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Phytochemical analysis of *Ficus carica* L. active compounds possessing anticonvulsant activity

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ABSTRACT

The anticonvulsant potential of *Ficus carica* methanol-extract (Fc) has been studied. It was found that Fc most active fraction is rich in oligosaccharides (OFG). ^{1}H, ^{13}C NMR and Nano-ESI, MALDI MS, and LC-MS techniques proved that OFG contains alpha-glucopyranoside oligomer in high amounts. Both Fc and OFG reduced strychnine (STR) convulsion-action. Fc and OFG fully protected the experimental-animals from STR-lethality. The intracerebroventricular-administration (ICV) of Fc or OFG in combination with glycine in ethanol-treated mice caused a dose-dependent returning to a 2nd-loss of righting-reflex (LORR), and was antagonized by STR. FC and OFG ICV injection counteracted STR-inhibition, confirming that Fc/OFG anticonvulsant mechanism of action was mediated by potentiation of glycine receptor. These results support Fc and OFG potential anticonvulsant-activity with good safety-profile.

1. Introduction

Family Moraceae is well recognized for its edible species, and it is divided into five tribes and about 750 species, characterized by milky-latex, anatropous ovules, unisexual flowers, and achene.[1] *Ficus* is one of the 35 genera of Moraceae family and it represents a rich source of nutrients which is important for health. Its value is as significant as to be named in Muslim Quran and Christian sacred-books.[1,2] Various species of *Ficus* have been utilized for many years in the Nigerian traditional-medicine to ameliorate depression, psychoses, pain, epilepsy, and inflammation.[3] *Ficus carica* L. (Family; Moraceae) is a deciduous medicinal-plant mainly present in the North African coast regions and was found to possess various pharmacological potentials, like the antibacterial, antiviral, and antioxidant activities.[1,4,5]

Earlier studies have shown that other species of *Ficus* contained sedative substances with analgesic, neuroleptic, and anti-inflammatory potentials.[3]

Oligosaccharides (low molecular-weight carbohydrates with 2–10° of polymerization) are an important type of bioactive compounds, which possess several physiological functions, and has a vital role in well-being, health improvements and reducing the risk of many serious disorders.[6]

Strychnine convulsion is one of the serious poisoning disorders which has been attributed to interfering with postsynaptic inhibition moderated by glycine.[7] Glycine is a vital inhibitory-transmitter to spinal cord inter-neurons and motor-neurons. Strychnine is a selective, competitive antagonist to the function of glycine at the glycine receptors.[8,9] Moreover, saccharides have shown to significantly potentiate the recombinant glycine receptor alpha-1, and reduction of glycine receptor EC_{50} values (Table 1).[10]

Moreover, recent *in vitro* experiments have shown that ethanol provokes chloride flux via the glycine receptor, which has been inhibited by glycine receptor antagonist, strychnine.[11] These studies prompted the exploration of the anticonvulsant drug interaction of glycine with oligosaccharides and ethanol by *in vivo* models.

Bioactive-substances that reverse the strychnine action have shown to possess anticonvulsant potentials. On the other hand, other bioactive-substances that aggravate strychnine action have demonstrated potentials to stimulate glycine receptors and might

Abbreviations: OFG, oligosaccharide rich fraction; Fc, *Ficus carica* methanol extract; EC_{50}, half maximal effective concentration; ECD, electrochemical detector; STR, Strychnine; TEC, tonic extensor convulsion; CNS, central nervous system.

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Table 1
Variability of recombinant alpha-1 glycine receptor EC_{50} values with saccharides, modified from (Breitinger et al., 2015).

Saccharide	Saccharide Concentration (mM)	Glycine receptor EC_{50} (μM)
Glucose	0	39 ± 15
	20	26 ± 8*
	50	14 ± 3**
	100	15 ± 2**
Fructose	0	39 ± 15
	50	8 ± 1**

N.B. *$p < .05$ and **$p < .01$.

have an anxiogenic action.[9] Therefore, the protective potentials of the tested substances to strychnine-induced convulsion has been utilized in this study as a model for investigating the anticonvulsant potentials of these substances as reported before.[7,12–16]

Furthermore, bioactive-substance in combination with glycine when administered intracerebroventricularly (ICV) would enhance the ethanol central depressant effect if the substance was glycine receptor potentiator, or blocked with glycine receptor inhibitors or strychnine.[17]

Since oligosaccharides and saponins which constitute the major components of Fc crude extract,[1] are thought to have potential central nervous system (CNS) effects, and it is claimed that either or both might be associated in the observed CNS effects of the *Ficus* extract.[3,10]

Consequently, in this study phytochemical investigation of the stem bark of *Ficus carica* L. has been performed leading to bio-guided characterization and isolation of its most neuroactive compound(s). Additionally, to assess the effect of *Ficus carica* extract (Fc) and its most active component on the anticonvulsant *in vivo* models in order to bridge earlier studies and to understand more about *Ficus carica* neuroactive potentials.

2. Materials and methods

The extraction, identification, quantification and isolation and the structure elucidation of the active component(s) of *Fc* extract using analytical and chromatographic methods have been done at the Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe-University Frankfurt am Main, Germany and Pharmaceutical Sciences Department, Beirut Arab University, Lebanon. All solvents and standards utilized in this study were commercially available (Sigma-Aldrich, Germany), and were of analytical grade and were used without further purification.

2.1. Extraction of the active component(s) of Ficus carica plant

Ficus carica stem barks were collected from Sidi-Krier private garden in Alexandria, Egypt. The latter leaves were identified and authenticated (*Ficus carica*, family Moraceae) by Prof. M. El-Olemy (Egypt) and a sample was kept in the Faculty herbarium with a voucher number (PS-16-29).

Fc stem bark coarse Fc powder 1000 g has been extracted with 10 l of 80% methanol. The resulting extract has been dried in vacuum utilizing rotary evaporator at 40 °C. The methanolic extract has been fractionated using column chromatography technique. Utilizing RP-silica, the extract has been partitioned using water and butanol mobile phases to provide butanol and aqueous fractions. The fractions were then dispersed in methanol and drop-wisely added to diethyl ether. The precipitates formed in different fractions were separated from the solution by centrifugation to give different fractions. Various fractions were examined for their potentials against the *in vivo* anticonvulsant model. The most active fraction was found to be rich in oligosaccharide, hence named oligosaccharide rich fraction (OFG).

2.2. Structure elucidation of OFG components

2.2.1. Sample preparation for ^{1}H and ^{13}C NMR analysis

OFG has been dissolved in 5 ml methanol. Then, it was evaporated using the heating block at 40 °C utilizing nitrogen gas. The dried extract was weighted to give 21.5 mg. The latter extract has been dissolved in 2 ml deuterated methanol (MeOH-d4) and evaporated. The latter step was repeated 2 times to ensure the deuteration of the sample. The 3rd dissolution in MeOH-d4 was added to NMR tube to be tested using Bruker ARX 300 spectrometer (Bruker, Germany).

2.2.2. Nano-ESI MS/MS^2 determination

One of the remarkable characteristics of the Nano-Electrospray ionization mass spectrometer (Nano-ESI MS) is its significant low-sample consuming.[18] Under Nano-ESI MS conditions, OFG was tested in the positive mode, as a result of the expected presence of sodium ions through-out the sample preparation process, and the strong-affinity of carbohydrates in the gas phase to sodium ions.[19]

2.2.3. MALDI-MS/MS^2 determination

Matrix Assisted Laser Desorption-Ionization Mass Spectrometer (MALDI-MS) is widely accepted for structure elucidation of micro- and macro-molecules. Furthermore, it has been utilized in this study to ensure the mass measured under Nano ESI-MS conditions and to determine these masses with high accuracy.[20]

2.2.4. LC-MS/MS^2 determination

LC-MS is an important analytical method that couples the HPLC physical separation and the mass spectrometric detection. LC-MS (Bruker, Germany) was used for an explicit detection and identification of OFG. Methanol and water 50:50 (v/v) have been used as a mobile phase and a MultoHigh 100 RP18-5μ (CS-GmbH, Germany) as an HPLC column. Moreover, OFG has been analyzed by HPLC using various detectors. These HPLC instruments were attached with a variety of detectors like UV, PDA and ECD (electrochemical detector). HPLC using UV and PDA detectors did not report detectable components in OFG possessing UV absorption. Furthermore, HPLC-ECD using various columns did not separate OFG extract components as all traces exist near the injection peak. The previous properties have been reported before for sugars.[21]

2.2.5. HPLC-PDA Fc whole extract

The Fc whole extract was analyzed utilizing MultoHigh 100 RP18-5μ (CS-GmbH, Germany) as an HPLC column at 30 °C. The mobile-phase consisted of gradient elution of (A) formic acid 0.1% (v/v) in double distilled and (B) methanol: 10% B 0 min; 28% B 5min; 45% B 30 min; 80% B 45 min. The injection volume has been 5 μL and the flow rate has been 1 ml/min. The UV wavelength range of PDA scan has been set at 200–600 nm focusing on 254 nm.

2.3. Animals

Male Swiss-Webster mice (Faculty of Pharmacy, BAU, Lebanon) have been habituated as long as seven days before experimentation. The housing of animals formed of standard-cages and a 12-h dark-light alternating cycle at room temperature. Mice had open-access to standard food (5% fat, 20% protein, and 1% multivitamin) and water. Animal-care and experiments have been done in compliance with animal experiment-legislations and with the approval: (2014A-004-P-R-0006) of BAU Institutional Review

Board.

2.3.1. Safety, tonic extensor convulsion and lethality experiments

Animals with 22–25 g average weight have been utilized in the safety, tonic extensor convulsion (TEC) and lethality experiments. Reference animals administered (IP) 2 mg/kg strychnine in order to measure time to TEC and time to death. The control mice have administered 200 μL saline followed half an hour later by 2 mg/kg strychnine administration. For safety tests, mice have administered only the highest doses of Fc (200 mg/kg) or OFG (50 mg/kg). Test animals have administered Fc (50, 100 or 200 mg/kg), or OFG (12.5, 25 or 50 mg/kg) and half an hour after administered strychnine (2 mg/kg). Animals have been monitored for a period of 1 h and the time till TEC and lethality have been measured. All values have been measured as means ± S.D (n = 4/group). By comparing all data obtained to the control. All tested compounds have been analyzed using Student's t-test followed by one way ANOVA test, and the *p*-value of ≤0.05 has been considered statistically-significant, as described before in literature.[22,23]

2.3.2. In vivo loss of the righting reflex (LORR) experiment

It has been reported that the bioactive-substance in combination with glycine when injected intracerebroventricularly (ICV) would enhance the ethanol central depressant effect if the substance was glycine receptor potentiator, or blocked with glycine receptor inhibitors or strychnine.[17]

In the presence of ethanol, glycine alone or in combination with Fc (50, 100 or 200 mg/kg), or OFG (12.5, 25 or 50 mg/kg) were ICV injected. The theory of this experiment was that Fc or OFG would potentiate or inhibit glycine provoked ethanol central-depressant effects. Strychnine, a glycine receptor inhibitor, has been injected in the presence of glycine alone or in combination to determine the mechanism by which Fc or OFG possesses their neuroactive properties.

Bicuculline, a GABA-receptor inhibitor, has been injected in the presence of glycine alone or combination to ensure that glycine receptor is the only functioning receptor.[17]

The LORR has been utilized to assess the degree of central-depression produced by ethanol utilizing a modified method reported before[17] (Table 2). Briefly, each mouse (n = 4/group) for each individual experiment received 20% ethanol (4.0 g/kg) as an i.p. injection for LORR-induction.[24] The time from administration to the ethanol-induced LORR has been recorded (seconds) and has been known as **time to LORR**. While **the LORR-end** (minutes) has been defined as the capability of mice to right themselves by rolling back to their feet three times within 15 s when positioned on their backs. Moreover, the time between the initial LORR and the following righting-reflex gaining has been recorded and known as the **ethanol-LORR**. A 2nd-period of LORR has been experienced by mice after test compounds ICV-injection. This 2nd-period has been monitored from ICV injection to the following righting-reflex regaining and has been known as the **return to LORR** (Fig. 1 and Table 2).

2.3.2.1. ICV drug administration. Twenty minutes after the animal lost the righting reflex following i.p. ethanol administration, a sagittal incision was made on the dorsal aspect of the head exposing the skull sutures. On an ethanol-anesthetized mouse (previous i.p.); a hole 3 mm deep was made 2 mm posterior to the bregma suture and 2 mm lateral to the sagittal suture using a 24-gauge needle. Immediately after the animals regained the righting reflex following the i.p. injection of ethanol, they were given an ICV injection of saline or drug (total volume of 10 μl) (Fig. 1 and Table 2). This ICV injection was administered over a period of 10 s. Upon ICV injection of saline or drug solution, a second LORR was recorded (return to LORR).[25] All values have been measured as means ± S.D. By comparing all data obtained to the control (Table 2), all tested compounds have been analyzed using one way ANOVA test, and the *p* value of ≤0.05 has been regarded as statistically significant, as described before.[22,23]

3. Results

3.1. Structure elucidation of OFG components

3.1.1. OFG ^{1}H NMR and OFG ^{13}C-NMR analysis

^{1}H NMR of oligosaccharides had been less assessed than ^{13}C NMR, giving rise to a very few accounts on oligosaccharides in literature.[26] The ^{1}H and ^{13}C NMR spectra of OFG was shown in (Table 1 and Fig. S1) (Table 3), and they ensure the presence of alpha-D-glucopyranose oligomer in high concentration in OFG. Other NMR spectra have been done like OFG ^{13}C-HMBC (Hetero-nuclear Multiple Bond coherence), ^{13}C-HSQC (Hetero-nuclear single quantum coherence), ROESY NMR (Rotating-frame Overhauser

Table 2
Protocol of *in vivo* LORR-ICV experiment.

Groups	n	Tested Substance(s)	Description
I	4	Control	Normal mice: Vehicle [sterile cold saline (0.9%)], ICV
II	4	Gly 1	Treated mice: Glycine 1 μmol/kg, ICV
III	4	Gly 15	Treated mice: Glycine 15 μmol/kg, ICV
IV	4	Gly 25	Treated mice: Glycine 25 μmol/kg, ICV
V	4	Gly 50	Treated mice: Glycine 50 μmol/kg, ICV
VI	4	STR 300	Treated mice: Strychnine 300 nmol/kg, ICV
VII	4	Gly15 + STR50	Treated mice: Glycine 15 μmol/kg and Strychnine 50 nmol/kg, ICV
VIII	4	Gly15 + STR100	Treated mice: Glycine 15 μmol/kg and Strychnine 100 nmol/kg, ICV
IX	4	Gly15 + STR300	Treated mice: Glycine 15 μmol/kg and Strychnine 300 nmol/kg, ICV
X	4	Gly15 + Fc50	Treated mice: Glycine 15 μmol/kg and Fc 50 μmol/kg, ICV
XI	4	Gly15 + Fc100	Treated mice: Glycine 15 μmol/kg and Fc 100 μmol/kg, ICV
XII	4	Gly15 + Fc200	Treated mice: Glycine 15 μmol/kg and Fc 200 μmol/kg, ICV
XIII	4	Gly15 + OFG12.5	Treated mice: Glycine 15 μmol/kg and OFG 12.5 μmol/kg, ICV
XIV	4	Gly15 + OFG25	Treated mice: Glycine 15 μmol/kg and OFG 25 μmol/kg, ICV
XV	4	Gly15 + OFG50	Treated mice: Glycine 15 μmol/kg and OFG 50 μmol/kg, ICV
XVI	4	Gly15 + Fc50 + STR300	Treated mice: Glycine 15 μmol/kg, Fc 50 μmol/kg and Strychnine 300 nmol/kg, ICV
XVII	4	Gly15 + Fc100 + STR300	Treated mice: Glycine 15 μmol/kg, Fc 100 μmol/kg and Strychnine 300 nmol/kg, ICV
XVIII	4	Gly15 + Fc200 + STR300	Treated mice: Glycine 15 μmol/kg, Fc 200 μmol/kg and Strychnine 300 nmol/kg, ICV
XIX	4	Gly15 + OFG12.5 + STR300	Treated mice: Glycine 15 μmol/kg, OFG 12.5 μmol/kg and Strychnine 300 nmol/kg, ICV
XX	4	Gly15 + OFG25 + STR300	Treated mice: Glycine 15 μmol/kg, OFG 25 μmol/kg, and Strychnine 300 nmol/kg, ICV
XXI	4	Gly15 + OFG50 + STR300	Treated mice: Glycine 15 μmol/kg, OFG 50 μmol/kg, and Strychnine 300 nmol/kg, ICV

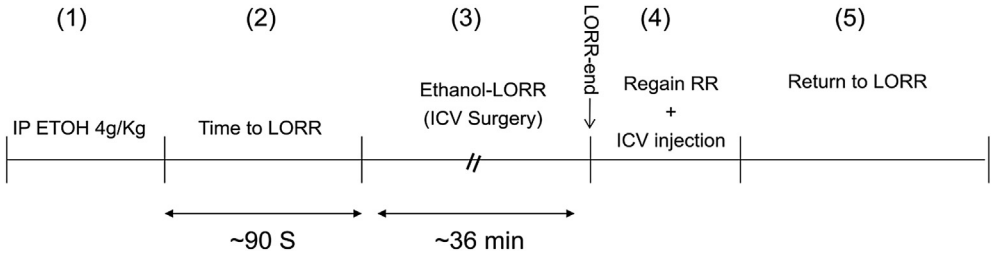

Fig. 1. LORR timeline.

Table 3
OFG ^{1}H NMR and OFG^{13}C-NMR data.

Sugar ring	Position	δ_H, m, (J in Hz)	δ_C
I	1	4.98, d (J = 3.6 Hz)	**105.32***
	2	4.01, m	75.69
	3	4.25, dd (J = 9.0, 9.8 Hz)	78.13
	4	4.28*, m	71.89*
	5	3.93, ddd (J = 2.3, 4.5, 10.2 Hz)	76.31
	6	3.81, m	62.72
	6′		
II	1	5.06, d (J = 3.6 Hz)	97.02
	2	3.60, dd (J = 3.6, 9.6 Hz)	73.69
	3	3.90, m	75.13
	4	**3.50***, dd (J = 9.3, 9.9 Hz)	71.89
	5	3.80, m	73.31
	6	3.73, m	62.27
	6′		
III	1	5.50, d (J = 3.5 Hz)	100.82
	2	3.56, dd (J = 3.7, 9.7 Hz)	73.20
	3	3.70, m	74.13
	4	3.40, dd (J = 9.4, 9.8 Hz)	71.89
	5	3.75, m	74.31
	6	3.83, m	62.17
	6′		

*Bold: Interring HMBC cross peaks are observed at the anomeric position.

Effect Spectroscopy) and COSY NMR (Correlation Spectroscopy).

In the ^{1}H NMR-spectrum results were illustrated in Table 3. The spectrum assures the characteristic peaks for alpha-D-glucopyranose oligomer present in OFG. The oligomer structure has been evidenced by the inter-ring HMBC cross peaks observed in the two positions (C1 and C4) of each reducing end D-glucose unit (Table 3). These results indicate that the C1 substituted D-glucose unit at the reducing end which is required for formation of (1–4) linked trisaccharide (Table 3), as reported before for other saccharides.[27]

The alpha-anomeric position of the glucopyranosyl groups has been concluded from the anomeric protons multiple peak at δ_H 3.81 (^{1}H, multiple) and (Table 3), as reported before.[28]

In the ^{13}C NMR-spectrum of OFG sample (Fig. S1A), exemplifies the area between 25 and 107 ppm and the results are shown in Table 3. The characteristic peaks in this spectrum assured the presence of α-D-glucose oligomer in OFG sample as reported before in literature for other extracts.[29] Furthermore, the analysis of the ^{13}C DEPT NMR spectrum experiment has revealed the presence of C_6 methylene group of the α-D-glucose oligomer. This methylene group appears as inverted peaks in the DEPT spectrum (Fig. S1B).

3.1.2. Nano-ESI MS/MS2 of OFG molecular mass determination

Under Nano-Electrospray ionization mass spectrometer (Nano-ESI MS) conditions, OFG has been tested in the positive mode. The full scan mass-spectrum in the positive ion mode was shown in (Fig. S2A), because of the expected presence of Na ions during sample preparation and the strong affinity of carbohydrates in the gas phase to Na ions.[19] All the Nano-ESI mass spectra were denominated by [M + Na]+and have exhibited negligible fragmentation. In (Fig. S2A), the major ion peak was displayed at m/z 365.1. By MS^2 analysis of the latter 365.1 ion peak, it has been fragmented to m/z 203 and 185 corresponding to two glucose moieties linked together (Fig. S2B).

3.1.3. MALDI-MS/MS2 determination

Furthermore, Matrix-Assisted Laser Desorption-Ionization-Mass Spectrometer (MALDI-MS) was utilized both to insure the mass measured under Nano ESI-MS conditions and to determine these masses with high accuracy. As expected, the main peak has been at m/z 365.16667 and 527.25001. By MS^2 analysis of 365.16667 peak it has been fragmented to m/z 203.08334 and 185. This MS^2 study confirms that the 365.16667 peak was formed of dimmer of glucose. While MS^2 has split the 527.25001 peak into m/z 365.16667 and 203.00001 corresponding to three glucose moieties linked together (Fig. S3).

3.1.4. Analyzing Fc whole extract and OFG by HPLC using various detectors

Fc whole extract has been analyzed by HPLC/PDA and the major peaks were identified and quantified by comparing to analytical standards and their standard calibration curves. The major peaks were, 3-Methoxy-hydroquinone-O-glucose (24.8%), 3,5-Dimethoxyhydroquinone-O-glucose (18.6%), Proto-catechuic acid-O-arabinose (14.9%), Chlorogenic acid (13.1%) and Vanillic acid-O-(rhamnose)3 (11.2%), and Rutin (13.6%) (Fig. S4A).

OFG extract has been analyzed by HPLC utilizing various detectors, like UV, PDA, ECD (electrochemical detector) and LC-MS.

HPLC using UV and PDA detectors seldomly reports detectable components possessing UV absorption. Furthermore, HPLC/ECD has exhibited one peak near the injection peak (Fig. S4B), as reported before for sugar oligomers.[21]

Finally, OFG extract has been analyzed using LC-MS. Methanol and water (50:50) have been utilized as a mobile phase, and a MultoHigh 100 RP18-5μ (CS-GmbH, Germany) as an HPLC column.

In the LC-MS analysis, OFG was used either as such (OFG unpurified), water of rinsing (OFG water), or residue after rinsing (OFG purified). The three samples of OFG were tested and superimposed along with blank methanol (Fig. S5). LC-MS chromatogram showed at 4.5–5.1 min the presence of m/z 203.1 and 365.1 peaks in the OFG water sample. These peaks ensure the presence of glucopyranoside oligomer in the OFG active fraction (Fig. S5).

3.2. In vivo studies-behavior effects of OFG on mice

The safety of various doses of Fc (50, 100 and 200 mg/kg), or OFG (12.5, 25 and 50 mg/kg) have been examined in male mice after test administration. No lethal or convulsive effects have been observed when the highest doses of Fc (200 mg/kg), or OFG (50 mg/kg) solely had been administered (Fig. 2).

3.2.1. Safety, tonic extensor convulsion, and toxicity experiments

Strychnine (2 mg/kg) solely caused TEC and death to all tested

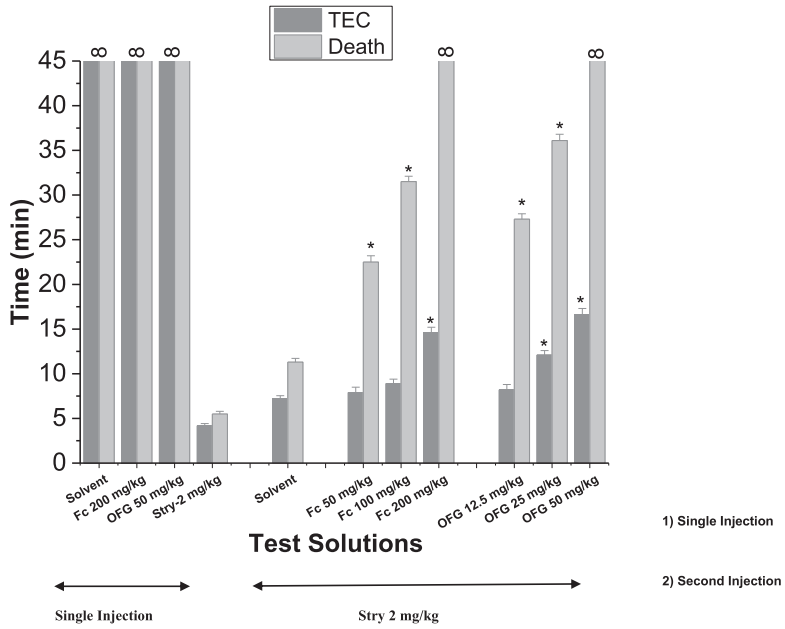

Fig. 2. Latency of tonic extensor convulsion (TEC) and death test. *In vivo* test of GlyR potentiation by Fc and OFG. See text for experimental conditions. Data are presented as mean ± SD, asterisks denote significant difference from control (one-way ANOVA, $p \leq .05$). Mice were treated with Fc (50, 100 and 200 mg/kg) and OFG (12.5, 25 and 50 mg/kg) or control (vehicle), 30 min later the mice administered 2 mg/kg strychnine nitrate (Stry). The time until occurrence of TECs and death is plotted (minutes ± SD), throughout a 60-min period. ∞ indicates no occurrence of tremors and/or survival of the animal.

mice. TEC was observed at 5.6 ± 0.6 min, while fatality provoked at 6.4 ± 0.5 min. The protective potentials to reverse strychnine induced convulsions have been utilized in this study as a model for the investigation of the anticonvulsant potentials of the tested compounds. Thus, pre-administration with Fc (50, 100 or 200 mg/kg), OFG (12.5, 25 or 50 mg/kg), or solvent (as control) followed by strychnine administration (2 mg/kg) half an hour later, have been monitored. As compared to control, animals that have been pre-administered Fc, the lethal effect of strychnine decreased in a dose-dependent pattern. Mice pre-treated with Fc highest dose (200 mg/kg), strychnine 2 mg/kg resulted in TEC after 14.35 ± 1.25 min (Fig. 2). Furthermore, full protection of strychnine lethality was recognized with the highest Fc dose (200 mg/kg) (Fig. 2). At lower doses, Fc (50 and 100 mg/kg) did not show significant improvement of TEC. On the other hand, Fc (50 and 100 mg/kg) resulted in decreasing strychnine lethality (time to death) from 6.3 ± 0.3 min (control) to 22.50 ± 1.30 min and 31.10 ± 1.00 min for Fc (50 and 100 mg/kg), respectively (Fig. 2).

Moreover, animals that have pre-administered OFG have ameliorated strychnine lethality, in a dose-dependent pattern, when correlated to control. At the highest dose of OFG (50 mg/kg, i.p.), 2 mg/kg strychnine resulted in TEC after 17.15 ± 1.05 min (Fig. 2). Furthermore, full protection of strychnine lethality was recognized with the highest OFG dose (50 mg/kg) (Fig. 2). At OFG (25 mg/kg), 2 mg/kg strychnine resulted in TEC after 12.05 ± 1.10 min (Fig. 2). At OFG lowest dose, OFG (12.5 mg/kg) did not show significant improvement of TEC. However, OFG (12.5 and 25 mg/kg) resulted in reducing strychnine lethality from 6.3 ± 0.3 min (control) to 27.1 ± 1.10 min and 35.3 ± 1.30 min for OFG (12.5 and 25 mg/kg), respectively (Fig. 2). The toxicity of strychnine has been decreased in animals that had administered Fc or OFG, in agreement with the *in vitro* data on recombinant glycine receptor reported before.[10]

3.2.2. Loss of the righting reflex (LORR) test

The timeline of the *in vivo* loss of the righting reflex (LORR) experiment was summarized in Table 2. All of the mice had shown LORR following ethanol IP injection (Table 2). The LORR onset has been recorded at 90.2 ± 4.05 s, and ethanol LORR endured for 36.5 ± 1.8 min. Among the experimental groups, there have been no differences in the LORR onset or in the ethanol-LORR.

3.2.2.1. Glycine effects on the ethanol-LORR in the absence or presence of strychnine. Glycine has augmented ethanol central depressant effects in mice, as evident by the immediate and dose dependant return to the LORR after glycine ICV injection at various doses (Fig. 3A).

The data in (Fig. 3B) has shown that when various doses of strychnine (50, 100, or 300 nmol/kg) have been ICV injected together with EC40 glycine (15 μmol/kg), strychnine has repressed glycine effects in a concentration-dependent manner.

ICV injection of strychnine 300 nmol/kg has completely abolished the glycine effects, implying that the only functioning receptor is the strychnine-sensitive glycine receptor (Fig. 3B).

Moreover, after strychnine ICV injection, mice have shown enormous abdominal flexions and head scratching-motions. After the ethanol-induced LORR, ICV injection of strychnine (300 nmol/kg) has been given alone in order to find out if strychnine could provoke LORR alone. The results have shown that no LORR has been noticed in any of the tested mice (Fig. 3C).

3.2.2.2. Glycine effects on the ethanol-induced LORR in the presence of fc or OFG and in the absence or presence of strychnine. The results have shown that various doses of Fc (50, 100 and 200 μmol/kg) when administered with EC40 glycine (15 μmol/kg), Fc potentiated glycine effects in a concentration-dependent manner (Fig. 3C), suggesting in that Fc is a potentiator of glycine receptor.

In the presence of glycine, strychnine (300 nmol/kg), the specific glycine receptor inhibitor, co-administration with Fc at low doses abolished their potentiating effects (Fig. 3C). Fc at the highest dose (200 μmol/kg) counteracted strychnine (300 nmol/kg) inhibition (Fig. 3C), strengthening the finding that Fc is a potentiator of glycine

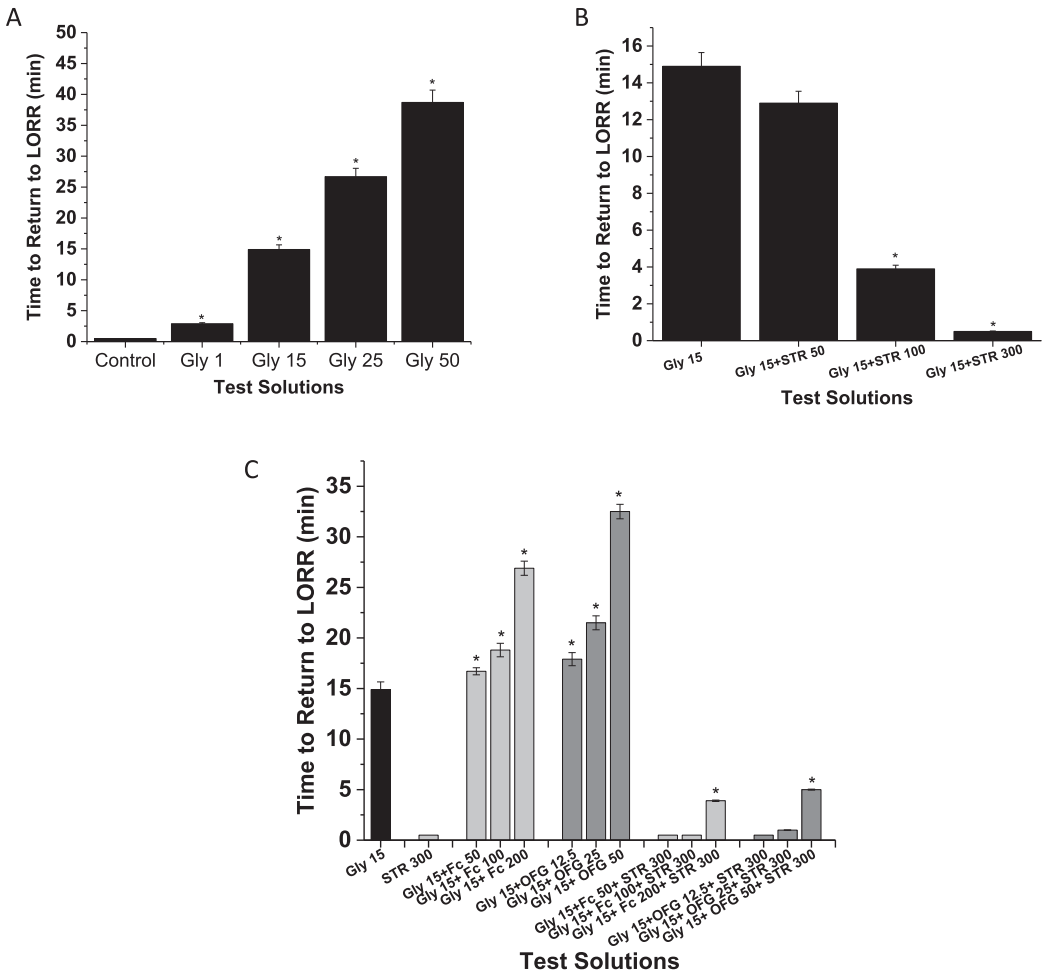

Fig. 3. Return to Righting Reflex (RR) experiment. (A) RR after ICV injection of glycine (Gly 1) Glycine 1 μmol/kg, (Gly15) Glycine 15 μmol/kg, (Gly 25) Glycine 25 μmol/kg, or (Gly 50) Glycine 50 μmol/kg (B) RR after ICV injection of (Gly 15) Glycine 15 μmol/kg, (Gly 15 + STR 50) Glycine 15 μmol/kg and Strychnine 50 nmol/kg, (Gly 15 + STR 100) Glycine 15 μmol/kg and Strychnine 100 nmol/kg or (Gly 15 + STR 300) Glycine 15 μmol/kg and Strychnine 300 nmol/kg (C) RR after ICV injection of (Gly 15) Glycine 15 μmol/kg, (STR 300) STR 300 nmol, (Gly 15 + Fc 50) Glycine 15 μmol/kg and Fc 50 μmol/kg, (Gly 15 + Fc 100) Glycine 15 μmol/kg and Fc 100 μmol/kg, (Gly 15 + Fc 200) Glycine 15 μmol/kg and Fc 200 μmol/kg, (Gly 15 + OFG 12.5) Glycine 15 μmol/kg and OFG 12.5 μmol/kg, (Gly 15 + OFG 25) Glycine 15 μmol/kg and OFG 25 μmol/kg, (Gly 15 + OFG 50) Glycine 15 μmol/kg and OFG 50 μmol/kg, (Gly 15 + Fc 50 + STR 300) Glycine 15 μmol/kg, Fc 50 μmol/kg and STR 300 nmol/kg, (Gly 15 + Fc 100 + STR 300) Glycine 15 μmol/kg, Fc 100 μmol/kg and STR 300 nmol/kg, (Gly 15 + Fc 200 + STR 300) Glycine 15 μmol/kg, Fc 200 μmol/kg and STR 300 nmol/kg, (Gly 15 + OFG 12.5 + STR 300) Glycine 15 μmol/kg, OFG 12.5 μmol/kg and STR 300 nmol/kg, (Gly 15 + OFG 25 + STR 300) Glycine 15 μmol/kg, OFG 25 μmol/kg and STR 300 nmol/kg, or (Gly 15 + OFG 50 + STR 300) Glycine 15 μmol/kg, OFG 50 μmol/kg and STR 300 nmol/kg. Data are presented as mean ± SD, asterisks denote significant difference ($p \leq .05$).

receptor.

Furthermore, the results have shown that when different doses of OFG (12.5, 25 and 50 μmol/kg) were administered with EC40 glycine (15 μmol/kg), OFG potentiated glycine effects in a concentration-dependent manner (Fig. 3C), suggesting in that OFG is the most active fraction in Fc, potentiating the glycine receptor.

Strychnine (300 nmol/kg), in the presence of glycine, co-administration with OFG at low doses abolished their potentiating effects (Fig. 3C). OFG at the highest dose (50 μmol/kg) counteracted strychnine (300 nmol/kg) inhibition (Fig. 3C), strengthening the finding that OFG is the most active fraction in Fc, potentiating the glycine receptor.

Moreover, ICV has proved to be eminent *in vivo* technique in screening GlyR modulators.

4. Discussion

The results have provided scientific-evidence that the *F. carica* stem bark ethanolic extract (Fc) and its most active fraction, the oligosaccharide-rich fraction (OFG) contained neuroactive principles that could significantly manage the strychnine-induced convulsive disorders.

The latter results encouraged us to find the neuroactive principles in the OFG. In the ^{1}H NMR spectrum of OFG sample, we have found characteristic peaks for alpha-D-glucopyranoside as reported for other extracts in literature.[30] The alpha-anomeric position of the glucopyranosyl moiety has been concluded from the anomeric-protons multiple peak at δ_H 3.81 (Table 3), as reported before.[28,31] Furthermore, the ^{13}C NMR normal and DEPT spectrum of OFG sample have confirmed the presence of characteristic peaks for D α-glucopyranoside as reported before in literature for other extracts.[29] The (1–4) linkage between various units of glucose were established by the C-1 long-coupling o (δ_C 105.32) of the terminal-glucose-unit to H-4 (δ_H 4.28) of the neighboring glucose-unit (Table 3), as reported before in literature with other oligosaccharides.[28,32,33] Under Nano ESI-MS, MALDI-MS and LC/MS conditions, the MS^2 study and the chromatograms confirmed that the OFG was formed of *O*-α-D-glucopyranosyl- (1–4) - α -glucopyranosyl- (1–4) -

alpha-glucopyranoside.

In safety tests, both Fc (200 mg/kg) and OFG (50 mg/kg), when administered alone in their highest doses, had shown no convulsive or lethal effects to the tested mice, indicating that Fc and OFG have good margins of safety.

Strychnine convulsion action is attributed to inhibition of the inhibitory postsynaptic glycine receptors. Bioactive substances which reverse strychnine action have shown to possess anticonvulsant potentials.[7–9] Experimentally, strychnine has shown convulsive and lethal effects at the concentration of (2 mg/kg). When animals were treated with various doses of Fc (50, 100 and 200 mg/kg) or OFG (12.5, 25 and 50 mg/kg) prior to strychnine administration, the strychnine lethality was significantly and dose-dependently decreased, as compared to animals administered solvent before strychnine administration (control). The highest doses of Fc (200 mg/kg) and OFG (50 mg/kg) had fully protected the tested mice from strychnine lethality. Moreover, the higher doses of Fc (200 mg/kg) and OFG (25 and 50 mg/kg) have elevated the time until TEC by about 2.0, 1.7 and 2.3 folds, respectively, compared to control. Thus, the convulsive and lethal effects of strychnine were significantly reduced by non-toxic doses of the Fc and OFG, concluding that Fc and OFG had anticonvulsant activity with good margins of safety.

In order to know the mechanism by which Fc and OFG possess their neuroactive response, LORR experiments were performed. After an ethanol-induced LORR, the ICV administration of glycine has provoked a concentration-dependent returning of the animals to a 2nd LORR (Fig. 3A). The 2nd LORR have been found to be counteracted by strychnine in a concentration-dependent manner. Indicating that glycine enhanced ethanol neuroactive effects by provoking the inhibitory strychnine-sensitive glycine receptor, as reported before.[34] These results validated the use of this model in screening strychnine-sensitive glycine receptor modulators by reaching the glycine-receptors within the CNS.

Fc has shown concentration-dependant potentiation of glycine provoked the return to 2nd LORR in comparison to the glycine EC40 single-shot, indicating that the Fc comprises a glycine receptor potentiator.

Furthermore, OFG has shown dose dependant potentiation of glycine provoked return to the LORR in comparison to the glycine EC40 single-administration, indicating in that the OFG is the most active fraction in Fc potentiating the glycine receptor.

Strychnine (300 nmol/kg) has shown to abolish the effects of Fc or OFG lowest concentrations towards glycine provoked the return to 2nd LORR. Moreover, FC and OFG highest concentrations counteracted strychnine inhibition, strengthening the finding that the *in vivo* protection of the tested mice from strychnine lethality was mediated by Fc/OFG potentiation of glycine receptor.

Thus, it could be concluded that Fc and OFG were *in vivo* potentiators to the glycine receptor. Consequently, ICV and LORR method have been proved to be a reliable method for investigation of glycine receptor modulators.

Taking together the high amounts of alpha-D-glucopyranoside oligomer in OFG, and the recent reports that oligosaccharides potentiated the glycine receptor (Table 1),[10] and our current finding of Fc and OFG reversing the stimulatory convulsive action of strychnine, and Fc and OFG potentiation of glycine return to the LORR, it could be concluded that the possible mechanism of Fc and OFG anticonvulsant activities might be moderated by potentiating the glycine receptor.

Therefore, the key finding of this study that *Ficus carica* and OFG have shown potential anticonvulsant activity mediated via potentiation of the inhibitory glycine receptor.

In conclusion, the findings of the current-study have supported that *F. carica* extract and its most active fraction, OFG had significant anticonvulsant activities with potential implications for the development of new treatments with good margins of safety for certain convulsive syndromes.

Author contributions

KR performed experiments; MW provided equipment and reagents; KR wrote the manuscript; MW made manuscript revisions.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jtcme.2018.01.007.

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