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Cone and leaf aqueous extract of Humulus Lupulus have neuroprotective and antiepileptogenic effects in mouse pentylenetetrazole kindling model

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ABSTRACT

Introduction. Despite existence of multiple synthetic drugs for epilepsy management and control, some patients suffer from treatment-resistant seizures and unwanted side effects of available medicine. Therefore, there is an urgent need to find new medications against epilepsy. The aim of current investigation was to assess anti-convulsive effects of cone leaves extracts of Humulus Lupulus (H.l) extract on chemical kindling model of epilepsy and its neuroprotective effects on CA3 region of hippocampus.

Methods. In this experimental study, 35 adult male mice divided into five groups (n=7) comprising, control group receiving distilled water, kindling group receiving only Pentylenetetrazole (PTZ) in dose of 45 mg/kg every 48 hours and 3 treatment groups that received cone leaves extracts of H.L in doses of 200, 400, 800 mg/kg by gavages 30 minutes before PTZ injection. Histological examination of hippocampal CA3 was carried out using Hematoxylin and eosin (H and E) staining at the end of experimental procedure.

Results. Data analysis indicated that treatment with cone leaves extracts of H.L has an inhibitory effect on chemical kindling. It significantly ($P<0.05$) retarded development of seizures and increased latency to seizure onset but did not have any significant effect on seizure duration. Histological examination demonstrated its neuro-protective effects on CA3 region of hippocampus.

Conclusion. Our findings suggest beneficial anticonvulsive effects for cone leaves extracts of H.L on PTZ kindling neuro-protective effects on hippocampus tissue. This medicinal plant may be beneficial for human seizure treatment so more investigation is proposed.

Keywords: hippocampus, histological study, epilepstogenesis, Humulus Lupulus, pentylentetrazole, kindling.

INTRODUCTION

Epilepsy, one of most common neurological disease, has afflicted millions worldwide. This disease primarily affects the brain and causes repetitive unpredictable electrical discharge in the brain [1]. Epilepsy puts tremendous economic pressure on the healthcare systems around the globe and affected individuals often are under mental pressure which forces them to seclusion [2]. Currently, there are more than fifty million afflicted individuals worldwide with higher incident in developing countries [3, 4].

Until the twentieth century, the primary cure for epilepsy included traditional herbal medications. Although, with advances in science and a more in-depth understanding of the etiology and molecular mechanisms of the disease, various therapeutic methods have developed. These include anti-seizure drugs, vagus nerve stimulation, and the surgical removal of the brain area in which seizure focus is located with the

anti-epileptic drugs (AED) being more common [5, 6]. Currently, there are more than 30 synthetic anti-seizure drugs available and the first line of treatment is using one or a combination of these drugs based on the severity of the ailment. More than 60 percent of the affected individuals respond to these treatments and may experience only a partial seizure while the remaining 40 percent show resistance to these drugs [7, 8].

Due to the resistance of some afflicted individuals and the severity of the side effects of synthetic drugs, researchers are trying to find more efficient treatments with fewer side effects, as such, plant-derived compounds are intriguing substances to find novel and effective treatments for epilepsy [5, 6]. Plants are a valuable source of a wide variety of secondary metabolites and are used in medical areas to prevent and treat various illnesses [9]. Humulus Lupulus (H.l), commonly known as Hops, has long been established to produce an anti-microbial and calming effect. A more in-depth study has suggested that this plant possess estrogenic and anti-cancer properties as well as having anti-apoptotic, anti-fungal, antioxidative, and anti-inflammatory substances [10, 11]. For example, H. L cones are a valuable source of Xanthohumol that exhibits strong antioxidative effects and can prevent the formation of tumors [12]. H. L cone has several functional groups of metabolites which can be classified into four categories: 1– bitter acids, which include α-acids – such as humulone, adhumulone, and cohumulone– 2 β–acids – including lupulone, cupulone, and adopulone– which make 5 to 20 percent of the Hops strobili. 3– Polyphenols including catechin, rotine, cumarine, cuestrine, and etc. that make 4 to 14 percent of the hops flower. 4– Volatile oils which have hundreds of terpenoid including humulone which is a sarcoid terpene, myrcene, which is a monoterpenoid, and β-caryophyllene that makes 0.3 to 1 percent of the plants strobili [13,14]. Studies have shown that terpenoids can ameliorate seizures by effecting GABA receptor activities and inhibition of voltage-dependent sodium channels [15]. Several distinct locations in the brain have been shown to possess higher susceptibility in the induction of seizures, the most important and best-known locations are the Temporal lobe and hippocampus [16].

The hippocampal formation consists of several interconnected components including Subiculum, hippocampus body, and dentate gyrus. The hippocampus body has four regions, namely CA1 to CA4. The vast majority of the hippocampus is made from CA1 and CA3 respectively. Hippocampus is a multi-layered tissue consisting of the molecular layer, the pyramidal layer, and the multiform layer. CA1 Pyramidal cells are small and compact although in CA2 and CA3 pyramidal cells are large and less compact [17]. The hippocampus is one of the primary tissues in which epileptic attacks are initiated, and hippocampus sclerosis is the most brain common tissue damage observed in the temporal lobe [18]. Numerous laboratory techniques are used in order to study the primary mechanisms of epileptogenesis and to find antiepileptic drugs [18, 19]. One of the most used models for induction and development of seizure in laboratory animals is kindling [20, 21]. Chemical kindling is a process in which general seizures can be induced in animals by consistent administration of a chemoconvulsant below the threshold, this process results in an increase in seizure activity and eventually leads to general seizure. [22- 24].

In this study, the anti-seizure effect of the cone leaf aqueous extract of H.L on pentylenetetrazole-induced epilepsy was studied, and its protective effect on hippocampus has been observed respectively.

MATERIALS AND METHODS

Preparation of the Aqueous Extract

The leaf of H.L cone (figure1: A) were dried in shade and stored at 30°C temperature (figure2: B), they were crushed further to obtain a powder and passed through a sieve (no. 40). In this test, water extract (soaking method) is used to extract from the hop plant, in which the powder of the hop plant is mixed in the amount of 5 grams in 50 ml of water and at a temperature of 35 degrees on a heater stirrer for 30 minutes. And in the next step, we placed it in the ultrasonic bath for 30 minutes, one of the important advantages of the ultrasonic device is that it breaks the plant's cell wall, and as a result of this process, the substances and metabolites of the plant are removed more easily. After removing the extract from the

Figure1. A. Humulus Lupulus cone, B. H.L dried cone and C. H.L extract

ultrasonic device, we pass the extract through a filter paper, and to separate the solvent, we add the solvent to the oven, which is set at a temperature of 40 degrees. Because the metabolites of the extract are sensitive to high temperature, we do not allow the temperature to exceed 40 degrees during the solvent separation. In this research, hop extract (figure1: C) was fed to mice through gavage. Before gavage, we dissolve each of the different doses of hop plant extract with distilled water relative to the average weight of the group, and give 0.5 cc orally to the groups 30 minutes before PTZ injection.

Animals

Swiss male albino mice (25–30 g) were procured from Tabriz University and were acclimatized in the animal house. Young healthy male mice were housed eight per cage and maintained at a temperature of 23 ± 2°C, at a humidity of 51 ± 10% and in a 12:12-h light/dark cycle with free access to rodent chow and water. The principles of working with laboratory animals approved by YASP were followed in all stages of the experiments.

PTZ kindling

Pentylenetetrazole (PTZ; Sigma Chemical Co., United States), were used for induction of epileptogenesis in the present study. PTZ was dissolved in normal saline solvent, and then injected intraperitoneally (45 mg/kg, i.p). Repetitive subthreshold of PTZ (45 mg/kg) were injected every 48 hours until the animals showed stage 4 or 5 seizures in three consecutive injections. Following the PTZ injection animals immediately were transferred to a glass box to monitor their behavior and were filmed with a camera.

After each PTZ injection, the convulsive behavior was observed for 30 min. The intensity of seizure response was scored according to chemical kindling scoring: Score 0 (no response); Score 1 (myclonic jerk); Score 2 (straub tail); Score 3 (clonic jerk without loss of righting reflex); Score 4 (clonic seizure with loss of righting reflex); Score 5 (tonic seizure), and Score 6 (death). Kindling occurs when animal gets Stage 4 or Stage 5 for consecutive 3 days after PTZ administration. The experimental groups taken were as follows, Group I normal control (vehicle only), Group II: kindling group (PTZ), Group III: H.L + PTZ (200 mg/kg H.L), Group IV: H.L + PTZ (400 mg/kg H.L), Group V: H.L + PTZ (800 mg/kg H.L).

Histopathological Examination

The dissected mice brains were fixed in a 10% formalin solution and embedded in paraffin. Coronal sections with 6 µm in thickness were obtained at hippocampus level, and fixed onto standard glass histological slides (75 × 25 × 1 mm). Each section was dewaxed in xylene, rehydrated through graded ethanol and underwent H&E staining. Then stained slides, and observed under a light microscope (Olympus, Japan) for histological examination.

Statistical analysis

In this study, the results are expressed as mean ± standard deviation. Statistical comparison between the experimental groups was carried out using SPSS, A Kruskal-Wallis test and Dunn's post hoc test were used to compare the maximum seizure stage among the experimental groups. To compare the delay in the seizure onset and seizure duration a one way ANOVA and Tukey's post-

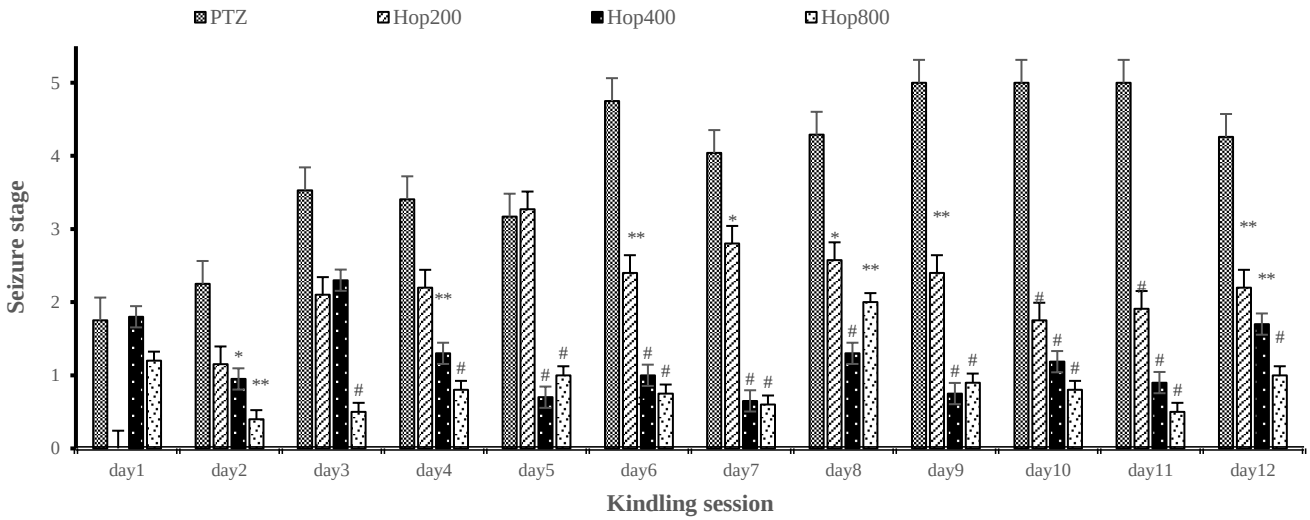

Figure2. The effects of aqueous extract of cone leave of H.L on seizure stages. These results demonstrated the extracts of Hop significantly inhibit the progression of seizure. *: P <0.05, **: P <0.01, #: P <0.01.

hoc test was used. A P <0.05 was considered statistically significant**.**

RESULTS

Effects of various doses of H.L cone leaf extracts on progression of seizure stages

Animals of kindling group were kindled with an average of 12 PTZ injections. Therefore, the animals in the treatment groups received 12 injections.

In figure 2, the progression of seizure stages in kindling and treatment groups (400, 600 and 800 mg/kg) were compared over the course of the day. As seen in the chart, H.L cone leaf extracts in the treatment groups has inhibitory effects on the kindling process. None of the treatment groups showed stage 5 seizure. There is a significant difference in the progression of seizure stages between kindling and treatment groups (P <0.001).

Effect of different doses of H.L cone leaves extracts on latency to onset of seizure

In figure. 3, the latency to onset of seizure was compared between the treatment groups and the kindle group. Data is presented as mean ± standard deviation. As shown in the diagram, H.L

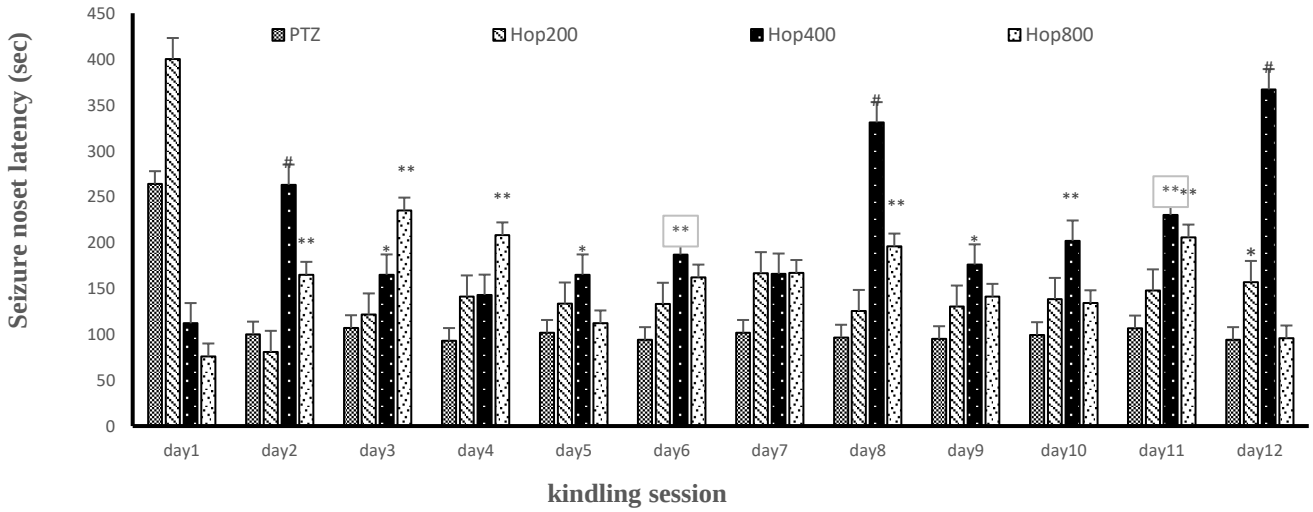

Figure 3. The effects of aqueous extract of cone leave of H.L on latency of seizure beginning. The results demonstrated the hope extracts significantly prolonged the initiation of seizure. *: P <0.05, **: P <0.01, #: P <0.01.

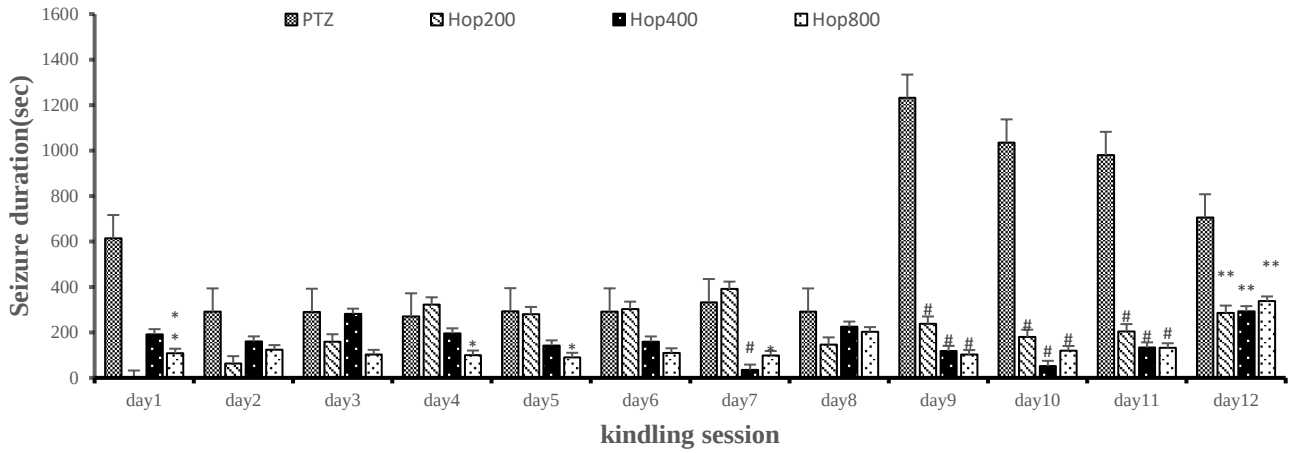

Figure 4. The effects of aqueous extract of cone leave of H.L on seizure duration. This finding showed hope extracts have significant reducing effects on seizure duration. *: P <0.05, $: P <0.01, #: P <0.01.

cone leaves extracts in the treatment groups has increased the duration of the onset of seizure compared to the kindle group. Statistical analysis shows that there is a significant difference between treatment groups and kindle group in all days (P <0.05).

The effect of different doses of H.L cone leaves extracts on the duration of seizure

In figure 4, the duration of seizure was compared between treatments and kindle groups. Data is presented as mean ± standard deviation. As shown in the diagram, H.L cone leaves extracts in the treatment groups has decreased the duration of seizure compared to kindle group. All doses of H.L cone leaves extracts had a nearly identical effect on seizure duration. Statistical analysis showed a significant difference between the treatment groups and the kindle group (P <0.001).

The H&E-staining the coronal sections of the hippocampal CA3 area showed the regular structures of the CA3 region where the pyramidal cell layer neurons (P) have a uniform size and distribution in the control group. Each neuron had a rounded central nucleus with a prominent nucleolus (Figure A). The equivalent section from kindling group did not exhibit uniform size and distribution of pyramidal neurons. Hyperchromatic nuclei (thick arrows) and vacuolated cytoplasm were detected in kindling group (Figure B).

Interestingly, administration of hope extract to treatment groups could protect CA3 pyramidal neurons against PTZ kindling, so that their appearance was similar to control group (Figure C).

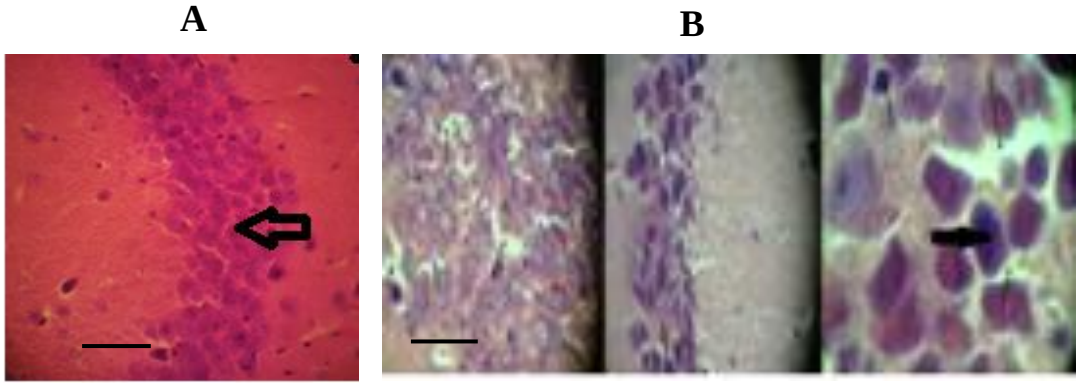

Figure 6. A: Pyramidal layer of CA3 region of hippocampus in control group has uniform size and distribution. Neurons that demonstrated by thick arrows. B: In kindle group hyperchromatic nuclei (dark arrows) and vacuolated cytoplasm showed in kindling gropes. Scale bar: 100µm.

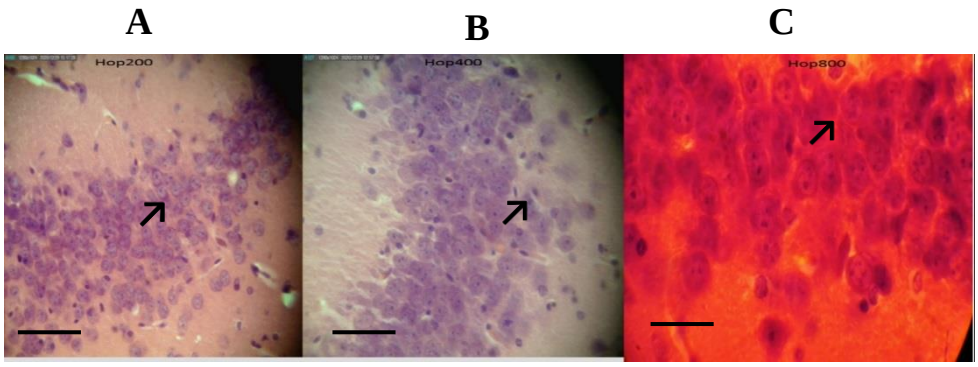

Figure 7. A: Pyramidal layer of CA3 region of hippocampus in dose of 200 mg/kg H.L, B: in dose of 400 mg/kg H.l and C in dose of 800 mg/kg H.l. Neurons have uniform of size and distribution in pyramidal layer. Scale bar: 100 µm.

DISCUSSION

The results of this study demonstrated that the hope extract led to a decrease in the severity and duration of the seizures while the initiation of seizures was delayed in comparison to the PTZ control group. Furthermore, results suggest that the Hops extracts with the dosage of 200 mg/kg doesn't produce effective anti-seizure effects, although, 400 and 800 mg/kg doses of the extract where more potent probably due to existence of secondary metabolites. The hops metabolite can affect the inhibitory receptors of GABA, and sodium, calcium, and potassium channels meaning that it may compete with anti-seizure therapeutics like diazepam and phenytoin in the inhibition of seizures in epilepsy. Furthermore, neuroprotective effect of the hops extract, was observed against PTZ kindling induced apoptosis of pyramidal CA3 area and then, it may prevent the hippocampal sclerosis due to repetitive seizures.

The hops extract exhibits anti-seizure effects on pentylenetetrazole-induced epilepsy, considering that 30 minutes before injection of the PTZ, the hops extract and the plant metabolites can enter into the brain through blood circulation and lead to inhibition of brain hyperactivity so injection of pentylenetetrazole and its binding to the picrotoxin site of GABA receptors will be less effective and thus anti-seizure effects can be achieved.

Results of this study have also suggested that the hops extract leads to a delay in the initiation of the seizure and progress of the seizure activity. In the initiation and spread of seizures, Cortical and subcortical circuits are involved in local and general seizures, deep frontal cortex is connected

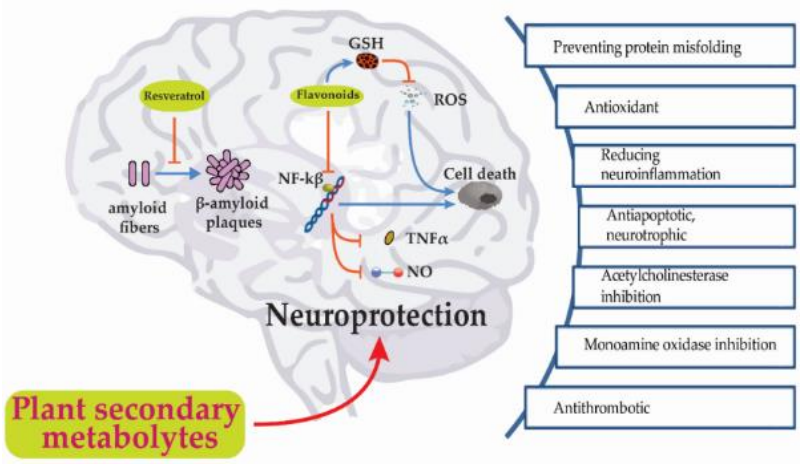

Figure 7. Neuron protection by plants secondary metabolites

to distinct regions of the primary olfactory cortex which seems to control initiation and the spread of the seizures, in addition, studies suggest that GABA antagonist or glutamate agonist injection in this region leads to initiation of convulsions [25].

Considering that in the hops flavonoids are the agonist of the GABA receptor, these compounds seem to affect the initiation and the spreading circuit by binding to GABA receptors thus inhibiting seizure events, furthermore, results suggest that some of the hops compounds lead to a decrease in brain stimulation and increase in brain inhibition leading to shorter duration of seizure events meaning that excitability of the brain which increase due to injection of PTZ, is reduced due to presence of the phytochemical agents that inhibit brain activity [26].

The effectiveness of plant's secondary derivatives on neurodegenerative disorders has been established in both in vitro and in vivo studies, recent studies have shown that secondary compounds including polyphenolic substances like flavonoids, phenolic acids, alkaloids, carotenoid' and terpenes possess great potential as neurodegenerative therapeutics as well as others complex pathophysiological disorders [27]. Studies conducted on the hops plant have established the presence of various compounds including terpenoids, flavonoids, and bitter acids (which include α and β acids) which leads to the hops sedative, anti-cancer, anti-inflammatory, anti-fungal, and anti-bacterial properties [14].

Studies have shown the existence of Xanthohumol in the hops plant which possesses anti-cancer, anti-angiogenesis, anti-inflammatory, and antioxidant properties, moreover, 1 to 50 micromole of Xanthohumol leads to tumor suppression through inhibition of the cell cycle and induction of apoptosis on several cancer type cells, Xanthohumol also regulates immune system response through inhibition of TNF-α which is known to cause necroptosis leading to inflammation, in addition, this compound can affect inflammation responses including HIF-1, iNOS, and the formation of free radicals leading to their inhibition and thus exhibiting neuroprotective effects [28].

Flavonoids are among the more important secondary metabolites of plants and can induce glutathione which is a strong antioxidant that prevents active oxygen species formation thus playing roles in oxidative protection of the cells. Flavonoids also inhibit NF-κB and TNF-α which leads to inhibition of inflammation thus preventing neural cell death [27]. Quercetin and routine are the most important flavonoids in the hops and their antioxidant effect has been established through studies, these compounds lead to the biogenesis of mitochondrion which results in the reduction of active oxygen species that leads to the protection of neurons, quercetin can also reduce the toxicity of 6-hydroxydopamine (6-OHDA) which is an organic compound used by researchers in order to induce selective neurodegeneration of the dopaminergic

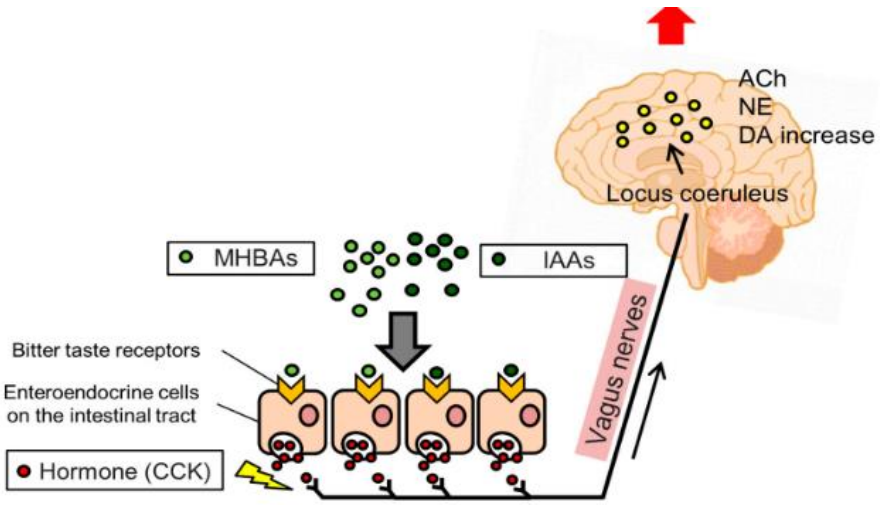

Figure 8. Mechanism of effect of the iso α-acids and bitter acids existing in the Hops

and noradrenergic neurons in the brain. Neural inflammation is among the most vital causes of brain injury, neurodegeneration, cerebral ischemia, and inflammatory diseases of CNS, furthermore, inhibition or reduction of pro-inflammatory substances or induction of anti-inflammatory cytokines is useful in order to prevent chronic inflammation and cell death [28]. Conducted studies on bitter acids existing in the hops have shown that these compounds possess vital functions, Isocohomulon, Isohomulon, Aldohomulon are all iso α-acids present in the hops and possess two cis and trans isomers, these metabolites enhance cognitive function through activation vagus nerve and dopamine signaling. In order to test the effect of iso α-acids on memory with Y-maze test which was used to study memory on the scopolamine-induced memory impairment in rat and mice models, the results suggested that iso α-acids can lead to amelioration of cognitive abilities [29].

Mechanism of the effect of the iso α-acids and bitter acids existing in the hops may be a binding to the bitter receptors existing on enteroendocrine cells and secretion of Cholecystokinin which can induce vagus nerve activity. The induction of vagus nerve by iso α-acids and mature hops bitter acids will lead to an increase in dopamine which in turn increase norepinephrine and acetylcholine that may result in improvement of cognitive functions [29], These are coherent with results observed from our experiment that showed ameliorating effects of the hops on neurons survival in the hippocampus.

CONCLUSION

Overall, it can be concluded that due to the effect of the hops extraction on various variables of the seizure as well as their neuro-protective results observed in the hippocampus, plant-derived compounds can be a more accessible and effective therapeutics with fewer side effects, furthermore, the hops plant is probably among the more promising plants for novel anti-seizure therapeutics.

DECLARATIONS

The authors of this manuscript declare no conflicts of interest whatsoever.

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