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Protective effects of *Origanum majorana* L. against neurodegeneration: fingerprinting, isolation and *In vivo* glycine receptors behavioral model

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Abstract

Extracts from Origanum majorana L. are used in Asia and Africa as sedatives and neurotonics. Few studies done to explore the active chemical constituents responsible for this apparent contrast. The inhibitory glycine receptors (GlyRs) are key mediators of synaptic signaling in spinal cord, brain stem, and higher central nervous system regions. Neurodegeneration may cause alteration of the GlyRs causing strychnine-like convulsions and stiffness. Here, modulation of GlyRs in-vivo was studied in a mouse model of strychnine toxicity. Total phenolics in Egyptian marjoram (Mj-eg) and Lebanese marjoram(Mj-lb) were calculated and fingerprinted. The Mj-eg and Mj-lb ethanolic extracts revealed to be potent modulators to GlyR; with potential anticonvulsant properties in low doses. Chlorogenic acid (CGA), p-coumaric acid (pCA) and p-hydroxy benzoic acid (pHBA) were detected in the active fractions via column chromatography and RP-HPLC fractionation. The active fraction phenolics at harmless low doses, showed anticonvulsant activity by reversing strychnine toxicity in mice. By applying Berenbaum isoblographic method, we confirmed that at low concentrations the protective effects of CGA and pCA on strychnine toxicity are synergistic. It could be concluded that both marjoram and active phenolics could be used as sedatives in low doses and as neurotonics in high doses. In order to fight against neurodegenerative diseases is to improve body antioxidant, marjoram provided to be good sources for antioxidant potential. In brief, the marjoram phenolics, especially CGA, pCA and pHBA, suggested to be novel GlyR modulators, good phytotherapy, pharmacological tool and a dose sensitive drug to treat convulsions, stiffness and neurodegenerative disorders.

Keywords: Phytotherapy; Glycine receptors; Origanum majorana L.; HPLC fingerprinting; Synergism; Neurodegenerative disorders.

Introduction

Historically, Marjoram was known as the "Herb of Happiness" to the Romans. Beside the condiment property, the Egyptian marjoram is used in the folk medicine for its neurotonic activity. On the other hand, the Lebanese marjoram is introduced in Asian folk medicine as a potent sedative [1]. This apparent contradiction raised many questions on the mechanism of marjoram action. Marjoram is rich in phenolic contents [2]. Phenolics are abundant in plants, in which they function in various protective roles. There is increasing evidence for many potential benefits through polyphenolic-mediated regulation of cellular processes such as inflammation and neuroactivity [3]. Inductive or signaling effects by phenolics may occur at concentrations much lower than required for effective radical scavenging [3]. Moreover, phenolics exhibit numerous biological and pharmacological effects, including anti-inflammatory, and anticarcinogenic [4-6], as well as cardioprotective, bacteriostatic, and secretory properties [7]. Some synthetic phenolics have been reported as $GABA_A$ receptor ligands [8]. These all raised serious questions on the content and concentration of effective phenolic compounds in Mj-eg and Mj-lb, and the effect of climate and ecological difference on the phenolic content and use of each. These issues are covered in the current study. The Folin and Ciocalteu method is the standard procedure for determining total phenolics through measuring reducing capacity of components of herbal extract samples [9, 10]. Chemically, chlorogenic acid (CGA) (Table. 1) is a hydroxycinnamate ester of caffeic acid and L-quinic acid [11] and it is mainly found in marjoram, nux vomica seeds, bamboo, and other plants [12]. CGA is used to treat bronchial diseases due to its spasmolytic and antimicrobial properties. Chlorogenic acid has been shown in *in-vitro* studies to inhibit the hydrolysis of the enzyme glucose-6-phosphatase in an irreversible fashion. In addition, *in vivo* studies on animal subjects have demonstrated that the administration of CGA lessens the hyperglycemic peak resulting from the glycogenolysis brought about by the administering of glucagon, a hyperglycemic hormone [13]. According to literature, it was found that the effect of CGA on the GlyR has not been reported yet. *p*-Coumaric acid (pCA) (Table. 1),

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structurally related to CGA, is a naturally occurring phenolic from marjoram as well as peanuts, tomatoes, carrots, and garlic [14-17]. It was found to decrease the excitability of capsaicin-sensitive nociceptive neurons, with much of this decrease occurring as a consequence of its inhibition of voltage-gated sodium channels [18]. *p*-Hydroxybenzoic acid (pHBA) (Fig. 1C) or (*4*-hydroxybenzoic acid) known pharmacological characteristics is its antibacterial properties[19, 20], especially in gastrointestinal infection. Many plants were recognized for their antibacterial effect to contain 4-hydroxybenzoic acid, including: *Pandanus odorus, Piper aduncum* and *Scrophularia frutescens* [21]. It is isomeric with the anti-inflammatory 2-hydroxybenzoic acid (salicylic acid), a precursor to the neuroprotective agent, aspirin [22].

In our previous work, it was proved that phenolics *in vivo* and *in vitro* have a potent modulatory actions on GlyRs [23]. The inhibitory glycine receptor (GlyR) is a member of the cysteine loop superfamily of ligand-gated ion channel receptors. It shares structural similarity with nicotinic acetylcholine [24]. The GlyR is mainly expressed in spinal cord, brain stem, and other regions of the central nervous system, where it mediates rapid synaptic inhibition. The convulsion action of strychnine is due to interference with postsynaptic inhibition mediated by glycine. Glycine is an important inhibitory transmitter to motor neurons and interneurons in the spinal cord, and strychnine acts as a selective, competitive antagonist to block the inhibitory effects of glycine at all glycine receptors [23]. Neurodegeneration may cause alteration of the GlyRs causing strychnine-like convulsions and stiffness [25]. Flavonoids, alcohols and local anesthetics have been shown to modulate GlyRs function. In this study, the aim of combination of marjoram ethanolic extracts or phenolics with strychnine, is to explore the effect of these extracts and phenolics on neuro-degeneration, taking GlyRs as *in vivo* model [23, 25]. Such combinations were used before in traditional medicine [23, 26]. Nevertheless, all extracts and phenolics under investigation were not reported to modulate the GlyR. The combined effects of the phenolic compounds on strychnine lethality may be synergistic or antagonistic. To answer this question we investigated the interaction of these substances via *in vivo* strychnine lethality test.

Here, we examined manipulation of GlyRs modulation by marjoram, and identifying the effective compound responsible of this action employing behavioral tests on mice. Using a strychnine lethality approach, modulation of GlyRs resulting from the coapplication of the marjoram or the effective compound together with strychnine was observed. Modulatory patterns were found *in vivo*, where the mode of modulation could be confirmed in behavioral tests on mice.

Materials and Methods

Plant material

Egyptian *Origanum majorana* L. (Mj-eg) was commercially purchased (Harraz herbalist, Cairo, Egypt). Lebanese *Origanum majorana* L. (Mj-lb) was commercially purchased (Ibn-Al-Nafess herbalist, Beirut, Lebanon). The plant was identified by direct comparison with an authentic sample kept at the Department of Pharmacognosy and Medicinal Plants, Faculty of Pharmacy, Beirut Arab University, Lebanon.

Sample preparation

Aerial parts of Egyptian *Origanum majorana* L. (Mj-eg, 250g), and Lebanese *Origanum majorana* L. (Mj-lb, 250g) were separately dry ground using TCM grinder (TCM, China). Both herbs were processed exactly the same way. Both fine powders were extracted using 2500 ml of 80% ethanol and were stirred for 24 hrs in their ethanol liquors. During which the flasks were covered by aluminum foil to prevent the light damage. After 24 hrs, the extracts were double filtered through a porcelain funnel using 20-25 μM filters. The filtered extracts were well dried using Rotavap (Buchi, Germany) at temperature 40 C under vacuum.

Chromatographic fingerprint of *Origanum marjoram* ethanolic extract

Optimization were done of the HPLC conditions and investigation of columns with different packing materials, different wavelengths from 200 nm to 400 nm and different mobile phase systems. An ideal chromatographic separation was achieved using RP C18 endcapped Lichrospher column (250 x 4.6 mm I.D.; 5 μM particle size) (Merck, Darmstadt, Germany), at 40 C. The separation was performed using a mixture of acetonitrile and phosphate buffer 34.1 mM, pH 2.1 with a gradient elution: 0-5 min (10% acetonitrile), 5-10 min (10-15% acetonitrile), 10-15 min (15-30% acetonitrile), 15-20 min (30-45% acetonitrile), 20-25 min (45-100% acetonitrile), and 25-30 min (100% acetonitrile). The flow rate was set at 1 mL/min. The temperature of the column was set at 40 C.

Bio-guided chromatographic fractionation and identification of the effective compound

The Lebanese marjoram (Mj-lb) was fractionated using column chromatography (CC). Preparative chromatography column was used. Elution was started using diethyl ether–acetone–water–formic acid (50:30:10:10) respectively and normal phase silica gel as stationary phase. During the entire chromatography process the eluent was collected in a series of fractions by time.

Each fraction was tested the same way as the test solutions in this study using *in vivo* behavioral studies on mice. The active fraction was analyzed using RP-HPLC.

HPLC analysis was carried out in a JASCO instrument (JASCO, Japan). Methanol/ phosphate buffer 34.1 mM, pH 2.1, (43:57) at a flow rate of 1 mL/min were used as isocratic mobile phase. Solvents were degassed by an on-line degasser of the ProStar System. The column used was a RP C18 endcapped Lichrospher column (250 x 4.6 mm I.D.; 5 μM particle size) was employed (Merck, Darmstadt, Germany), at 30 C. The injection volume was 20 μL and UV detection was performed using UV detector at 214 nm, which have the highest absorptions for the tested active

fraction as tested using JASCO spectrophotometer (JASCO, Japan).

Calibration and control samples

In order to quantify the amount of major polyphenols of the extract in Mj-eg and Mj-lb, calibration curves of these phenolics were performed. Series of commercial phenolics standard (Sigma-Aldrich, Germany) known concentrations (1-30 μg/ml) in methanol were injected using the RP-HPLC and calibration curve for concentrations versus peak areas were plotted. Methanol/ phosphate buffer 3.41 mM, pH 2.1, (43:57) at a flow rate of 1 mL/min were used as isocratic mobile phase and measured at 254 nm.

Specificity and limit of quantitation

Specificity of the method was verified by comparing the chromatograms of six blank samples before and after each run. In blanks, no signal at the retention times of the compounds under investigation was detected. The lowest concentration of the analyte measured with acceptable precision (relative standard deviation 10%) is defined as lower limit of quantification (LLOQ). The LLOQs were 1.0 μg/mL for the phenolics under investigation.

Linearity, precision and accuracy

Linearity and precision were proven comparing the slope and the correlation coefficient of four calibration curves on four different days. Good linearity for the assay ($r^2 > 0.998$) was found over the investigated calibration range of 1.0–30 μg/mL for phenolics under investigation. The relative standard deviations of the slopes of the four calibration curves were below 10%.

The intra-day precision and accuracy as well as the inter-day precision and accuracy were proven by comparing three different extracts and/or phenolic samples (n = 4). The values were situated within the range of the calibration curve. Intra- and inter-day precision and accuracy were found acceptable, with relative standard deviations lower than 15%.

Behavioral studies

Male Swiss-Webster mice (Faculty of Pharmacy, Beirut Arab University (BAU), Beirut, Lebanon) were housed for 1 week prior to experimen-tation. The environment consisted of standard mouse cages with a 12-h light/dark cycle. The temperature was 22 ± 1 C, animals had free access to water and standard laboratory pellets (20% proteins, 5% fats, and 1% multivitamins [23]. All animal care and experiments were done in accordance with animal experiment legislation and with approval of the local ethics commission. All data were tested for significance using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

Tonic extensor convulsion and toxicity tests

Male mice with an average weight of 18–22 g were used. Reference mice received a single ip injection of 2 mg/kg strychnine to determine the pharmacological end point for tonic extensor convulsions (TECs) and lethality. Control mice were injected ip with 0.2 ml Dimethyl sulfoxide (DMSO) followed 30 min later by an ip injection of 2 mg/kg strychnine in DMSO [23]. Test mice were injected ip with Egyptian marjoram (Mj-eg), Lebanese Marjoram (Mj-lb) or Mj-lb active fraction in doses ranging from 0.1 to 10 mg/kg, and 30 min later injected ip with 2 mg/kg strychnine. Mice were observed over a period of 1 h and the time until occurrence of TECs and death recorded. The first presence of tonus in the hind limbs with stretching was taken as TEC onset, whereas death, preceded by clonic convulsions and tonic seizures, was the pharmacological end point. Another control group of mice was injected with Mj-eg, Mj-lb or Mj-lb active fraction only; in doses up to 10 mg/kg, neither of the test compounds (except strychnine) alone produced convulsions or toxicity. Synergism effects of CGA and pCA on the strychnine lethality were calculated by the algorism of Berenbaum [27], where *IF* is the interaction factor:

$$\frac{\text{Concentration of CGA in combination}}{\text{Equi-effective concentration of CGA alone}}+\frac{\text{Concentration of pCA in combination}}{\text{Equi-effective concentration of pCA}}=IF$$

The equi-effective concentrations of the drugs are taken from the lethality graphs and represent those concentrations at which CGA or pCA alone showed the same effect on strychnine lethality as in combination. An interaction factor of <1 indicates synergistic, a factor of 1 additive, and a factor of >1 antagonistic effects [28].

Determination of total phenolic compounds in the extracts

Generally, measurement of color occurred by reaction between Folin-Ciocalteu's phenol reagent [29, 30], and this method is a preferred method for the determination of the phenolic compounds present in plants, because the majority of plant antioxidants are polyphenols [10, 29]. Total contents of the phenolic compounds in the extracts were determined by Folin-Ciocalteu's method[30] as gallic acid equivalents (GAE) [10, 31]. Briefly, 250 μl of Folin-Ciocalteu's phenol reagent was mixed with 50 μL of the samples, and 500 μl of 20% water solution of Na_2CO_3 was added to the mixture. Mixtures were vortexed and completed with water to 5 mL. As control, reagent without adding extract was used. After incubation of the samples at room temperature for 30 min, their absorbance was measured at 765 nm. The calibration curve created by using fresh prepared gallic acid solutions was used as a base in calculations of total phenolic compound contents in the extracts. Experiments were repeated 3 times for every extract and the total phenolics were given in average values as GAE (mg gallic acid/g extract) [32]. For the calibration curve, 10 mg of gallic acid was dissolved in 10 mL of MeOH using an ultrasonic bath (stock solution). Different dilutions of stock solution were prepared and were determined by Folin-Ciocalteu's method [30, 33]. Experiments were repeated three times for every dilution and a calibration curve was created [31].

Determination of Antioxidant Activity with the 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

The antioxidant activity of Mj-eg and Mj-lb were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. A methanolic stock solution of Mj-eg and Mj-lb (concentrations of stock solutions were 15, 10, 5, 1, 0.1 and 0.05 mg/mL) was put into a cuvette, and 2 mL of 4 mg/ml methanolic solution of DPPH was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined with a JASCO spectrophoto-meter (JASCO, Japan) at zero time, 20, 40 and 60 mins for all samples. Methanol was used to zero the spectrophotometer. Absorbance of the DPPH radical without antioxidant, i.e. the control, was measured daily. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution. Percent inhibition of the DPPH radical by the samples was calculated according to the formula of Yen & Duh: % inhibition = [(AC(o) – AA(t)) / AC(o)] * 100. Where AC(o) is the absorbance of the control at t = 0 min and AA(t) is the absorbance of the antioxidant at t = 1 h.

Results and Discussion

The variability of phenolic acid concentrations in plants under various physiological stress situations raises questions concerning the ecological significance of such behavior [34]. Phenolic compounds, like CGA, *p*CA and *p*HBA (Table 1) have been shown to be present in Egyptian and Lebanese *Origanum majorana*. *Origanum majorana* has been used in folkloric medicine as sedative and neurotonic. The mechanism of this apparent contradiction was not well understood. We studied the effects of the Egyptian and Lebanese *Origanum majorana*, fractions, effective fractions, single phenolics alone, and in combination with strychnine. The inhibitory glycine receptors (GlyRs) is a member of the cysteine loop superfamily of ligand-gated ion channel receptors. Preliminary electro-physiological studies using patch-clamp and the Mj-eg shown that the later modulates the *in vitro* homomeric 1 GlyR transfected on HEK 293 cells. This effect was mainly investigated in this study using *in vivo* behavioral studies on mice.

Chromatographic fingerprint of *Origanum marjoram* ethanolic extract

Mj-eg and Mj-lb ethanolic extracts were injected in the HPLC instrument to study their fingerprint pattern. We obtained 21 chromatographic peaks, from which 13 peaks were confirmed using commercial reference standards and were in accordance with previously reported *Origanum marjoram* ethanolic extract samples [2]. They were CGA (Peak No.1), pCA (Peak No.2), pHBA (Peak No.3), Quercetin-3-O-glucoside (Peak No.4), Gallocatechin (Peak No.5), Luteolin-7-O-glucoside (Peak No.6), Ferulic acid (Peak No.7), Phloridzin (Peak No.8), Apigenin-7- O-glucoside (Peak No.9), Rosmarinic acid (Peak No.10), Quercetin (Peak No.11), Apigenin (Peak No.12), Luteolin (Peak No.13) (Fig 1A for Mj-eg and 1B for Mj-lb). Both Mj-eg (Fig. 1A) and Mj-lb (Fig. 1B) ethanolic extracts have identical fingerprint peaks except in concentrations and the presence of Apigenin-7- O-glucoside (Peak No.9) and Rosmarinic acid (Peak No.10). It was found that Apigenin-7- O-glucoside and Rosmarinic acid are only abundant in Mj-eg ethanolic extract (Fig. 1). The concentrations of the major polyphenols were measured using the area under peaks, where the sample peak areas are directly related to the amount of analyte in each sample. These peak areas were used with the calibration curves to quantify the amount of analyte in each sample (Fig. 2 and Table 1).

Bio-guided chromatographic fractionation and identification of the effective compound

The Lebanese marjoram (Mj-lb) was fractionated using column chromatography (CC). During the entire chromatography process the eluent was collected in a series of fractions by time. Each fraction was tested the same way as the test solutions in this study using *in vivo* behavioral studies on mice. The active fraction was analyzed using RP-HPLC . Methanol/ phosphate buffer 3.41 mM, pH 2.1, (43:57) at a flow rate of 1 mL/min were used as isocratic mobile phase. The *in vivo* active fraction of Mj-eg found to contain about (4.83 ± 0.02 % CGA, 5.82 ± 0.01 % pCA and 6.92 ± 0.03 % pHBA), while Mj-lb contain about (12.37 ± 0.03 % CGA, 2.93 ± 0.03 % pCA and 5.54 ± 0.05 % pHBA) using standard calibration curve at 254 nm (Table 1).

TEC and Lethality Glycine Receptors Behavioral Model

When administered alone, Egyptian and Lebanese *Origanum majorana* did not produce any convulsions. Inhibition by simultaneous application of strychnine, and effective fractions and single phenolics. None of the tested animals showed any TECs, and test compounds (except strychnine) alone were not otherwise toxic even in highest concentration tested (10mg/kg) or lethal in any of the test animals. In contrast, an injection of strychnine alone (2 mg/kg ip) was lethal in all tested animals (Fig. 2 A-C). TEC set in after 7.85 ± 0.35 min and death occurred after 8.5 ± 0.42 min (n = 4). In mice that had been preinjected with either Egyptian marjoram (Mj-eg) or Lebanese Marjoram (Mj-lb) (0.1, 1 and 10 mg/kg ip). The toxic effects of strychnine were significantly reduced to about 2 folds in the relatively low concentrations (0.1 mg/kg ip) of either Mj-eg (TEC 15.6 ± 0.8 min and lethality 23.3 ±1.3 min) or Mj-lb (TEC 16.5 ± 1.1 min and lethality 24.45 ± 1.2 min) (Fig. 2A). The reduction of toxicity was significantly lowered with increasing the concentration of Mj-eg (1 mg/kg ip) (TEC 9.85 ± 0.2 min and lethality 17.23 ± 0.5min). The Mj-lb (1 mg/kg ip) TEC's produced by strychnine was not significantly different from the control, while strychnine lethality was decreased. Surprisingly, the highest dose of Mj-eg (10 mg/kg ip) the TEC's of strychnine was significantly aggravated (4.45 ± 1.5 min) and lethality, but strychnine lethality was decreased (11.24 ± 1.4 min). On the other hand, the highest dose of Mj-lb (10 mg/kg ip) TEC's produced by strychnine was not

significantly different from the control, while strychnine lethality was decreased (13.3 ± 1.0 min). Therefore, as expected form GlyRs modulators, it could be concluded that either Mj-eg or Mj-lb when given together with strychnine, in low concentrations reduce strychnine toxicity, as expected from sedatives to potentiate the inhibitory GlyRs. Phenolic compounds, like CGA, pCA and pHBA were detected in the Mj-eg and Mj-lb active fractions via RP-HPLC fractionation and separation method. These phenolics evidenced to be a novel GlyRs modulator via *in vivo* strychnine lethality test. CGA, pCA and/or pHBA (0.005, 50 and 500 μg/ml) alone or in combination were tried as the previous extracts. Phenolics did not show alone any TECs or death even in the highest tested concentration (500 μg/kg ip) (Fig. 2A-B). The tested phenolics concentrations are chosen according the data derived from phenolics HPLC standard calibration curve. The toxic effects of strychnine were significantly reduced to about 1.5 folds in the relatively low CGA concentration (0.005 μg/kg ip) (TEC 11.9 ± 0.9 min and lethality 13.3 ± 1.1 min). The increase of toxicity was not significantly elevated with increasing the concentration of CGA (50 μg/kg ip) (TEC 7.2 ± 0.6 min and lethality 8.1 ± 0.5 min). The highest CGA (500 μg/kg ip) the TEC's and lethality of strychnine was significantly aggravated (TEC 3.18 ± 0.2 1.5 min and lethality 5.25 ± 0.3 min) (Fig. 2A). The toxic effects of strychnine were significantly reduced to about 4 folds in the relatively low pCA concentration (0.005 μg/kg ip) (TEC 23.06 ± 1.3 min and lethality 24.17 ± 1.2 min). The increase of toxicity was not significantly elevated with increasing the concentration of pCA (50 μg/kg ip) (TEC 7.5 ± 1.0 min and lethality 11.5 ± 1.28 min). The highest pCA (500 μg/kg ip) the TEC's and lethality of strychnine was significantly aggravated (TEC 3.98 ± 0.9 min) and lethality 6.75 ± 0.7 min). The toxic effects of strychnine were significantly reduced to about 3 folds in the relatively low pHBA concentration (0.005 μg/kg ip) (TEC 20.06 ± 1.0 min and lethality 32.17 ± 1.1 min) (Fig. 2B). The protection effect was significantly decreased with increasing the concentration of pHBA (50 μg/kg ip) (TEC 11.5 ±1.2 min and lethality 25.5 ± 1.2 min). Surprisingly, the highest pHBA concentration (500 μg/kg ip) the TEC's and lethality of strychnine was significantly decreased (TEC 19.0 ± 0.9 min and lethality 21.8 ± 0.7 min) (Fig. 2B).

Synergistic *In Vivo* dose dependent modulation of Glycinergic Transmission

In order to find the protective effect of combination of the active polyphenols towards strychnine toxicity, the effect of different concentrations of CGA and pCA was analyzed by isoblographic method of Berenbaum. Using isoblographic method of Berenbaum [27]. CGA was combined at concentrations of 5–10 ng/kg with pCA at concentrations from 5 to 10 ng/kg the effects were synergistic (Fig.2C; 3A-B). Mediating its protection towards strychnine toxicity, we could show in our experiments that at low concentrations the protective effects of 5 ng/kg (EC50) and 10 ng/kg (EC95) of both CGA and pCA on strychnine toxicity are synergistic (Fig. 3 A-B). Independent the exact mechanisms by which CGA is this synergism could allow to decrease the lethality of strychnine without increasing the dose, which indeed will not increase the protection as the synergistic combination will do.

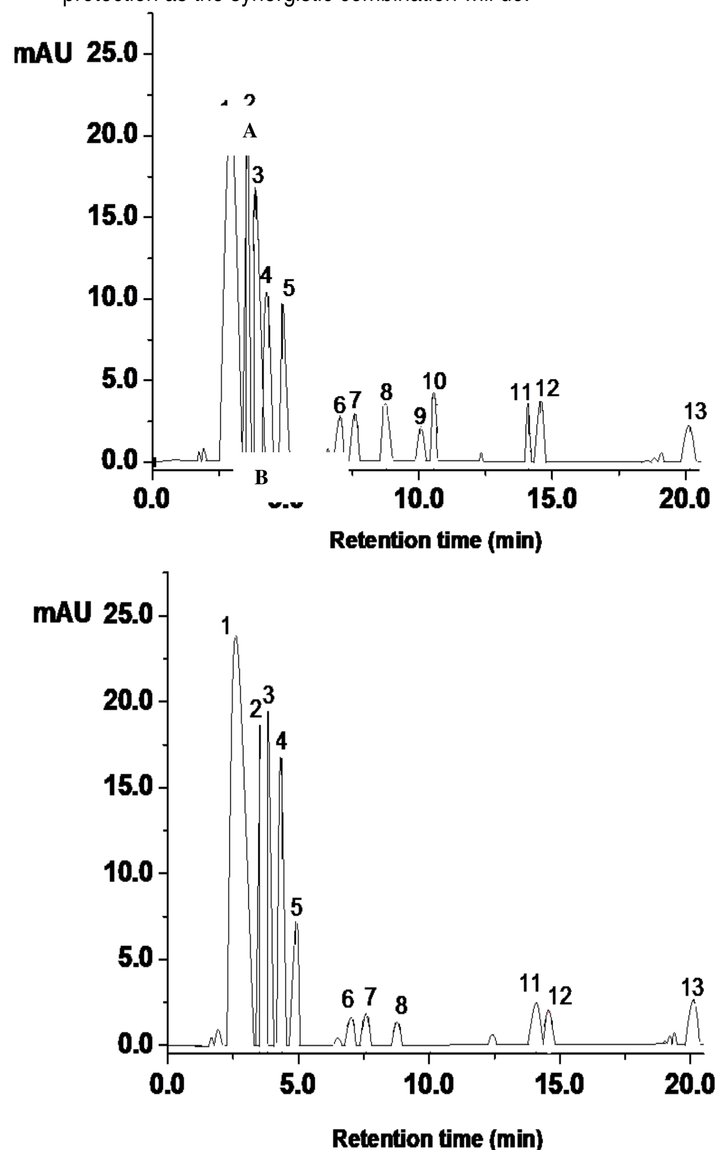
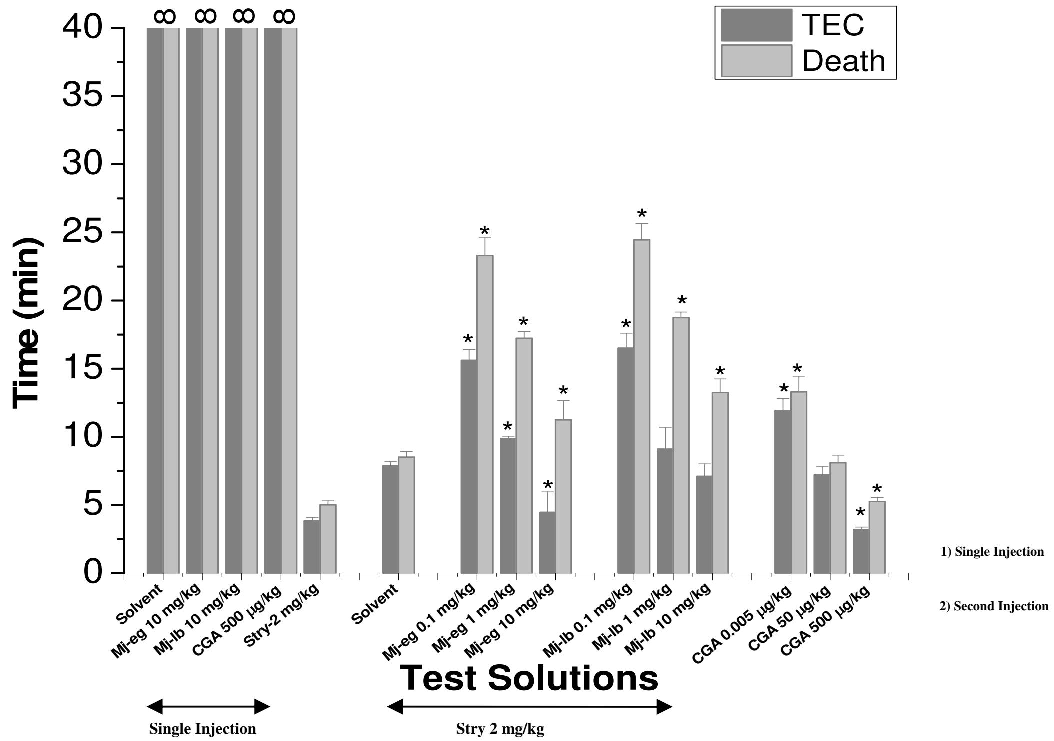


Figure.1 The HPLC fingerprint pattern of *Origanum marjoram.*
1---CGA 2---pCA 3---pHBA
4---Quercetin-3-*O*-glucoside 5---Gallocatechin
6---Luteolin-7- *O-glucoside* 7---Ferulic acid
8---Phloridzin 9---Apigenin-7- *O-glucoside*
10---Rosmarinic acid 11---Quercetin
12--- Apigenin 13---Luteolin
(A)--- Mj-eg (B)---Mj-lb

A



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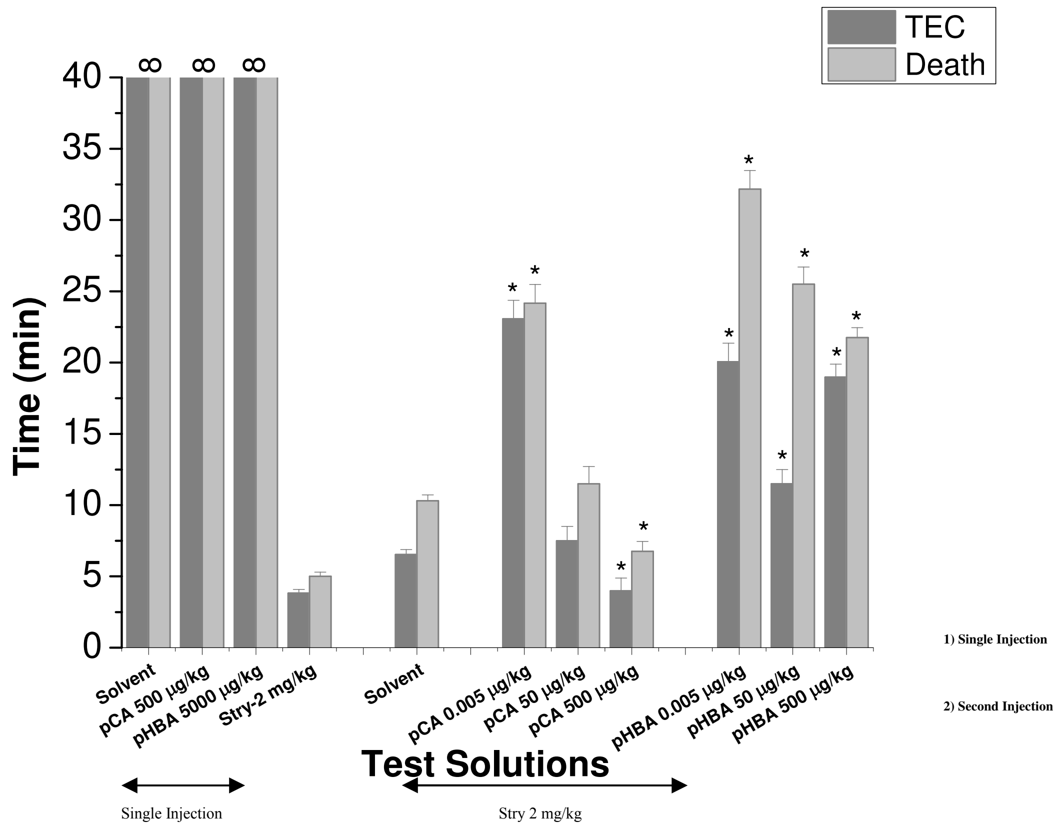

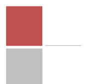

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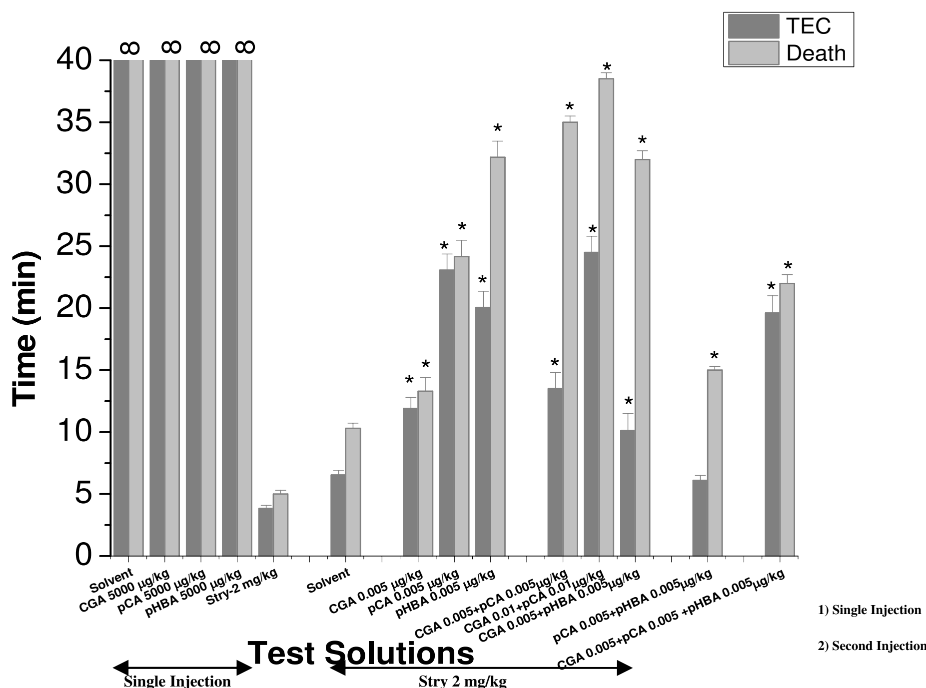
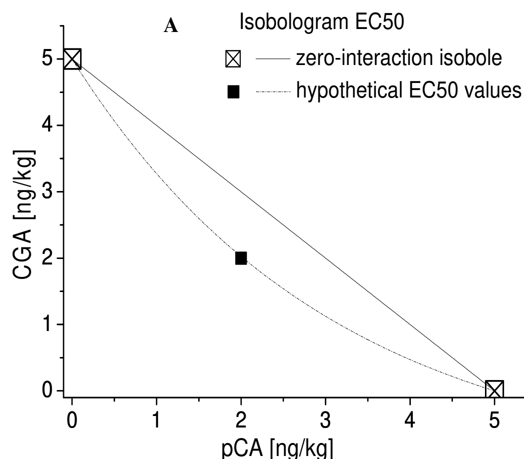


Figure. 2. Tonic extensor convulsion (TEC) an death test. See text for experimental conditions. Data are presented as mean ± SD; asterisks denote significant difference from control (solvent) (one-way ANOVA, p 0.05). indicates no occurrence of tremors and/or survival of the animal. Taurine (Tau) 0.1 mg/kg as positive control. The time until occurrence of TECs and death is plotted (minutes ± SD), throughout a 60-min period. (A) In vivo test of GlyRs potentiation by marjoram isolated from Origanum majorana leaves and chlorogenic acid (CGA). Mice were treated ip with Mj-eg (0.1, 1 and 10 mg/kg), Mj-lb (0.1, 1 and 10 mg/kg) or CGA (0.005, 50 and 500 g/ml) or control (vehicle), 30 min later the mice were injected with 2 mg/kg strychnine nitrate ip. (B) In vivo test of GlyRs potentiation by p-Coumaric acid (pCA) or p-Hydroxybenzoic acid (pHBA) . Mice were treated ip with pCA (0.005, 50 and 500 g/kg), pHBA (0.005, 50 and 500 g/kg) or control (vehicle), 30 min later the mice were injected with 2 mg/kg strychnine nitrate ip. (C) In vivo test of GlyRs potentiation by chlorogenic acid (CGA) and/or p-Coumaric acid (pCA) and/or p-Hydroxy Benzoic acid (pHBA). Mice were treated ip with solely or in combination of CGA (0.005 g/kg) and/or pCA (0.005 g/kg) and/or pCA (0.005 g/kg) or control (vehicle), 30 min later the mice were injected with 2 mg/kg strychnine nitrate ip.

Phenolics and Strychnos Nux vomica

Neuroactive phenolics under investigation
(CGA, pCA and pHBA) proved to show the same *in vivo* behavior towards strychnine lethality as before with marjoram extracts under investigation. Mj-eg when given together with strychnine, in high concentrations increase strychnine TEC, as expected from neurotonics to inhibit the inhibitory GlyRs. The Mj-lb showed to be more potent protecting agent to strychnine toxicity in relatively low concentrations, in comparison with Mj-eg. Moreover, both Mj-eg and Mj-lb relatively have the same pattern of modulating the GlyRs, so it could be concluded that they have the same effective phenolic compounds (CGA, pCA and pHBA). *Strychnos nux- vomica* seeds,LD_{50} = 256 mg/kg [35], contain both strychnine, LD_{50} = 0.98 mg/kg [36], and neuroactive phenolics, like *Origanum*. By comparing our results with the previous facts and due to presence of the neuroactive phenolics in comparatively low doses in the unprocessed nux vomica seed powder, we can conclude that these phenolics may be the cause of this 12 folds safety compared to using strychnine alone.



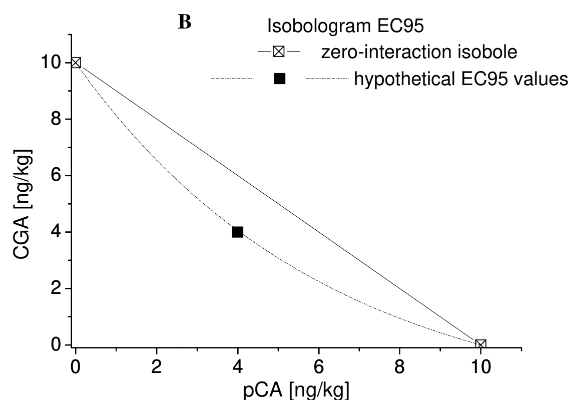


Figure. 3. The synergism (at low concentrations) of the combination of CGA and pCA on the altering strychnine lethality is shown in (Figure. 4). Isobolograms of the effective concentrations EC50 and EC95 with the zero-interaction isoboles and hypothetical EC (A-B) are shown.

Table. 1. Major phenolic content using RP-HPLC of the ethanolic extracts of Egyptian Marjoram (Mj-eg) and Lebanese Marjoram (Mj-lb)

Peak No.	Major olyphenols	Chemical Structure	RT[a](min)	Mj-eg conc.using RP- PLC(%)[b]	Mj-lbconc.using RP-HPLC(%)[b]
1	CGA		3.0	4.83 ± 0.02	12.37 ± 0.03
2	pCA		3.5	5.82 ± 0.01	2.93 ± 0.03
3	pHBA		3.8	6.92 ± 0.03	5.54 ± 0.05
4	Quercetin-3-O-glucoside		4.4	8.31 ± 0.05	10.53 ± 0.06
5	Gallo-catechin		4.9	2.57 ± 0.03	4.86 ± 0.03
6	Luteolin-7- O-glucoside		7.0	1.55 ± 0.01	1.14 ± 0.01
7	Ferulic acid		7.6	1.59 ± 0.01	1.42 ± 0.01
8	Phloridzin		8.8	1.50 ± 0.01	0.82 ± 0.01
9	Apigenin-7- O-glucoside		10.0	1.58 ± 0.01	0.01 ± 0.01
10	Rosmarinic acid		10.6	1.44 ± 0.01	0.01 ± 0.01
11	Quercetin		14.1	2.50 ± 0.03	2.15 ± 0.01
12	Apigenin		14.6	2.86 ± 0.02	1.49 ± 0.01
13	Luteolin		20.2	1.11 ± 0.01	4.66 ± 0.03

[a] RT = Retention Times compared to commercial reference standards
[b] Expressed as the mean percentage (n=4 for each value) ± standard deviation (SD)

Table. 2. Total phenolic content using Folin–Ciocalteu Reagent (FCR), and Strychnine (STR) protective (TEC and lethality) capacity (*In vivo*) at the lowest concentration, of the ethanolic extracts of Egyptian Marjoram (Mj-eg) and Lebanese Marjoram (Mj-lb)

	Total Phenolics using FCR Assay (mg gallic acid/g extract)	STR-TEC Protective Capacity (*In vivo)* at Mj- Lowest Concentration (%)	STR-Lethality Protective Capacity (*In vivo)* at Mj- Lowest Concentration (%)
Mj-eg	17.15 ± 0.10	49.7 ± 0.6	63.5 ± 0.7
Mj-lb	7.48 ± 0.06	52.4 ± 0.7	65.2 ± 0.7

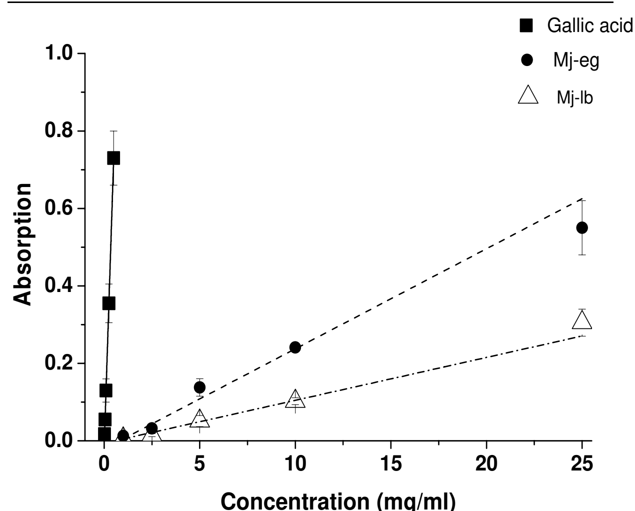


Figure. 4. Folin-Ciocalteu assay of Egyptian Marjoram (Mj-eg) and Lebanese Marjoram (Mj-lb) and relate its results to standard Gallic acid. (Solid squares and solid line) Standard Gallic acid in concentrations (0.025, 0.05, 0.1, 0.25 and 0.5 mg/ml), straight line equation: Y=1.5X-0.02. (Solid circles, dashed line) Mj-eg in concentrations (1, 2.5, 5, 10 and 25 mg/ml), straight line equation: Y=0.026X-0.02. (Open circles, dashed-dotted line) Mj-lb in concentrations (1, 2.5, 5, 10 and 25 mg/ml), straight line equation: Y=0.011X-0.006. The total phenolics in Mj-eg = 17.154 ± 0.100 mg gallic acid/g extract. The total phenolics in Mj-lb = 7.483 ± 0.060 mg gallic acid/g extract. All absorbance were measured at 765 nm. Results are (mean ± SD) of three parallel measurements. Gallic acid curve R^2 of 0.9999. $P < 0.01$, when compared to the control.

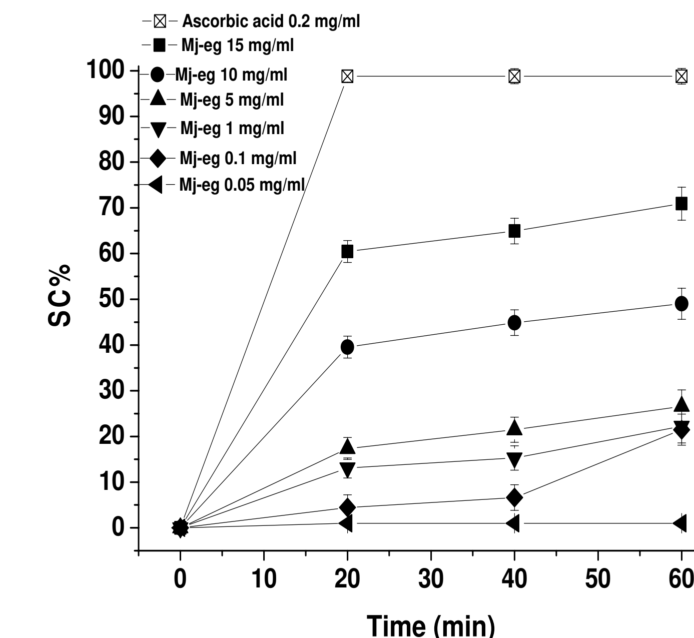
Total phenolic compounds Using FCR Assay

It was found that total phenolic compound (Table 1) extracted from Mj-eg is 17.154 ± 0.100 mg gallic acid/g Mj-eg extract. While that of Mj-lb is 7.483 ± 0.060 mg gallic acid/g Mj-lb extract (Fig. 4).
Total phenolic compounds as Gallic acid equivalent (GAE) of Mj-lb was even lower than that of Mj-eg which was reflected as difference on their activity.

This variation may be due to climate differences between Lebanon and Egypt. Lebanon has a Mediterranean climate characterized by a long, hot, and dry summer, and cool, rainy winter [37] while, Egypt has an arid desert climate. It is hot or warm during the day, and cool at night [38]. Surprisingly, we found that Mj-lb, although having lower phenolic compounds we found that it has relatively higher protecting ability towards strychnine TECs than Mj-eg, with higher phenolic content (Fig. 2A). This protecting ability is attributed to that marjoram phenolics, in low doses potentiates the inhibitory glycine receptor which is reflected in marjoram protection towards strychnine TECs. While, in relatively high doses, marjoram, inhibits the inhibitory glycine receptor which is revealed in its aggravation to strychnine lethality (Fig. 2A).

Origanum marjoram radical scavenging activity on DPPH

A concentration-dependent assay was carried out with Mj-eg and Mj-lb, and the results are presented in Fig. 4 A-B. These results provide a direct comparison of the antioxidant activity with ascorbic acid (AA). Both, Mj-eg and Mj-lb possessed significant scavenging activity on the DPPH radical and acted as an antioxidant. The scavenging effect was increased with increasing concentration and reaction time (Fig. 6). Mj-eg showed a higher scavenging activity than Mj-lb at all concentrations (0.05, 0.1, 1, 5, 10 and 15 mg/ml). At steady state, when the absorbance of the antioxidant at t = 1 h, after t the same concentrations (0.05, 0.1, 1, 5, 10 and 15 mg/ml) respectively, Mj-eg exhibited a DPPH scavenging activity of 1.0 ± 0.6%, 21.5 ± 3.4%, 22.2 ± 3.6%, 26.6 ± 3.6%, 49.0 ± 3.4%, and 70.9 ± 3.6 whilst Mj-lb showed 0.75 ± 0.4%, 4.8 ± 3.4%, 9.0 ± 2.6%, 15.0 ± 3.0%, 37.3 ± 3.6 and 58.8 ± 3.6%. IC_{50} (Mj-eg) = 9.63 ± 0.80 mg/ml, while the IC_{50} (Mj-lb) = 13.10 ± 1.10 mg/ml. Hence, the Mj-eg had higher scavenging potential than Mj-lb, when absorbance were measured at 517 nm, IC_{50} (Mj-eg) = 9.63 ± 0.80 mg/ml, while the IC_{50} (Mj-lb) = 13.10 ± 1.10 mg/ml. Results are (mean ± SD) of three parallel measurements. $P < 0.01$, when compared to the control AA. Both, Mj-eg and Mj-lb had approximately the same speed of radical scavenging, which is leading the way that they both had the same effective phenolic compounds but with different concentrations (Table 1). The latter concentration was evidenced in the next section when calculating the total phenolic compounds using FCR. Active fraction phenolics possessed significant scavenging activity on the DPPH radical and acted as an antioxidant. The scavenging effect was increased with increasing concentration and reaction time (Fig. 6).



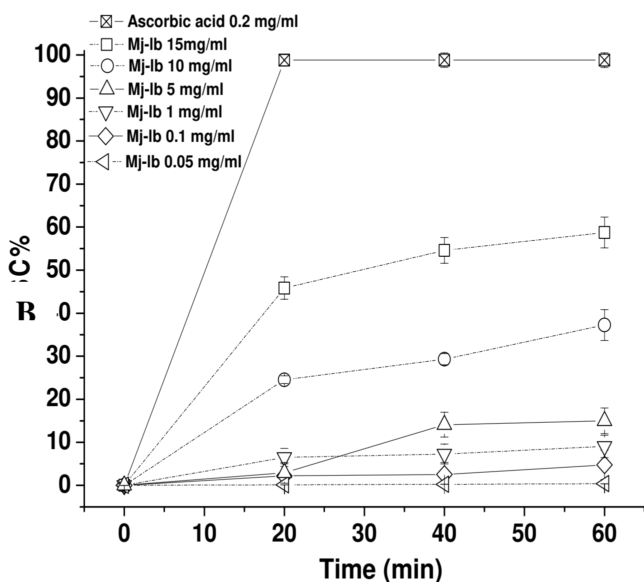


Figure. 5. DPPH free radical scavenging activity of Egyptian Marjoram (Mj-eg), Lebanese Marjoram (Mj-lb) and positive control Ascorbic acid. (A) (Solid squares and solid line) 15 mg/ml Mj-eg, (Solid circles and solid line) 10 mg/ml Mj-eg, (Solid up-triangles and solid line) 5 mg/ml Mj-eg. (Solid down-triangles and solid line) 1 mg/ml Mj-eg, (Solid diamonds and solid line) 0.1 mg/ml Mj-eg, (Solid left-triangles and solid line) 0.05 mg/ml Mj-eg and (Open crossed-squares and solid line) 0.2 mg/ml Ascorbic acid as positive control. (B) (Open squares and dashed-line) 15 mg/ml Mj-lb, (Open circles and dashed-line) 10 mg/ml Mj-lb, (Open up-triangles and solid line) 5 mg/ml Mj-lb. (Open down-triangles and dashed-line) 1mg/ml Mj-lb, (Open diamonds and solid line) 0.1 mg/ml Mj-lb, (Open left-triangles and dashed-line) 0.05 mg/ml Mj-eg and (Open crossed-squares and solid line) 0.2 mg/ml Ascorbic acid as positive control. SC% (percentage of scavenging activity on DPPH radical) = [(absorbance of control)-(absorbance of sample)/(absorbance of control)] * 100. All absorbance were measured at 517 nm. Results are (mean ± SD) of three parallel measurements. *P* < 0.01, when compared to the control.

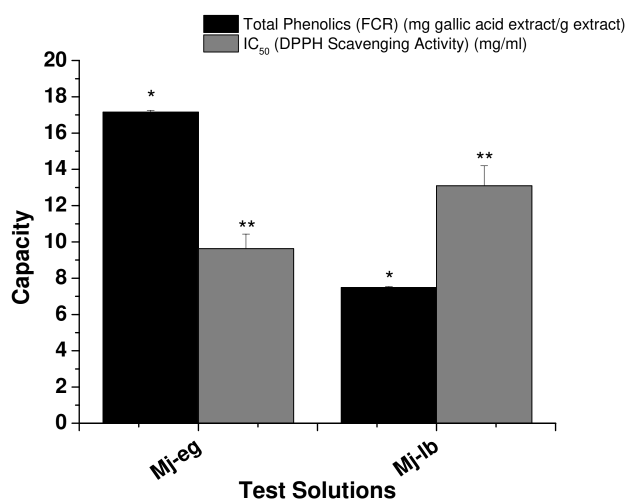


Figure. 6. Results of the Folin–Ciocalteu reagent (FCR) assay and 2, 2-diphenyl-1-pricylhydrazyl (DPPH) IC_{50} radical scavenging activity from the methanolic extracts of Egyptian marjoram (Mj-eg) and Lebanese marjoram (Mj-lb). Data replotted from table 1. Left axis provide capacity of total phenolic content using Folin–Ciocalteu Reagent (FCR) or IC_{50} of 2, 2-diphenyl-1-pricylhydrazyl (DPPH) radical scavenging activity. "asterisks" denote significant difference (p 0.05) between the different extracts in FCR total phenolics (*) and DPPH IC_{50} (**).

Using the same method, CGA have shown previously radical scavenging activity with IC_{50} (CGA) = 3.09 μg/ml [39]. Likewise, *p*CA has shown to possess lower radical scavenging activity than CGA with IC_{50} (pCA) = 7.8 μg/ml [40] and *p*HBA has shown to possess the lowest radical scavenging activity with IC_{50} (*p*HBA) = 43.02 ± 0.01 μg/ml [41].

Conclusion

We have presented scientific evidence that marjoram extracts (Mj-eg and Mj-lb) contain a potent modulator to GlyRs; with potential anticonvulsant properties in low doses, which may be attributed to its content of phenolics. The low concentration combinations of these phenolics, especially, CGA and pCA, were synergistic. We concluded that the presence of low concentration of the phenolics may be the cause that the unprocessed nux vomica seeds are 12 folds safer than using strychnine alone. Marjoram phenolics, especially chlorogenic acid, *p*-coumaric acid and *p*-hydroxy benzoic acid are suggested to be a novel GlyRs modulator, good phytomedicine, antioxidant, pharmacological tool and a dose sensitive drug to treat convulsions, stiffness and neurodegenerative disorders.

Conflict of interest

None of the authors have a financial relationship with a profitable entity that has an interest in the content of this study.

Authors' contributions

Karim Raafat; Did the experimental part, data analysis and wrote the manuscript. Hasan Jassar; Did some measurements. Maha Aboul-Ela and Abdalla El-Lakany; Revised the manuscript.

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