2 protein expression and increasing p53 protein expression. Previous studies have shown that antioxidant attenuated ischemic neuronal apoptosis through Bcl-2 up-regulation and Bax down-regulation [36]. We opined that Jobelyn’s anti-apoptotic property is a critical input in minimising the neurodegenerative deficit associated with programmed cell death. The role of apoptosis in neuronal loss secondary to CNS assault is well known [37, 38]. Additionally, the high lipid and oxygen content of the CNS makes it highly susceptible to LP-related cellular damage [39]. To answer the question whether the neuroprotective effect of Jobelyn is related to its antioxidative property, we estimated the MDA level in samples of the PFC. It is well known that an increased tissue level of MDA is directly proportional to the degree of LP and hence the extent of damage [40, 41]. This study shows that Jobelyn significantly reduced the level of MDA in the tissue. We, therefore, concluded that the protective effect of Jobelyn in AUD is plausibly associated to its anti-lipid peroxidative properties. In pathological conditions in the CNS, free radicals are overproduced and overwhelm the endogenous antioxidant defences, leading to LP, which subsequently induce membrane damage, cell lysis, organelle dysfunction, and calcium dyshomeostasis [39]. Without any doubt, in the quest of combating the menace of free

<table>
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<th>alcohol</th>
<th>Jobelyn</th>
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<tbody>
<tr>
<td>Anti-NSE</td>
<td>Positive (+)</td>
<td>Increased expression (++)</td>
<td>Positive (+)</td>
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</tr>
<tr>
<td>Anti-p53</td>
<td>Barely detectable</td>
<td>Expressed in certain cells (+)</td>
<td>Expressed in almost all cells (+++)</td>
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**Table 2. Cellular response of neuron to selected antibodies in the Jobelyn treatment of binge EtOH-induced AUD**

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radical assault, it is critically important to identify agents that could provide an exogenous antioxidant source into the milieu interior of the CNS to compensate for the endogenous depletion of antioxidant. This study suggests that Jobelyn is capable of providing such exogenous source of antioxidant.

Conclusion

This study has demonstrated the potentials of Jobelyn (a nutraceutical) in the quest for a remedy for AUDs. Till date, acamprosate, disulfiram, and naltrexone, which are the approved pharmacotherapeutic interventions for the treatment of AUDs have left much to be desired [2]. Jobelyn’s strategy of preventing neurodegeneration from within the neurons safeguards them ad initium. Coupled with this, it also safeguards the neuropil by neutralising the assault of free radical. Therefore, the neuroprotective mechanisms of Jobelyn in alcohol-induced cortical neurodegeneration involve an increase in p53 protein expression, a decrease in \(\tau\)-enolase proteins expression, and an alleviation of oxidative stress.

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This article complies with International Committee of Medical Journal editor’s standard requirements for manuscript.

Authors’ Contributions

O.A.C. was involved in the conception, design, animal experimentation, tissue analysis (biochemical, histological, and immunohistochemical), and interpretation of data, statistical analysis, and manuscript write-up. I.S.F. participated in the design, immunohistochemical analysis, interpretation of data, and manuscript revision for significant intellectual content. A.O.G. participated in the design, histological and immunohistochemical analysis, interpretation of data, and manuscript revision for significant intellectual content.

Disclosure Statement

The authors declare that they have no competing financial/ non-financial interests.

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