The Potential Role of Carotenoid Pigment Isolated from a New Rhodotorula Species in Ameliorating Cerebral Ischemic Stroke Experimentally

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ABSTRACT

Objectives: Natural antioxidants particularly carotenoids have been associated with a lowered risk for stroke and cerebrovascular diseases. The present study proposes a new microbial source of carotenoid-rich bio-pigment and investigates its potency in mitigating ischemic stroke in an animal model.

Methods: A yeast isolate rich in carotenoid pigment was defined morphologically and physiologically, recognized by 18S rDNA as Rhodotorula mucilaginosa G20 with a similarity of 100%, then submitted to Gen Bank (accession number KY271337.1). The extracted pigment was analyzed using high-performance liquid chromatography in vitro. Furthermore, an in-vivo rat model was assessed to investigate the effect of the extracted pigment against Endothelin-1 (ET-1) induced focal cerebral ischemia. Rats were assigned into four groups: Group (1) normal control rats received saline. Group (2): sham-operated rats received saline, Group (3): rats received ET-1.Group (4): rats received extracted pigment and ET.

Results: HPLC results revealed that the extracted yeast pigment consists mainly of neoxanthin and β-carotene. In vivo, pretreatment with extracted pigment significantly enhanced the grip strength, restored impaired vertical forelimb use induced by ET-1 in rats, halted oxidants biomarkers, and triggered antioxidant mediators. Moreover, it suppressed inflammatory and apoptotic markers. The histological and structural deterioration in the ventral subiculum was deterred.

Conclusions: Based on these encouraging results, The extracted microbial carotenoid pigment production can be considered efficient and economical and proves to be a promising approach for ameliorating cerebral ischemic injuries.

Key Words: Carotenoid, Endothelin, Neoxanthin, Oxidative Stress, Rhodotorula, Stroke

INTRODUCTION

Many synthetic coloring agents were banned by the U.S. Food and Drug Administration (FDA) and the European Food Standards Authority (EFSA) due to their toxicological problems and carcinogenicity. Efforts were made to produce natural pigments not only for their role in industries like cosmetics, food, textile, and plastic but also in medical uses for their antioxidant, anti-inflammatory, anticancer, and anti-microbial activities. Micro-organisms are a promising natural color source besides; they are versatile tools in biotechnology. Many bio-pigments were produced using microorganisms including carotenoids, melanins, flavins, or quinines. Carotenoids are the most prevalent natural pigment with many significant biological activities and manufacturing applications. A growing interest in this class of pigments is directing the search for new. Rhodotorula sp., Blakeslea trispora, Phaffia sp. Streptomyces chrestomyceticus, Flavobacterium sp, and Phycomyces blakesleeanus are acknowledged for producing carotenoids. Rhodotorula yeast strains are efficient in utilizing the whole biomass and produce a high yield of carotenoids. The pharmacological value of carotenoids in many diseases involving cardio- and cerebrovascular diseases is due to their lipid-soluble anti-oxidant nature. Stroke is a major cerebrovascular disorder leading to acquired adult disability and death. Only a minority of patients suffering from acute ischemic stroke benefit from thrombolytic intervention with recombinant tissue plasminogen activator since it must be taken within the first 5 hours of ischemic...
injury onset. Stroke mostly results in dysfunction with a permanent or reversible neurological deficit. Infiltration of inflammatory cells, oxidative molecules, excitotoxicity, and apoptotic mediators through ischemia-impaired blood-brain barrier lead to lesion progression and eventual neuronal cell death. Dietary intake of antioxidants is considered a modifiable risk factor that can reduce the severity of injury after an ischemic stroke. Neuroprotective treatments during or following exposure to ischemic stroke interrupt the cellular, biochemical, and metabolic elaboration of injury and ameliorate brain injury. Several studies revealed the beneficial capability of carotenoids such as α- and β-carotene, zeaxanthin, lycopene, and lutein on suppression of stroke risk.

The present study utilized Rhodotorula species for the production of carotenoid-rich pigment which can be applied in clinical therapeutics. The produced pigment was investigated biologically for its neuroprotective impact on the brain ischemic injury rat model. Focal injection of endothelin (ET)-1 is considered a reproducible model of ischemic stroke. Modulating therapeutic effects were investigated behaviorally, biochemically, and histopathologically.

**MATERIALS AND METHODS**

**Drugs**

Endothelin-1 powder ≥97% was obtained from Sigma-Aldrich (St. Louis, MO, USA) (Lot#102M4762V). Ketamine, xylazine, astaxanthin, α- and β-Carotene, lycopene were purchased from Sigma-Aldrich Fine Chemicals (Zeaxanthin, Violaxanthin, Antheraxanthin, Neoxanthin) were obtained from Fluka (Buchs SG, Switzerland).

**Isolation and identification of carotenogenic yeast**

The isolated yeast strain that produces carotenoid, was isolated from the surface of cold yogurt by the particle plating method. The isolated yeast was identified by determining its morphological properties. Also, The produced pigments and shape of colonies of yeast were examined.

Yeasts biomass was collected from grown YPD cultures. One ml of broth was centrifuged at 10,000 rpm for 10 min. DNA was extracted using a DNA extraction Kit and its quality was evaluated on 1.2 % agarose gel where a single band of high Mw DNA was observed. The extracted DNA was used as a template for phylogenetic analysis. The 18S rDNA gene was PCR-amplified using ITS1 primer (5'-TCCGTAGGTAGACCTGGG-3') and ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3'). Then, partial sequencing was performed at Macrogen Company, Korea. Nucleotides sequences were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/) by using the BLASTN program followed by a sequence alignment. A phylogenetic tree was constructed using the Neighbor-Joining algorithm version 5.2 of Molecular Evolutionary Genetics Analysis (MEGA) and 1,000 re-samplings.

**Production of carotenoid-rich pigment**

Carotenoids were produced using yeast malt broth medium with the following ingredients: yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l, glucose 10 g/l at pH 5. The medium was sterilized at 121°C for 20 minutes. The production of cell biomass was performed in three successive steps. Firstly, 500ml of yeast malt broth medium was inoculated with yeast cells (OD 0.5 at 610 nm) and incubated for 5 days at 30°C and 160 rpm. At the end of the incubation period, the entire 500ml production medium was added to 4.5L of yeast malt broth medium and incubated under the same conditions. Finally, a total of 5L was used for inoculation of 45L of sterile yeast malt broth medium in a 75L total volume bioreactor. The culture was incubated at 30°C for 5 days with aeration at 0.2 v/v/m and 200 rpm agitation speed.

**Extraction of bio-pigment**

Yeasts were harvested by continuous centrifugation using CEPA continuous centrifuge. The cells harvested from the 50L production medium were hydrolyzed with (1N) HCl for one and half hours in a water bath at 70°C. Excess acid-washed away with water and then cells get immersed in acetone: methanol (1:1) overnight. The pigment was extracted from the cells using acetone until all color was removed. The extract of acetone was allowed to dry out in the air.

**HPLC analysis of carotenoids extract**

Extracted carotenoids were dried and concentrated under nitrogen and re-dissolved in methanol. 10 μL of the carotenoid extract was analyzed using reversed-phase HPLC (C18; 25 cm×4.6 mm, 5 μm) and isocratic mobile phase composed of (dichloromethane: acetonitrile: methanol) (20:70:10, v) at a flow rate of 1.0 ml/min. Peak recognition and λ max values of these components were accepted by their retention times and standard chromatogram characteristic spectra put down with a Shimadzu SPD-10AVP photodiode array detector (Shimadzu, Japan) the spectra were recorded from 400-600 nm scan range. The mass spectra were obtained using 130°C heated source APCI, and the probe was held at 500°C. The voltages were optimized for corona (5 kV), HV lens (0.5 kV), and cone (30 V). At 100 and 300 L/h, respectively, nitrogen was used as a sheath and drying gas. In positive mode, the spectrometer was calibrated, and [M+H]+ ions were captured. Mass spectra of carotenoids with m/z 400-700 were acquired and verified with respective standards.

**Animals**

40 adult female Wistar rats weighing 160±20g (NRC, Egypt) were used in this study. Rats were kept in plastic wire-
Experimental design
Animals were assigned into 4 groups (n=10). Group 1 (normal control group) rats received saline (1 ml/kg, i.p/day for 14 days), Group 2 (sham group) rats received saline (1 mg/kg, i.p/day for 14 days) followed by saline intracerebroventricular (4 μl, ICV, once). Group 3 (Induced group) rats received saline (1 mg/kg, i.p/day for 14 days) followed by ET-1 (4 μl, ICV, once). Group 4 (Bio pigment group) rats were pretreated with carotenoid extract (50 mg/kg, i.p/day for 14 days) followed by ET-1 administration.

Twenty-four hours after ET injection, rats were behaviorally examined for grip strength and activity in cylinder test. Then rats were euthanized, decapitated, and brains were harvested and hippocampi were dissected for biochemical and histological examination.

Endothelin-1 (ET-1) induced cerebral ischemia
All rats were anesthetized with a ketamine/xylazine cocktail (1 ml/kg i.p.). The skin above the skull was sterilized using a 5% povidone-iodine solution. Each rat was mounted on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). At midline, an incision was made to expose the skull. A hole was drilled above the intended lesion site through the skull using a dental burr. The coordinates for the injection were determined based on the rat brain atlas. 20 AP-2.7 mm, ML +3.2 mm, and DV -3.1 mm. (4 μl) 100 pmol/l of ET-1 solution in sterile saline, at the rate of 0.5 μl per minute was delivered. A 10 μl Hamilton syringe was lowered into place and allowed to remain undisturbed. In sham control animals, saline solution was applied instead of ET-1 solution and the same procedures were followed.

Neurobehavioral assessment:
At the end of the experimental period, motor behavioral impairment for all rats was assessed using the wire hanging test and the cylinder test.

Wire hanging test
The wire hang test was carried out to evaluate neuromuscular strength. Each rat was placed with its forelimbs on a 20 cm long wire adjusted horizontally 50 cm above the surface. Latency time to fall was recorded. 30 seconds cut-off time was taken. 21

Cylinder test:
The cylinder test is used to measure locomotor activity and behavioral deficits in the rats by calculating rearing frequency. The spontaneous movement was estimated by inserting each rat in a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm) for 5 min. The number of rears was recorded after each treatment. A rear was counted when an animal made a vertical movement with both forelimbs removed from the ground. 22

Biochemical analysis:
Oxidative stress biomarkers
Lipid peroxidation was evaluated by estimating thiobarbituric acid reactive substances (TBARS) in brain tissues as per. 23 Catalase (CAT) and superoxide dismutase (SOD) activities were estimated following 24,25 respectively. Bioassay for xanthine oxidase (XOD) activity was performed according to Gulec et al. 26 Glutamine Synthetase (GtSx) activity was evaluated as per Sunil et al. 27 and tyrosine hydroxylase (TH) activity was determined using enzyme linked-Immunosorbent technique using (ELISA) kit provided by BioSource, Inc. San Diego, USA.

Brain inflammatory and apoptotic mediators
Matrix metalloprotein-9 (MMP-9), Nuclear factor Kappa B (NF-κB), and Caspase-3 and Nuclear factor erythroid -2 (NRF-2) were estimated using ELISA kits provided by BioSource Inc., USA according to the manufacturers’ instructions.

Statistical Analysis
Data were displayed as mean±SEM. All experiments were tested for significance using Tukey’s post hoc analysis following one-way ANOVA. Differences were considered significant at p<0.05.

RESULTS
Phylogenetic Analysis and Identification of Carotenogenic Yeast
The isolated yeast strain G20 has rapid growth and the ability to produce a high quantity of pigments. G20 isolate has a mucous, smooth surface and a color that ranges from orange to red on the YPD agar plate. Cells were oval under the microscope. (Figure 1)

The ITS sequence obtained from isolated G20 strain was compared with the sequences in the GeneBank database and presented that the G20 strain possessed 100% similarity to Rhodotorula mucilaginosa. The nucleotide sequence was placed under the KY271337.1 accession number in the GeneBank (http://www.ncbi.nlm.nih.gov). The phylogenetic tree
of *Rhodotorula mucilaginosa* KY271337.1 was constructed with closely related sequences accessed from the GenBank as shown in Figure 2.

**HPLC-APCI-MS analysis of the carotenoid extract**

The results revealed that the extracted yeast pigment consists mainly of neoxanthin (40%) and β-carotene (30%), according to retention time and absorption spectra as compared to respective reference standards. Neoxanthin was eluted at 2.66 min, β-carotene was eluted at 3.47 min. Positive ion mass spectra were obtained for the peaks of maximum absorption and [M+H]$^+$ dominant for neoxanthin was 601.2 of and fragments of 509.1 [M+H-18]$^+$, and 491.2 [M+H-18-92]$^+$ The mass spectra for β-carotene was mass peak of 537.2 [M+H]$^+$, 519.2 [M+H-18]$^+$, and 445.2 [M+H-92]$^+$.

**Neurobehavioral assessment**

**Wire hanging test:**

As indicated in table (1), ET-1 treated rats exhibited a shorter latency period in the wire hanging test as compared to the sham group. Pretreatment of ET-1 rats with carotenoids extract almost normalized the latency period as compared to the control and sham group.

**Cylinder test**

Data presented in table 1 revealed that ET-1 treated rats had impaired rearing frequency versus sham group, whereas, pre-administration of ET-1 rats with carotenoids extract restored normal rearing frequency as compared to control and sham group.

**Oxidative stress status**

The current data demonstrated a significant elevation in brain MDA level following ET-1 induced ischemia (1.61±0.15 nmol/mg protein) as compared to the sham control group (0.75±0.03 nmol/mg protein). Meanwhile, pretreating ischemic rats with carotenoids extract significantly ameliorated brain MDA level as compared to ET-1 injected rats (0.90±0.04 nmol/mg protein). XOD could serve as a source for reactive oxygen species (ROS) and was found to be triggered after ET-1 ischemia production (0.19±0.04 vs. 0.07±0.03 nmol/mg protein). However, carotenoids extract reduced its content to a normal level (0.07±0.02 nmol/mg protein). In the same manner, GtSx was significantly elevated after ET-1 treatment (25.2±4.2 vs 14.8±2.8 μmol/mg protein) as compared to the sham group, carotenoids extract treatment significantly attenuated the GtSx level (15.5±2.1 μmol/mg protein) as compared to ischemic group (Fig.3A).

Brain SOD and CAT activities were significantly down-regulated (68%, 74.3% respectively) in ischemic rats as compared to the sham group. However, rats that received carotenoids extract prior to ET-1 administration exhibited significant enhancement in brain antioxidant enzymes activities (104.2% for SOD, 222.2% for CAT) as compared to ET-1 injected group. Also, A significant depression in TH activity in rat brains was observed following ET-1 administration (16.3±4.2 vs. 38.4±6.9 U/mg protein) as compared to the sham group. Carotenoids extract pre-administration in ischemic rats significantly ameliorated TH decline in comparison with the ET-1 group. (Fig. 3B)

Data are indicated as mean±sem. Significance at p<0.05. Different characters indicate significant differences

**Inflammatory and apoptotic biomarkers**

Neuroinflammation and inflammatory changes of brain tissue trigger most neurodegenerative disorders. Brain inflammatory mediators values, MMP-9 and NF-κβ (Fig. 4A,4B) as well as apoptotic marker caspase -3 level (Fig.4A) were significantly elevated in ischemic rats in comparison with the sham group. Carotenoid extract pre-treatment revealed both significant anti-neuroinflammatory and anti-apoptotic activities via significantly decreasing the level of MMP-9, NF-κβ, and caspase-3 (34%, 73.7%, 50% respectively) as compared to the ischemic group. Furthermore, brain NRF-2 level was significantly inhibited by ET-1 treatment (0.24±0.1 vs 2.9±0.7 nmol/mg protein) as compared to the sham group, carotenoid extract pre-treatment almost restored NRF-2 normal activity (2.1±0.6 nmol/mg protein) (Fig.4C).

**Histological investigation**

Examining brain slices of sham control animals under a microscope revealed the entrance of a syringe with minimal diffusion and congestion. However, after ET-1 perfusion, the capillary plexus was congested. Nuclear pyknosis and degeneration were observed in neurons of the subiculum, fascia dentate, and hilus associated with congested blood vessels. The striatum showed focal hemorrhage with nuclear pyknosis and degeneration in some neurons as well as diffuse gliosis. Pretreatment with carotenoids extract showed the normal histological structure of the cerebral cortex, as well as the subiculum and fascia dentate and hilus with congested blood vessels in the latter. Diffuse gliosis was detected in between the neurons of the striatum.

**DISCUSSION**

In comparison to chemical and inorganic pigments, microbial pigments have numerous advantages. Pigment generation by microorganisms is an efficient and beneficial process as compared to the chemical synthesis of pigments. Microbes can grow easily and fast in the cheap culture medium and independent from weather conditions. Microbial pigments are available in different shades. These pigments are biodegradable.
and environment friendly. They also have a number of clinical properties, such as antioxidant, anticancer, antiproliferative, immunosuppressive, diabetes treatment, and so on. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications.29

Currently, there is no available approved therapy that could minimize cerebral damage and neurological disability caused due to ischemic insult.30 Experimental studies on many neuroprotective agents are still ongoing. The current investigation aimed to investigate the possible neuroprotective effect of carotenoid pigment extracted from Rhodotorula yeast strains against focal cerebral ischemia in rats concerning locomotor, biochemical, and histopathological assessment findings.

Several yeast species can synthesize natural carotenoids, particularly the genera Rhodotorula, Xanthophyllomyces, Phaffia, and Sporobolomyces.31 Carotenoids produced by Rhodotorula species are preferred over other carotenoids—producing microorganisms owing to their high growth rate and unicellular nature.6 Moreover, Cells may be grown in traditional bioreactors and biomass and used directly in pharmaceuticals.32 Carotenoid pigment production was reported in multipleRhodotorulaisolates such as R. acheniorum, R. minuta, R. mucilaginosa, R. graminis, and R. glutinis.3 Carotenoid pigment production was reported in multipleRhodotorulaisolates such as R. acheniorum, R. minuta, R. mucilaginosa, R. graminis, and R. glutinis.3 Rhodotorula mucilaginosa is one of the most important pigment-producing yeasts. It is considered one of the best potential candidates for biotechnological production of β-carotene and another carotenoid.33 The current study showed a mass production of carotenoid pigments with high biological effect from species R. mucilaginosa.

R. mucilaginosa produces four main pigments (torulene, torularhodin, β-carotene, and γ-carotene),34 which are synthesized at different rates depending on the strain.35 Isolated R. mucilaginosa F-1 from a biodiesel plant tank and the main components of its carotenoid content were β-carotene (28.8%), torulene (40.0%) and torularhodin (23.2%). The current study showed that the main bio-pigment components were neoxanthin (40%) and β-carotene (30%). Other carotenoids were also detected in minor quantities.

In the current study, cerebral ischemia was induced by microinjection of a potent vasoconstrictor ET-1. Cerebral injury induced by ET-1 resulted in impairing neuromuscular strength and locomotor activity, triggering oxidative stress, upsurging inflammatory cascade, and inducing apoptotic damage.

Ischemic stroke is a cascade of events initiated by a decline in blood flow leads to depletion of oxygen and glucose supply, Ca2+ ion overload, free radical formation, and mitochondrial and DNA damage in neuronal cells.36 Hippocampus is particularly sensitive to stroke and ischemia due to dense innervations.37 The medial nucleus accumbens, a crucial node in brain circuits that service components of motivation and emphasis spatial information, receives input from the hippocampus’s subiculum area.38 Histological examination of brain tissue revealed that ET-1 ischemia was localized in the subiculum region and caused nuclear pyknosis and degeneration in neurons of the subiculum, fascia dentate and hilus which explains the impairment in neuromuscular and locomotor activity observed in ET-1 treated rats.

Brain energy depends mainly on oxidative phosphorylation.39 During Ischemia, oxidative stress arises from the impaired mitochondrial electron transport chain40, where oxygen-free radicals and oxidants are generated and consume the natural defense anti-oxidants such as GSH, SOD, or CAT.41 Additionally, brain high lipid content is susceptible to free radical peroxidation resulting in increased content of MDA (a marker of lipid peroxidation)42 and suppression of TH expression (mRNA and protein) and down regulation of enzyme activity due to accumulation of toxic aldehyde which induces dysfunction of tyrosine hydroxylase and attenuates synthesis of catecholamine neurotransmitter such as dopamine and triggered neuron cell damage.43 Cerebral ischemia injury induces degradation of ATP levels through the generation of toxic oxygen metabolites. Ischemia is allocated to an accelerated increase in the interstitial concentration of hypoxanthine and adenosine which become substrates for the xanthine oxidase pathway. Elevation in xanthine oxidase results in the induction of oxygen radicals. High levels of oxygen radicals lead to an increase of xanthine oxidase activity.44 Moreover, An increase in extracellular glutamate associated with a decrease in glutamine levels was observed during cerebral ischemia.45 Thus, enhancing brain metabolism of glutamate which in turn upregulates the level of glutamine synthetase enzyme essential in restoring extracellular glutamate following ischemia to a normal level and prevent the neurotoxic effects of this excitatory amino acid on the brain.46

Studies have found that inflammatory processes, excitotoxicity, and depolarizations due to infarcts and apoptosis are associated with cerebral ischemia.47 Ischemia causes activation and accumulation of microglial cells and leukocytes in brain tissues, leading to inflammatory injury and release of inflammatory cytokines Oxidative stress leads to multiple changes including increased levels of pro-inflammatory and pro-oxidant molecules in brain cells, such as NF-kB, XOD, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and MDA.48 Free radicals produced activate MMPs thus allow more damage to BBB and flow of neurotoxins and inflammatory cells.49

The transcription factor NF-kB plays a regulatory role in, metabolism and cell growth, apoptosis, proliferation, aging, survival, synaptic plasticity, and memory.50 The NF-kB signaling pathway is associated with oxidative stress and
Inflammation. Its activation ensues perturbation of cellular homeostasis and triggers either necrosis or apoptosis. Cytochrome c translocates from the cytoplasm after brain ischemia and interacts with apoptotic protease activating factor (c-APAF-1) and deoxyadenosine triphosphate (dATP)/ATP, leading to activation of caspase-9 which in turn activates caspase-3.

On the other hand, the NRF-2 level is significantly down regulated during ischemia. NRF-2 transcription factor is a downstream target of NF-κB and mediates both inductive and constitutive expression of antioxidant response element regulated genes, including antioxidant proteins and phase II detoxifying enzymes which protect the cell against oxidative stressors.

Pre-treatment cerebral ischemic rats with carotenoid-pigment extract significantly improved the neurologic and locomotor deficits induced by ET-1, halted oxidative stress biomarkers: MDA, GtSX, and XOD. Meanwhile, enhanced antioxidant enzymes SOD, CAT, and TH. Also, reduced inflammatory mediators such as MMP-9, NF-kB, the apoptotic marker caspase-3, while, promoted NRF-2.

The current data demonstrated that the main carotenoids bio-pigment components extracted from R. mucilaginosa yeast were β-carotene and neoxanthin. β-carotene a lipid-soluble carotenoid that was reported for its antioxidant effect in vivo. β-carotene effectively functions as a chain-breaking antioxidant and a neuroprotective agent in the spinal injury-induced rat model through interfering with both the stimulation of pro-inflammatory mediators and the activation of the NF-κB pathway. Additionally, β-carotene ameliorated the cognitive impairment model and acted via modulating oxidative stressors and inhibiting acetylcholinesterase enzyme.

There is scarce data about the bioactivity of the less common carotenoid, neoxanthin. However, its chemical structure is xanthophyll class carotenoid, which is linked to effective neuroprotective antioxidants such as astaxanthin. Moreover, previous studies reported its potent protective activity against oxidative stress and inhibition of DNA damage.

Carotenoids (β-carotene and neoxanthin) are involved in scavenging singlet molecular oxygen and peroxyl radicals. β-carotene can restore the activity of the antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase), inhibit lipids peroxidation, and prevent the infiltration of neutrophils. Restored antioxidants especially SOD may decrease, attenuate or prevent the ischemic damage progress via halting the superoxide-anion dependent glutamate release, which in turn, suppresses glutamine synthetase activity.

In the present study, carotenoids extract prevented the increase in XOD. β-carotene and tocopherol in palm oil are potent antioxidants that can inhibit lipid oxidase as manifested by depression in xanthine oxidase and xanthine dehydrogenase enzymes activities. Current data illustrated an elevation in TH expression when the carotenoid extract was administered prior to ET-1, suggesting a neuroprotective effect of the extract on dopaminergic neurons. This could be attributed to the antioxidant capacity of the extract. As reported recently, apoptotic death of DA neurons may be initiated by oxidative stress, neuroinflammation, and decreased glutathione levels, which induces a suppression in dopamine and its biosynthetic enzyme, tyrosine hydroxylase (TH).

The present investigation propose that the neuroprotective effect and the reduction of infarcted area in rat stroke model by carotenoid pigment extract involves NF-κB deactivation with a subsequent decrease in MMP 9 and caspase 3. Bai et al. reported that carotenoids blocked the NF-κB-dependent expression of inflammatory genes, such as TNF-α, IL-1β, iNOS, and COX-2 in LPS-administrated animals via inhibiting IkBα degradation and nuclear translocation of the cytosolic NF-κB p65 subunit. Besides its potent antioxidant activity which directly suppresses NF-κB activation and NF-κB-dependent inflammatory products including MMP-9, β-carotene was reported to inhibit apoptosis via activating the PI3K/Akt/mTOR pathway.

Moreover, carotenoids can enhance NRF-2 by interacting with the NRF-2 pathway, accelerating its translocation into the nucleus, and enhancing phase II enzymes and antioxidants. Interruption of oxide.

**CONCLUSION**

It could be concluded that carotenogenic yeast R. mucilaginosa can be considered as an indispensable source for carotenoid pigment of unique composition under the investigated growth conditions. Moreover, this study supported the neuroprotective, antioxidant, anti-inflammatory, and anti-apoptotic activities of the examined extract against cerebral ischemia.

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**Conflict of interests**

The authors declare no conflict of interests
Author contributions
GI, SE and MA conducted experiments; NS and GI assisted with the analysis of results and prepared the manuscript.

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Salem et al: Carotenoid pigments ameliorate cerebral ischemic stroke

Figure 1: Yeast strain *Rhodotorulamucilaginosa* G20 cultivated on YPD media.

Figure 2: Phylogenetic tree of *Rhodotorulamucilaginosa* G20 (KY271337.1) obtained by neighbour-joining analysis of 18 rDNA.

Figure 3: Effect of carotenoids extract pre-treatment on brain oxidant (A) and antioxidant (B) status of ET-1 cerebral ischemic rats.

Figure 4: Effect of carotenoids extract pre-treatment on brain inflammatory markers (A) MMP-9, B) NF-κβ and apoptotic markers A) caspase-3, C) NRF-2. Data are indicated as mean±sem. Significance at P<0.05. Different characters indicate significant differences.

Figure 5: Photomicrographs showing rat hippocampal tissues stained with H&E (x100). (A) sham group showing normal brain architecture (B) ET-1 ischemic group revealing congestion of the capillary plexus at the cerebral cortex (arrow1) and nuclear pyknosis and degeneration in neurons of subiculum (arrow2), fascia dentate (arrow3) and hilus (arrow4). (C) Carotenoids extract group showed normal histological structures and mild congestion in dentate (arrow 5&6).
Table 1: Effect of carotenoids extract pretreatment on the behavioral activity of ET-1 cerebral ischemic rats

| Group                  | Normal control     | Sham control        | ET-1       | Carotenoids extract/ET-1 | F value |
|------------------------|--------------------|---------------------|------------|--------------------------|---------|
| Wire hanging test      | 23.01±4.24        | 26.12±3.48          | 10.16±2.11 | 21.18±2.47               | 4.742   |
| (seconds)              | CI(12.1,33.9)     | CI(17.1,35.08)     | CI(4.7,15.5) | CI(14.8,27.5)          |         |
| Cylinder test (num-    | 27.50±2.69        | 23.67±1.68          | 12.83±2.66 | 21.67±2.01               | 7.27    |
| ber of rears)          | CI(20.5, 34.4)    | CI(19.3,28)        | CI(5.9, 19.6) | CI(16.5, 26.8)         |         |

Data are indicated as mean±sem. Significance at P<.05. Different characters indicate significant differences.