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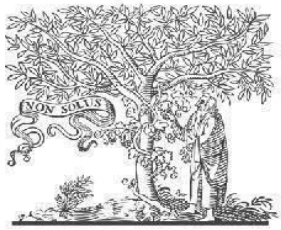

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PHARMACOLOGICAL SCREENING OF *Origanum sipyleum* LEAF EXTRACT FOR NEUROPROTECTIVE EFFECTS IN A RAT MODEL OF ISCHEMIC STROKE

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ABSTRACT

This study aimed to assess the beneficial effects of Extract of *Origanum sipyleum* (EOS) pre-supplementation in a middle cerebral artery occlusion (MCAO) model of ischemic stroke. Ischemic stroke was induced in rats by inserting an intraluminal suture for 90 minutes, followed by 24 hours of reperfusion injury. The animals were evaluated for neurobehavioral changes, which were associated with decreased acetylcholinesterase activity, increased oxidative stress (evidenced by enhanced lipid peroxidation), and reduced thiol levels. Pre-supplementation with EOS (200 and 400 mg/kg body weight) for 21 days effectively restored acetylcholinesterase activity, reduced lipid peroxidation, and normalized thiol levels, thereby alleviating MCAO-induced behavioral deficits. EOS significantly decreased cerebral infarct volume and improved cerebral ischemia. The study demonstrated the protective effects of EOS supplementation in ischemic stroke, suggesting its potential application in stroke management.

Keywords: Cerebral ischemia, *Origanum sipyleum*, Antioxidant, Oxidative stress.

INTRODUCTION

Stroke remains a significant cause of mortality in developed countries, ranking third among leading causes of death. Despite extensive research, effective neuroprotective treatments for stroke are still lacking. Oxidative stress plays a crucial role in neuronal death following stroke, primarily mediated by reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, hydrogen peroxide, and peroxynitrite generated during ischemic reperfusion (IR) injury. These radicals initiate lipid peroxidation and trigger neuronal death. Induction of inducible nitric oxide synthase (iNOS) post-IR injury leads to excessive nitric oxide (NO) production, which, in combination with superoxide, forms peroxynitrite, a potent radical contributing to neuronal demise by causing rapid hydroxylation and nitration of cellular components. Antioxidants, including curcumin and quercetin, have shown promise in mitigating ROS-mediated damage and protecting neurons in animal models of cerebral ischemia.

Stroke poses a significant global health burden due to its high morbidity, mortality, and disability rates, particularly as populations age worldwide. Its incidence is rising, especially in developing countries, with younger adults also experiencing elevated mortality rates, potentially linked to modern stressors. The aftermath of stroke places substantial economic

and social burdens on families and society. Despite ongoing efforts, many experimental medications for stroke treatment and prevention have not proven successful in clinical trials. Tissue plasminogen activator (t-PA) stands out as an FDA-approved thrombolytic for acute ischemic stroke, yet its efficacy is transient, and it carries bleeding risks, limiting its broader application.

Various forms of circulatory dysfunction are associated with different types of brain ischemia. Numerous experimental models, ranging from transient to permanent interruptions of blood flow at local or global levels, have been developed to explore these variations. The primary focus lies in utilizing these models to understand the origins of injuries and to devise effective treatment strategies for individuals with cerebrovascular diseases. It is crucial that the pathophysiology of these models accurately reflects real-world conditions to ensure that experimental findings can be translated effectively into clinical practice. Many previous clinical trials have failed due to interventions and therapeutic concepts that were tailored to the model rather than the disease itself, underscoring the importance of selecting appropriate animal models, establishing reliable methods to quantify ischemic injury, and accurately evaluating outcome data.

Animal models play a pivotal role in studying human diseases, particularly stroke. Over several decades, researchers have heavily relied on animal models to study ischemia and develop novel therapeutics. Among these models, the middle cerebral artery occlusion (MCAO) model has been extensively used, constituting over 40% of neuroprotection research due to its close resemblance to human ischemic stroke. Conversely, models involving craniotomy for inducing cerebral ischemia may lead to increased rates of brain infections. Thus, the intraluminal suture-based MCAO model in rats is widely accepted for its simplicity and consistent infarct volume.

Reperfusion exacerbates tissue damage by reintroducing blood flow and oxygen to ischemic tissues, potentially worsening the initial hypoxia-induced injury. This phenomenon, akin to global hypoxic injury, is observed in focal ischemic injury related to stroke. This review compiles comprehensive literature on ischemia-reperfusion injury, elucidating its underlying causes and therapeutic approaches. Recent advancements in multimodal imaging have enabled precise monitoring of serial perfusion changes, offering insights into the dynamic nature of reperfusion injury in acute ischemic stroke. Understanding these dynamics may pave the way for developing protective strategies against ischemia-reperfusion damage in clinical settings.

Fig1: Leaves of *Origanum sipyleum*

Figure 2: Leaves and Plant of *Origanum sipyleum*

Acute oral toxicity study (OECD 423)

The oral toxicity test procedures followed the OECD Guideline 423. Each test phase involved systematic use of three animals of the same gender per group. Toxicity of the test substance was assessed based on mortality rate or the dosage causing harm (50, 300, or 2000 mg/kg body weight), providing straightforward evaluation options. This method is advantageous as it minimizes animal use while yielding meaningful data compared to other approaches. Data analysis utilized standardized and categorized information. In this study, ethanol-based extracts of EOS were orally administered at 2000 mg/kg body weight. Initially optional, this dose level became mandatory to detect any adverse effects on water consumption in observed rats. A subsequent 72-hour experiment replicated the initial setup with three additional rats, monitoring various parameters including skin tone, salivation, bowel movements, sleep patterns, tremors, spasms, and impacts on cardiovascular, autonomic, and central neurological systems.

Middle cerebral artery occlusion induced focal cerebral ischemia

This study utilized a modified version of the middle cerebral artery occlusion (MCAO) technique, originally described by Longa et al., to induce localized brain damage through reduced blood flow. Here's a concise summary of the procedure:

Anesthesia and Preparation:

Rats were initially administered atropine sulfate (0.5 mg/kg) via abdominal cavity injection for pre-anesthesia, followed by anesthesia induction with chloral hydrate (350 mg/kg) via abdominal cavity injection. A midline incision was made at the nape of the neck to expose the trachea and left common carotid artery.

Surgery:

The left external carotid artery was dissected after identifying its confluence with the internal carotid artery. The external carotid artery was surgically manipulated and incised following meticulous preparation. A 3-0 nylon monofilament coated with poly-L-lysine (0.01%

solution) was introduced through the external carotid artery and advanced up to 21 mm or until encountering resistance, effectively occluding the middle cerebral artery. The filament was secured in place with ligature, and the incision was closed with sutures. Ischemia was maintained for 2 hours, after which the filament was carefully withdrawn under anaesthesia to prepare for reperfusion.

Post-Operative Care:

Rats received a 1 mL intraperitoneal injection of sterile 5% dextrose solution at 4 and 12 hours post-MCAO to support recovery.

Neurological Assessment and Sample Collection:

Neurological scoring was conducted post-reperfusion, with a score below 3 indicating absence of immediate neurological deficits. After 22 hours of reperfusion, rats were euthanized, and their brains were extracted for analysis. Blood collection was performed via retro-orbital sinus puncture under light ether anesthesia on the final day of the experiment.

Biochemical Analysis:

Serum samples were centrifuged and used for biochemical tests including LDL, HDL, VLDL, triglyceride, and total cholesterol levels using Erba kits. Antioxidant enzyme levels such as glutathione (GSH) and superoxide dismutase were assessed using standard methods.

Protein Estimation:

Protein content in brain homogenates was quantified using the Biuret method. Bovine serum albumin (BSA) was used to establish a standard curve correlating protein concentration with optical density at 540 nm.

Measurement of Decreased Glutathione Levels (GSH):

GSH levels were determined using the Ellman method. Serum samples were treated with TCA, and after centrifugation, DTNB and phosphate buffer were added. Absorbance was measured at 412 nm, and GSH concentration was expressed as nmol/mg protein. This protocol ensured standardized induction of focal cerebral ischemia and rigorous evaluation of neurological outcomes and biochemical parameters in experimental rats.

RESULTS

Phytochemical Screening

Table 1: Phytochemical screening of *Origanum sipyleum*

Sr. No.	Metabolite	Tests	Ethanolic Extract
1	Phenols	i) Ferric Chloride Test	+
2	Alkaloids	Dragendroff's Test Mayer's Test Wagner's Test	+ + +
3	Tannins	Ferric Chloride Test Lead Acetate Test	+ +
4	Lipids	i) Acrolein Test	-
5	Amino acids	i) Ninhydrin Test	-
6	Saponins	i) Foam Test	-
7	Flavonoids	Ferric Chloride Test Lead Acetate Test	+ +
8	Resins	HCL Test Ferric Chloride Test	+ +
9	Steroids/Terpenes	Salkowaski Test Liebermann-Burchard Test	- -

Table 2: Acute toxicity study of EOS at a dose of 2000 mg/kg

Parameters	1st hr	2nd hr	3rd hr	4th hr
Piloerection	-	-	-	-
Edema	-	-	-	-
Urine stains	-	-	-	-
Alopecia	-	-	-	-
Loss of writhing reflex	-	-	-	-
Circling	-	-	-	-
Nasal sniffing	+	+	+	+
Lacrimation	-	-	-	-
Seizures	-	-	-	-
Righting reflex	+	+	+	+
Grip strength	+	+	+	+
Eye closure at touch	+	+	+	+
Rearing	+	+	+	+
Straub tail	-	-	-	-

Effect of Extract of Origanum sipyleum Leaves on Body Weight

In this study, significant changes in body weight were observed across different treatment groups:

1. **MCAO-treated Groups:**
   - There was a noticeable increase in body weight compared to the normal control group.
2. **EOS-treated Groups:**
   - Groups treated with Extract of Origanum sipyleum (EOS) at doses of 200 mg/kg and 400 mg/kg showed a significant decrease in body weight compared to the MCAO group.
3. **Vitamin E Group:**
   - Vitamin E treatment also led to a significant decrease in body weight compared to the MCAO group.
   - However, when compared to the normal control group, vitamin E treatment restored body weight to levels similar to those of the control group.

These findings indicate that EOS and vitamin E treatments have distinct effects on body weight in the context of middle cerebral artery occlusion (MCAO) in experimental models.

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Table 3: Effect of Extract of *Origanum sipyleum* leaves on Body Weight

Group	Body Weight (grams)	
	Before treatment	After Treatment
Normal Control (NC)	201±5	213±3
Middle carotid artery occlusion (MCAO)	203±9^{ns}	315±10$^{@}$
Extract of Origanum sipyleum leaves (EOS Low)	202±4^{ns}	213±6**
Extract of Origanum sipyleum leaves-high (EOS High)	199±5^{ns}	216±5**
Vitamin E (Vit E)	198±6^{ns}	217±4**

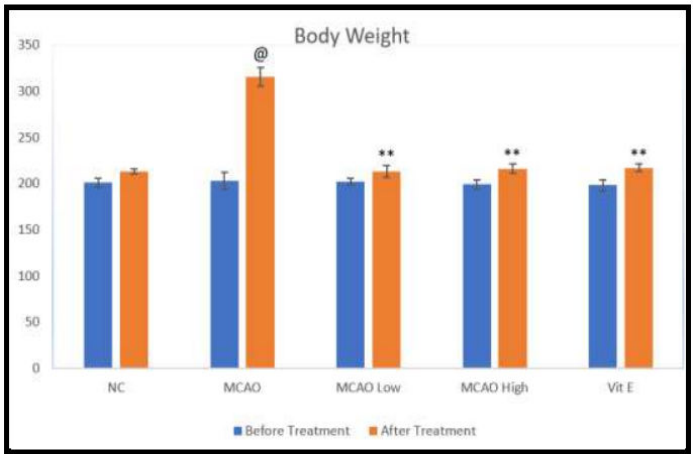

Figure 3: Effect of Extract of *Origanum sipyleum* leaves on Body Weight

Table 4: Effect of Extract of *Origanum sipyleum* leaves on AChE level

Group	AChE level (mmol/min/mg of protein)
Normal Control (NC)	0.168±0.019
Middle carotid artery occlution (MCAO)	0.345±0.022 $^{@}$
Extract of Origanum sipyleum leaves-low (EOS Low)	0.21±0.015 **
Extract of Origanum sipyleum leaves-high (EOS High)	0.181±0.012 **
Vitamin E (Vit E)	0.171±0.018 **

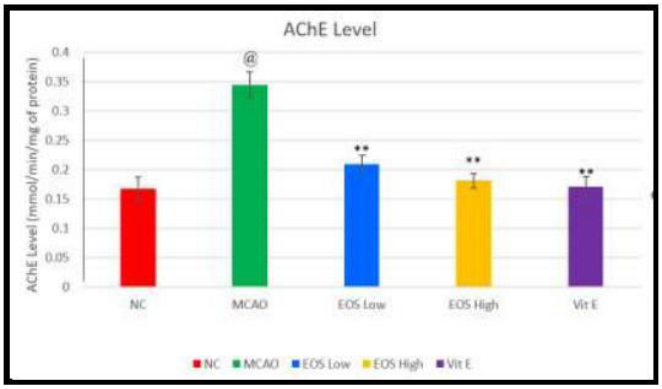

Figure 4: Effect of Extract of *Origanum sipyleum* leaves on AChE level

All values are expressed as Mean ± SEM (n=6). A significant increase in AChE level was observed compared to the normal control group ($p < 0.01$). Additionally, a significant decrease in AChE level was observed compared to the diseased control group ($p < 0.01$).

Table 4.4 and Figure 4.2 illustrate the effect of Origanum sipyleum on AChE activity in cerebral ischemic stroke rats. The diseased control group showed a significant ($p < 0.01$) increase in brain AChE activity compared to the normal control group. Conversely, the groups treated with EOS at doses of 200 mg/kg and 400 mg/kg, as well as Vitamin E at 10 mg/kg, exhibited a significant ($p < 0.01$) decrease in brain AChE activity.

Effect of Extract of Origanum sipyleum Leaves on Lipid Profile

In the serum of MCAO-treated groups compared to the normal control group, there was a significant decrease in HDL levels observed. However, in the serum of EOS-treated groups at 200 mg/kg and 400 mg/kg, there was a significant increase in HDL levels compared to the MCAO group. Vitamin E also significantly elevated HDL levels compared to MCAO and restored them to levels comparable to the normal control group.

Furthermore, significant increases in LDL, VLDL, TG, and TC levels were observed in the serum of MCAO-treated groups compared to the normal control group. In contrast, the serum of EOS-treated groups at 200 mg/kg and 400 mg/kg showed significant decreases in LDL, VLDL, TG, and TC levels compared to the MCAO group. Similarly, Vitamin E significantly reduced LDL, VLDL, TG, and TC levels compared to MCAO and restored them to levels similar to the normal control group.

DISCUSSION & CONCLUSION

This study aimed to investigate the effects of pre-supplementation with Extract of *Origanum sipyleum* (EOS) on behavioral abnormalities and biochemical changes associated with neuronal injury in an experimental stroke paradigm. The results from various behavioral tests clearly demonstrated that MCAO-induced ischemia-reperfusion injury significantly impaired both motor and cognitive functions in the subjects.

The phytochemical analysis (refer to Table 1) revealed a diverse array of beneficial compounds present in EOS plants. Moreover, based on the results of the acute toxicity test, it was concluded that EOS extract did not pose any health risks even at doses as high as 2,000 mg/kg. This data provides crucial information for accurately determining safe dosages for future studies.

Herbal medicines are increasingly gaining popularity due to their therapeutic benefits without causing adverse effects. In this context, our study utilized an animal model of focal cerebral ischemia to evaluate the efficacy of ethanol extracts from Origanum sipyleum in preventing and treating stroke episodes.

The brain's susceptibility to reactive oxygen species (ROS) during ischemia-reperfusion is well-documented, owing to its low levels of antioxidative enzymes such as SOD, GSH-Px, and CAT, coupled with high concentrations of oxidizable unsaturated fatty acids prone to lipid peroxidation. ROS generated during reperfusion can cause oxidative damage to DNA, lipids, and proteins, leading to cellular dysfunction and death.

EOS demonstrated potential as a free radical scavenger by inhibiting xanthine oxidase activity, thereby reducing superoxide and hydrogen peroxide production during cerebral ischemia-reperfusion. Previous studies have shown EOS's effectiveness in preventing LDL oxidation and lipid peroxidation, further highlighting its antioxidant properties.

Our findings also indicated that EOS effectively modulated the LDL/HDL ratio, consistent with other research suggesting its potential in slowing atherosclerosis progression and reducing serum cholesterol levels. Additionally, EOS was observed to decrease cholesterol levels in rats by inhibiting lipid oxidation and enhancing antioxidant enzyme activity.

In conclusion, these results underscore the promising therapeutic potential of Origanum sipyleum extract in mitigating ischemia-reperfusion injury through its antioxidant properties and modulation of lipid profiles. Further research is warranted to elucidate its precise mechanisms and clinical applications in stroke management.

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