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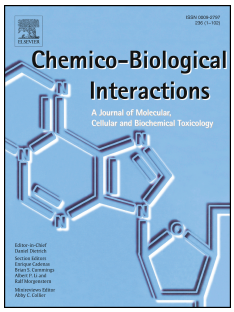

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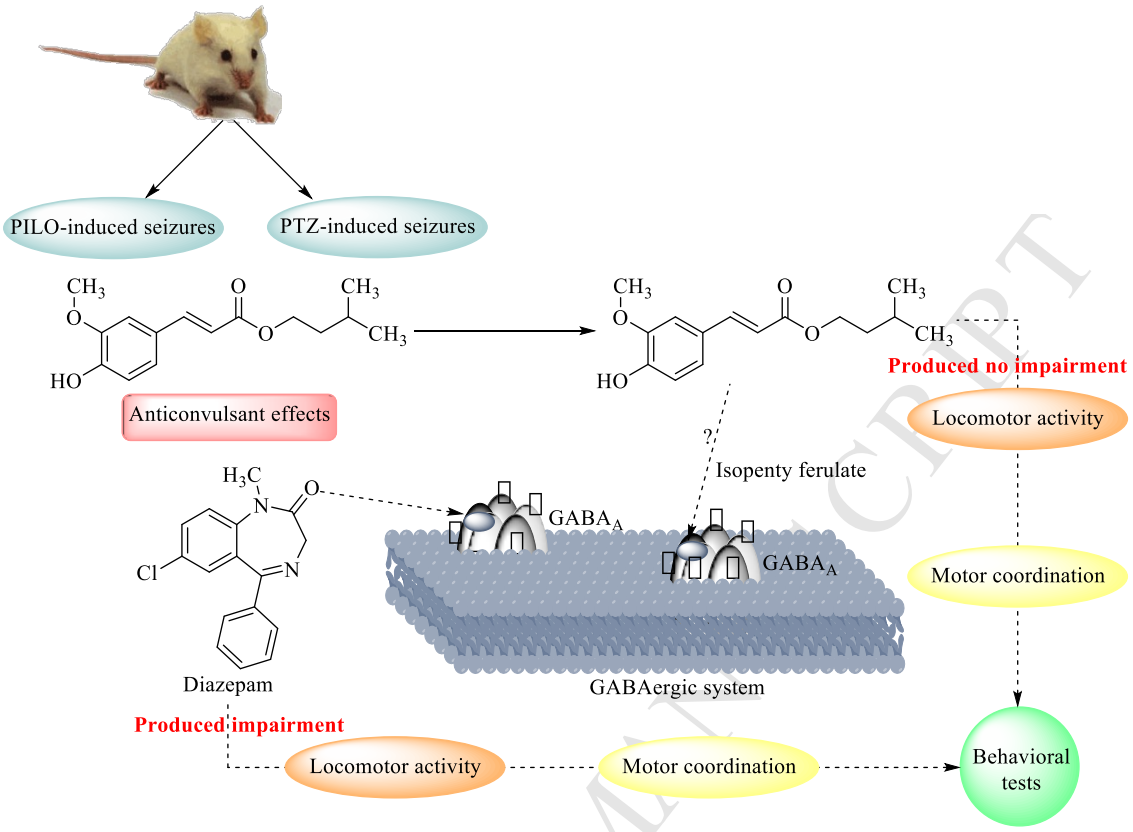
Graphical abstract



Anticonvulsant and behavioral effects observed in mice following treatment with an ester derivative of ferulic acid: Isopentyl ferulate

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Abstract

The objective of this study was to evaluate the potential anticonvulsant effect of isopentyl ferulate, a new ester derived from ferulic acid in mice (*Mus musculus)* subjected to two models of induced seizures. According to the results obtained, the IF at doses of 25, 50 and 75 mg/kg (i.p.) showed protective effect against induced seizures by pilocarpine (400 mg/kg, i.p.) and pentylenetetrazole (70 mg/kg, i.p.). In the two animal models of seizures, the pretreatment of the IF (25, 50 and 75 mg/kg) with flumazenil blocked the anticonvulsant effect, suggesting that the mechanism of action of this ester derived of ferulic acid may be related to activity in the benzodiazepine-binding site of the $GABA_A$ receptor (γ-aminobutyric acid, type A). In addition to the anticonvulsant effect, behavioral changes as neurotoxicity indication were assessed by using the rota rod and open field tests. The results obtained showed that the IF (25, 50 and 75 mg/kg) does not induce significant changes in locomotor activity and motor coordination when compared with the control group, unlike the results presented by diazepam. Thus, these results demonstrate a new pharmacological knowledge of IF with potential application against epileptic seizures. However, further studies are needed to elucidate other neurobiological mechanisms underlying epilepsy.

Keywords: Anticonvulsant effect; epileptic seizures; isopentyl ferulate; locomotor activity; motor coordination.

1. Introduction

Epilepsy is a severe neurological disorder characterized mainly by recurrent seizures due to an abnormal neuronal hyperexcitability in the brain and according to the World Health Organization, affects approximately 50 million people worldwide [1,2]. Even with the significant advances made by several research groups for the treatment of epilepsy, approximately 30% of patients treated with antiepileptic drugs showed recurrence of uncontrolled seizures [3]. Furthermore, the main problems associated with antiepileptic drugs (e.g. - carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone, and valproate) involve to exhibit some side effects such as cognitive dysfunction, ataxia, sedation, hypersensitivity or worsening of seizures [4, 5].

Way forward, there is a need for the development of new research with the objective of evaluating new molecules with a better therapeutic effectiveness than antiepileptic drugs already existing. The natural products derived from plants represent a great opportunity for the discovery of new substances with potential therapeutic interest for the treatment of epilepsy [6, 7]. Among the groups of substances from natural origin with potential antiepileptic effect, the essential oils play an important role in scientific research due to their high level of bioactive components, by which can be detached the derivatives of phenylpropanoid and terpenoid [8].

The phenylpropanoids are a group of compounds derived from the carbon skeleton of phenylalanine, which have various neuropharmacological activities [9, 10, 11]. Among these compounds, stands out the ferulic acid that presents several pharmacological activities such as neuroprotective [12], antidiabetic [13], anticancer [14] anti-inflammatory [15], antidepressant [16, 17] and mainly antioxidant [18]. In addition, the development of their derivatives that comprise similar chemical structure are promising in relation to a range of new pharmacological properties on the central

nervous system, which may be related to neuroprotective action [19, 20, 21]. Despite its promising pharmacological activities, few researches have directed a pharmacological study on phenylpropanoids for the treatment of epilepsy.

Thus, the present study has the objective of providing information on potential anticonvulsant effect and the possible mechanism of action of Isopentyl ferulate (IF), a ester derivative of ferulic acid in mice subjected to two seizure models (Pilocarpine-induced seizures and Pentylenetetrazole-induced seizures). This compound was also tested for the muscular relaxation effect and locomotor activity using the rota rod test and open field test, respectively.

2. Materials and methods

2.1. Reagents and drugs

Polyoxyethylene sorbate (Tween 80), pilocarpine, pentylenetetrazole and flumazenil were obtained from Eg Sigma Chem Ex. Co. St. Louis, Missouri, USA. Diazepam was purchased from Union Chemical (Brazil). All the other chemicals were of the analytical grade.

2.2. Preparation of the substance

Isopentyl ferulate (IF, Fig. 1) has a molecular formula of $C_{15}H_{20}O_4$, refractive index of 1.544 ± 0.02, surface tension of 40.3 ± 3.0 dyn/cm and density of 1.104 ± 0.06 g/cm^3. The process of developing the product consists of a reaction of esterification of ferulic acid [22, 23]. The process of esterification of ferulic acid consists in a stirred mixture of ferulic acid (5 mmol) in isoamyl alcohol (200 ml). Ethanol was added to concentrated sulfuric acid (0.067 mL, 1.25 mmol) and the reaction mixture was subjected to reflux for 3 hours in a 500 ml flask. After cooling to 25 °C, ethyl acetate was added and the solution was washed with water and brine. The ethyl acetate fraction

was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column using 20% ethyl acetate in hexane to produce isopentyl ferulate (yield 55%) [24]. Subsequently, the IF was emulsified with 0.05% Tween 80 dissolved in 0.9% saline and administered via intraperitoneal (i.p.) in doses of 25 mg/kg (IF 25), 50 mg/kg (IF 50) and 75 mg/kg (IF 75).

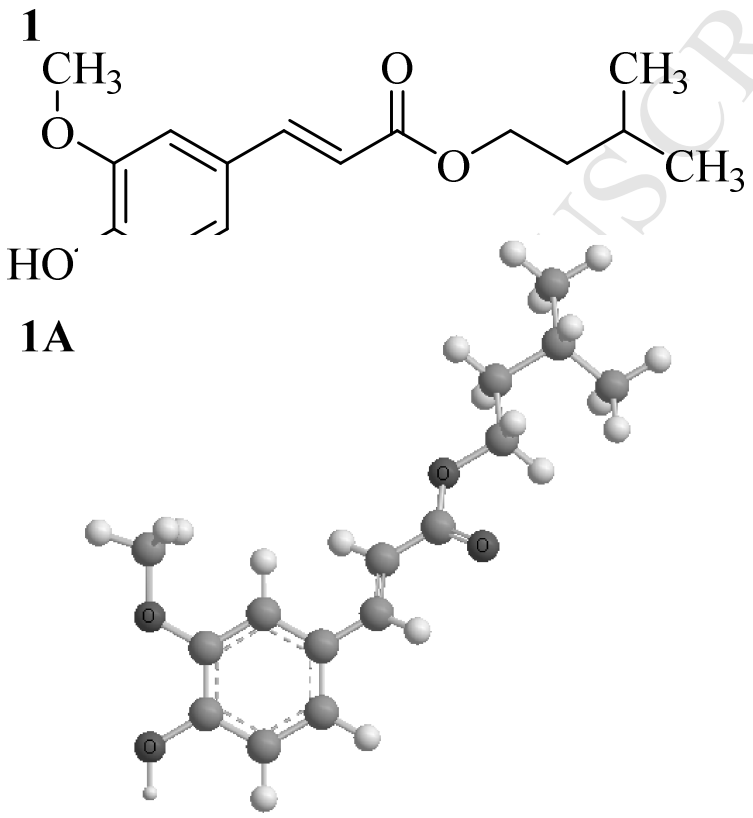

Fig. 1. Chemical structure of isopentyl ferulate (3-methylbutyl (E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate (**1** and **1A**).

2.3. Animals and evaluation of behavioral seizure

Male Swiss (*Mus musculus*) adult mice (25–30 g; 2 months-old) were used in this study (Central Animal Laboratory of the Federal University of Piaui). Animals were housed in cages (35 cm L × 52 cm W × 17 cm H) with free access to food (Purina® pellets) and water and were kept under standard artificial light–dark cycle (lights on at

07:00 a.m.) with controlled temperature (25 ± 2°C). The animals were acclimated for three days before the experiments and were housed in groups during the experiments. Animals were tested during the light period and observed in a closed room with controlled temperature (25 ± 2°C).

Protocol and procedures were approved by the Ethics Committee in Animal Experimentation of UFPI (CEEA/UFPI - authorization number: #030/13). All protocols were designed aiming to reduce the number of animals used to a minimum, as well as to minimize their suffering. The experiments were performed according to the Guide for Care and Use of Laboratory of US Department of Health and Human Services, Washington, DC (1985).

To investigate the effects of IF, an acute treatment to the test animals received by intraperitoneally. Vehicle group was treated with 0.05% Tween 80 dissolved in 0.9% saline (0.1 mL/kg, negative control). IF 25, IF 50 and IF 75 groups were treated with emulsified IF in the vehicle mentioned at doses of 25, 50 and 75 mg/kg, respectively. The doses used were based on a preliminary pharmacological screening protocol [25] with different doses of the IF administered to mice. At all doses used, no signs of acute toxicity or behaviors suggestive of neurotoxicity were observed (data not shown). Diazepam (DZP) group was treated with diazepam (2 mg/kg, positive control) emulsified in vehicle. The selection of the dose of diazepam was based on previous studies [26, 27].

In turn, to clarify the mechanism of action of IF, other groups were treated with flumazenil (FLU), DZP and IF at a dose of 75 mg/kg and associations. The FLU group was treated with flumazenil (5 mg/kg) emulsified vehicle. The DZP + FLU group was pretreated with flumazenil (5 mg/kg) and, after 15 min treated with DZP (2 mg/kg). The

IF 75 + FLU group (n = 8) was pretreated with FLU (5 mg/kg) and, after 15 min, treated with IF 75.

2.4. Pilocarpine-induced seizure

This model was developed by Turski et al. [28]. Briefly, after 30 min from the doses of 25, 50 and 75 mg/kg (i.p.), all the groups received pilocarpine (400 mg/kg, i.p.). Direct observation was made for 4 h to monitor latency to the first seizure (tonic-clonic seizures, with or without raising) and the number of animals that seized and/or death [29, 30, 31].

2.5. Pentylenetetrazole-induced seizure

Pentylenetetrazole (PTZ) was used to induce clonic convulsions [32]. Briefly, after 30 min from the doses of 25, 50 and 75 mg/kg (i.p.), all groups received PTZ (70 mg/kg, i.p.) and animals were observed for 4 h to monitor the same parameters of the previous test [7].

2.6. Open Field Test

The open-field apparatus Archer [33], was made of acrylic (transparent walls with black floor, 30 x 30 x 15 cm) divided into nine squares of equal area. After 30 min of treatments with vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p.), DZP (2 mg/kg; i.p.) or IF (25, 50 and 75 mg/kg, i.p.), the number of squares crossed with four paws (spontaneous locomotor activity) was measured during 5 min. Furthermore, it was observed the frequency of self-cleaning behavior (grooming) and number of surveys (rearing) for 5 minutes [25].

2.7. Rota-Rod Test

The rota rod test was used for the evaluation of motor coordination and muscle relaxation produced by drugs in animals [34]. After 30 min of treatments with vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p.), DZP (2 mg/kg; i.p.) or IF (25, 50 and 75 mg/kg, i.p.), mice were placed with the four paws on a bar of 25 mm of diameter at a rotation speed of 17 rpm for a period of 3 min for each animal. The time of permanence on the bar (second) and the number of falls was measured with three renewals at most.

2.8. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA for multiple comparisons and followed by Student–Newman–Keuls as post hoc test by GraphPad Prism (version 6.0; GraphPad San Diego, California, USA. copyright © 1994-1999). Chi square test was used to analyse percentage of animals with seizures and mortality rate. Differences were considered statistically significant when $p<0.05$.

3. Results

After administration of pilocarpine and pentylenetetrazol, the tested animals (n=8) showed peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea and orofacial movements), and stereotyped movements (grooming and rearing), which persisted until seizures (Table 1 and 2).

3.1. Anticonvulsant effect in pilocarpine-induced seizures

IF effects in pilocarpine-induced seizures in mice are presented in Table 1. The groups of animals treated with IF 25, IF 50 and IF 75 showed an increased in latency to first seizure of 159.6%, 189% and 245.8% when compared with the vehicle group, respectively ($p<0.05$). The results of the IF 75 and DZP were reversed when pretreated with the FLU.

The percentages of the number of animals with seizures were 75%, 50% and 25% for the IF 25, IF 50 and IF 75 groups when compared with the vehicle group, respectively ($p<0.05$). In the pilocarpine-induced seizure model, the IF 25, IF 50 and IF 75 significantly reduced the percentage of mortality when compared with the vehicle ($p<0.05$).

Table 1. Effects of IF (25, 50 and 75 mg/kg, i.p.) on pilocarpine-induced seizure test in mice.

Treatments	Dose (mg/kg)	Latency (s)	Seizures (%)	Death animals (%)
Vehicle	-	11.46 ± 0.89	100	100
DZP	2	31.11 ± 1.69[a]	25[b]	25[b]
FLU	5	11.84 ± 1.43	100	100
IF	25	29.76 ± 0.98[a]	75[b]	75[b]
	50	33.12 ± 1.59[a]	50[b]	50[b]
	75	39.63 ± 1.36[a,g,h]	25[b]	25[b]
FLU + DZP	5 + 2	11.54 ± 1.87[c]	100[e]	100[e]
FLU + IF	5 + 75	11.39 ± 1.16[d]	75[f]	75[f]

Values are the mean ± S.E.M. for 8 mice (per group). [a]$p<0.05$ (ANOVA followed by *t*-Student-Neuman-Keuls as *post hoc* test), when compared with vehicle; [b]$p<0.05$ (χ^2 test), significantly different from vehicle; [c]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the DZP group; [d]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the IF 75 group; [e]$p<0.05$ (χ^2 test), significantly different from the DZP group; [f]$p<0.05$ (χ^2 test), significantly different from the IF 75 group; [g]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the IF 25 group; [h]$p<0.05$ (ANOVA followed by t-Student–Neuman–Keuls as post hoc test), significantly different from the IF 50 group. IF = Isopentyl ferulate (25, 50 and 75 mg/kg, i.p.); DZP = Diazepam (2 mg/kg, i.p.); Vehicle = pilocarpine (400 mg/kg, i.p.).

3.2. Anticonvulsant effect in pentylenetetrazole-induced seizures

The evaluation of IF effects in PTZ-induced seizures in mice is shown in Table 2. The IF 25, IF 50 and IF 75 groups showed an increase of 96.5%, 96.7% and 269% in latency to first seizure when compared with the vehicle group, respectively. Similarly to the previous test, the pretreatment with FLU reversed the anticonvulsant effects in animals treated with DZP and IF 75.

The percentages of the number of animals with seizure were 75%, 50% and 50% for the IF 25, IF 50 and IF 75 groups when compared with the vehicle group, respectively ($p<0.05$). In the PTZ-induced seizure model, the IF 25, IF 50 and IF 75 significantly reduced the percentage of mortality when compared with the vehicle ($p<0.05$).

Table 2. Effects of IF (25, 50 and 75 mg/kg, i.p, IF) on PTZ-induced seizure test in mice.

Treatments	Dose (mg/kg)	Latency (s)	Seizures (%)	Death animals (%)
Vehicle	-	103.25 ± 1.78	100	100
DZP	2	885.27 ± 2.91[a]	00[b]	00[b]
FLU	5	101.89 ± 1.67	100	100
IF	25	202.92 ± 1.18[a]	75[b]	75[b]
	50	203.16 ± 1.79[a]	50[b]	50[b]
	75	381.25 ± 1.82[a,g,h]	50[b]	50[b]
FLU + DZP	5 + 2	105.78 ± 2.55[c]	100[e]	100[e]
FLU + IF	5 + 75	109.67 ± 3.08[d]	100[f]	100[f]

Values are the mean ± S.E.M. for 8 mice (per group). [a]$p<0.05$ (ANOVA followed by Student-Neuman-Keuls as *post hoc* test), when compared with vehicle; [b]$p<0.05$ (χ^2 test), significantly different from vehicle; [c]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the DZP group; [d]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the IF 75 group; [e]$p<0.05$ (χ^2 test), significantly different from the DZP group; [f]$p<0.05$ (χ^2 test), significantly different from the IF 75 group; [g]$p<0.05$ (ANOVA followed by Student–Neuman–Keuls as post hoc test), significantly different from the IF 25 group; [h]$p<0.05$ (ANOVA followed

by t-Student–Neuman–Keuls as post hoc test), significantly different from the IF 50 group. IF = Isopentyl ferulate (25, 50 and 75 mg/kg, i.p.); DZP = Diazepam (2 mg/kg, i.p.); Vehicle = Pentylenetetrazole (70 mg/kg, i.p.).

3.3. Effect of isopentyl ferulate on locomotor activity

The group treated with DZP showed a reduction of 38%, 58.5% and 54% in the number of crossing, rearings and groomings, respectively, when compared with the vehicle ($p<0.05$) (Fig. 2A, 2B, 2C). However, the mice treated with IF 25, 50 and 75 mg/kg did not present any difference in number of crossing, rearings and groomings when compared to the vehicle ($p>0.05$).

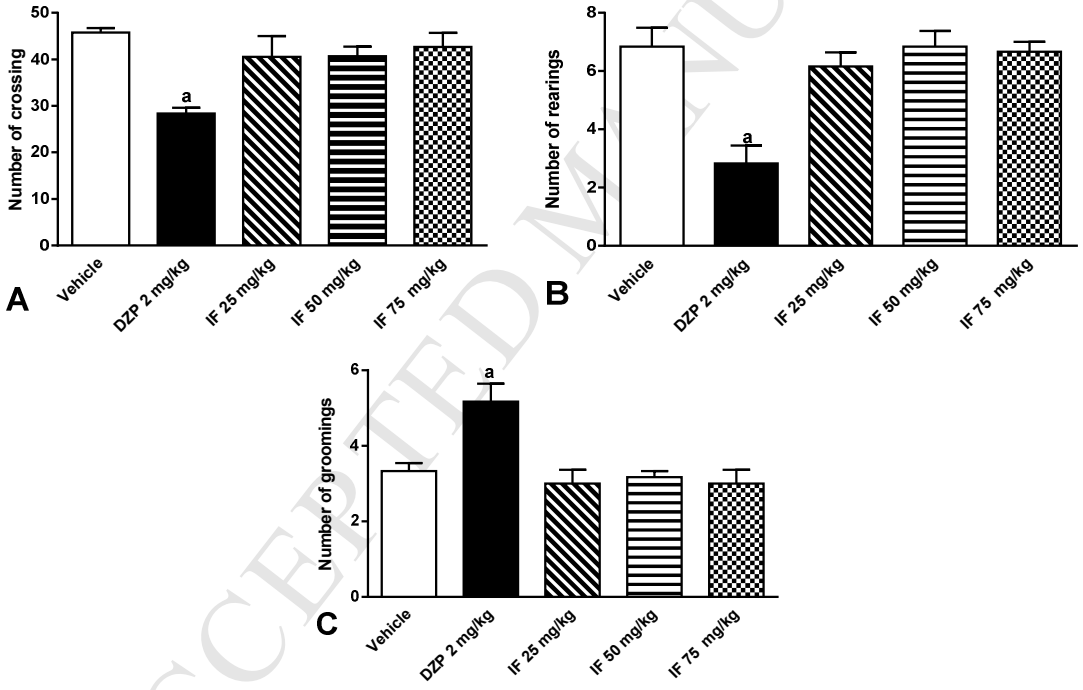

Fig. 2. Effects on the number of crossing (A), rearings (B) and groomings (C) in the open field test. Values are expressed as mean ± S.E.M. for the number of crossing, rearings and groomings of 8 mice (per group) used in the experimental protocols. The differences in the experimental groups were determined by analysis of variance (ANOVA) followed by *Student-Neuman-Keuls* as *post hoc* test. IF = Isopentyl ferulate

(25, 50 and 75 mg/kg, i.p.); DZP = Diazepam (2 mg/kg, i.p.); Vehicle = 0.05% Tween 80 dissolved in 0.9% saline. [a]$p<0.05$, compared to the vehicle group.

3.4. Effect of isopentyl ferulate on motor coordination test

The animals treated with DZP 2 mg/kg reduced 32.58% the time of permanence on the swivel bar when compared to the vehicle ($p<0.05$), while the IF 25, IF 50 and IF 75 mg/kg had no statistical difference when compared to the vehicle ($p>0.05$) (Fig. 3A). Considering the number of falls, the mice tread with DZP 2 mg/kg had this parameter increased by 129.31% when compared to the vehicle ($p<0.05$), while the IF 25, IF 50 and IF 75 mg/kg did no statistical difference when compared to the vehicle ($p>0.05$) (Fig. 3B).

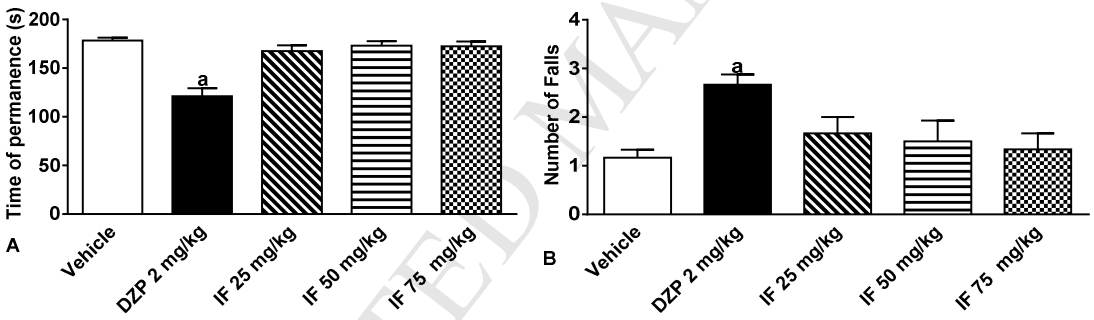

Fig. 3. Effects of IF at doses of 25, 50 and 75 mg/kg in mice on the remaining time on the rotating bar (A) and number of falls (B) on rota rod test.Values are expressed as mean ± S.E.M. of the length of stay on the rotating rod and the number of falls (n = 8 mice per group). Differences between experimental groups were determined by analysis of variance (ANOVA) followed by *Student-Neuman-Keuls* as *post hoc* test. Isopentyl ferulate (25, 50 and 75 mg/kg, i.p.); DZP = Diazepam (2 mg/kg, i.p.); Vehicle = 0.05% Tween 80 dissolved in 0.9% saline. [a]$p<0.05$ compared to vehicle group.

4. Discussion

The demand for more effective, safer and cheaper therapeutic alternatives has lead to decrease in the use of medicinal plant combinations and an increase in the use of isolated and/or synthetic therapeutic substances. For this reason, different constituents found in essential oils are synthesized and used as raw material for research of their pharmacological activities [35, 36]. To our knowledge this is the first time, we are demonstrating that IF exerts significant anticonvulsant effects in two separate models of seizure.

The experimental procedure of seizure induced by pilocarpine in rodents (*Rattus norvegicus* and *Mus musculus*) is one of the main model used to study epilepsy and still reproduces the main characteristics of temporal lobe epilepsy in adult humans [37, 38]. In the present study, after the administration of pilocarpine 400 mg/kg, a cholinergic muscarinic agonist, the animals showed behavioral changes that have evolved for installation of seizures including clonic movements of the upper extremities and which was observed in 100% of animals. In the same group, the seizures progressed to the development of status epilepticus in 100% of animals with the survival rate by 0%.

When treated with isopentyl ferulate, an increase in the time of installation of the first seizure, as well as the reduction in the number of animals with seizures and the number of dead animals was observed. Similar result was obtained in the study conducted by Santos et al. [39], in which it was demonstrated that the caffeic acid (phenylpropanoid) has anticonvulsant effect in the pilocarpine-induced seizures in Wistar rats. Despite the differences between the experimental procedures, the IF when compared with the caffeic acid proved to be more effective at reducing the number of animals that presented seizures, as well as in the percentage of mortality.

Pentylenetetrazole-induced seizure model is another experimental procedure which is widely used for epilepsy study. In this model, PTZ induces convulsions by

inhibiting the γ-aminobutyric acid (GABA) pathway in the central nervous system via the inhibition of $GABA_A$ receptor [40, 41]. The IF at low doses attenuated the onset of PTZ-induced seizures, decreased the number of seizures and the number of dead animals. DZP acts directly by activation of $GABA_A$ receptors and therefore is highly effective in this animal model of seizures, as observed in the present study (Table 2).

Similar result was demonstrated in the study conducted by Nugroho et al. [42] with the caffeic acid (10-20 mg/kg) in PTZ-induced seizures (70 mg/kg, i.p.). The result obtained for the IF is also according to the study conducted by Coelho et al. [43], in which it was demonstrated that the rosmarinic acid (ester of caffeic acid) at low dose increased the latency to first seizure and decreases the number of PTZ-induced seizures (50 mg/kg) in mice. It is important to highlight that the ester of caffeic acid (2 mg/kg) showed a better anticonvulsant effect at a lower dose than caffeic acid (4 mg/kg), which may suggest that the ester compounds are promising anticonvulsants.

When the results obtained by Coelho et al. [43] are compared with the results obtained in the present study, the IF was more effective in reducing the number of animals with seizures. In addition, the caffeic acid and rosmarinic acid were tested in three doses and only the intermediate dose was effective in reducing seizures, while the IF showed a dose dependence of the anticonvulsant effect.

The results obtained for IF was similar to the study conducted by Ilhan et al. [44], in which it was demonstrated that caffeic acid phenethyl ester at a dose of 100 μmol/kg (i.p.) administered in mice showed a high anticonvulsant effect and prevented oxidative stress induced by PTZ.

This study also evaluated the possible effect of the IF on the GABAergic system during the induced seizures by pilocarpine and PTZ, since this system is directly involved in control of neuronal activity. It was demonstrated that the pretreatment of

mice with flumazenil, a GABAergic antagonist, reversed significantly the anticonvulsant action of DZP and the IF at a dose of 75 mg/kg, suggesting that this ester derived from ferulic acid can act on modulatory site of benzodiazepine in $GABA_A$ receptor (γ-aminobutyric acid, type A) in the brain of mice (Fig. 4). Even being observed a possible action on $GABA_A$ receptor, the development of future research is needed to determine if the antiseizure effect of the IF was mediated by a direct interaction in the same site which acts the DZP or at other allosteric sites of $GABA_A$ receptor (Fig. 4).

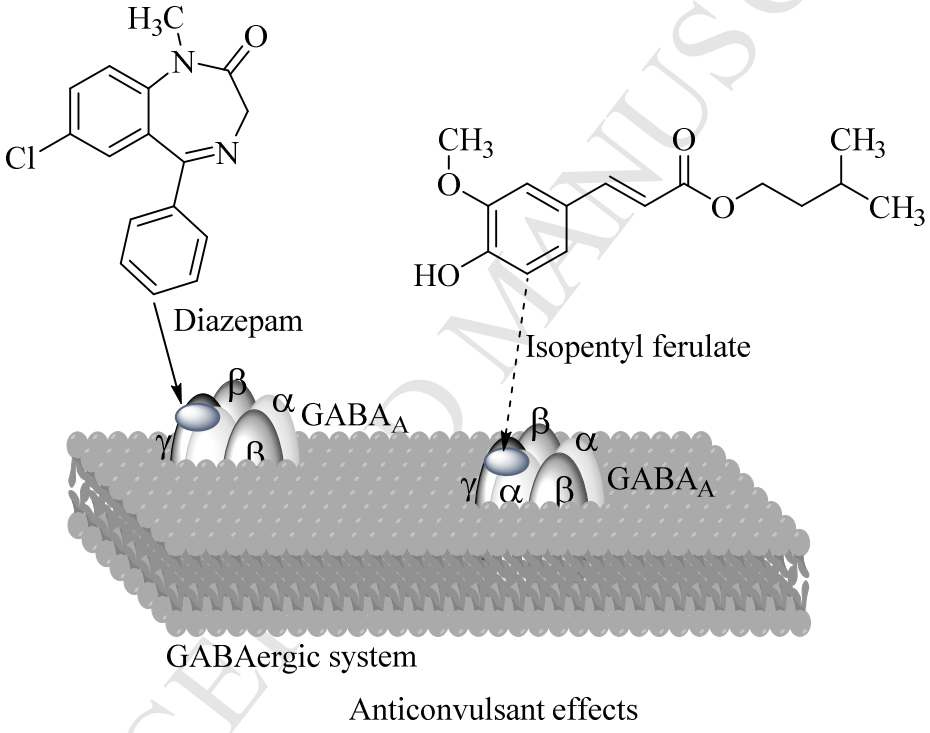

Fig. 4. Possible action mechanism of IF in the $GABA_A$ receptors. The arrow with a solid line indicates that the Diazepam modulates $GABA_A$ receptors (αγ) and the arrow with dotted line indicates that the protective effect of the IFF against seizures may be related to a possible modulation of $GABA_A$ receptors (αγ).

A study conducted by Yoon et al. [10] demonstrated that the sinapic acid (phenylpropanoid) may act on the $GABA_A$ receptor. Kim et al. [45] demonstrated that the anticonvulsant effect of this phenylpropanoid was blocked by the treatment with

FLU, thus suggesting the possible activation of $GABA_A$ receptors, as also observed for the IF.

The ferulic acid is a powerful antioxidant with neuroprotective properties in several models of studies [12], but its anticonvulsant effect has not been reported in previous studies. But analyzing the studies of Zhang et al. [16], it was demonstrated that the ferulic acid (25 and 50 mg/kg) did not show affect the locomotor activity (number of crossings) of mice in open field test. Using another experimental model (YLS-1ª Multi-autonomous Activity Instrument), Tu et al. [46], has shown that the ferulic acid significantly reduced the locomotor activity at doses of 15 and 30 mg/kg. Thus, only the study of Zhang et al. [16], is in agreement with the results obtained for the IF, in which it was demonstrated that there were no significant changes to the dose of 25, 50 and 75 mg/kg in groomings, rearings, and crossings, indicating the absence of changes in locomotor activity.

Additionally, this ester derivative of ferulic acid did not cause deficits of the motor coordination and muscle relaxation activities in mice by rod route test when treated in doses that produced an anticonvulsant effect, unlike observed for DZP. Thus, the observed anticonvulsant effect of IF may not be due to its muscle relaxation effects.

5. Conclusion

In conclusion, this is the first study that demonstrates a protective effect of isopentyl ferulate against induced seizures in mice and that this ester derived from ferulic acid may act through the GABAergic system. In addition, the neurotoxicity study demonstrates that there were no changes in motor coordination and locomotor activity, which is commonly seen in antiepileptic drugs such as diazepam. Thus, these results demonstrate a new pharmacological knowledge of isopentyl ferulate with

potential application against epileptic seizures. However, further studies are needed to elucidate other neurobiological mechanisms underlying epilepsy.

Conflict of interest

The authors declare that they have no conflict of interest.

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Highlights

- ⇨ Isopentyl ferulate (IF) acts as an anticonvulsant agent in Pilocarpine-induced seizure.
- ⇨ IF acts as an anticonvulsant agent in Pentylenetetrazole-induced seizure.
- ⇨ The anticonvulsant effects may be related to the activation of $GABA_A$ receptors.
- ⇨ IF produced no impairment in locomotor activity and motor coordination.

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