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Research Report

Anxiolytic-*like* effects of phytol: Possible involvement of GABAergic transmission

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ABSTRACT

Phytol, a branched chain unsaturated alcohol, is particularly interesting because it is an isolated compound from essential oils of different medicinal plants. The aim of this study was to evaluate the anxiolytic-*like* effects of phytol in animal models to clarify their possible action mechanism. After acute intraperitoneal treatment with phytol at doses of 25, 50 and 75 mg/kg behavioral models of open-field, elevated-plus-maze, rota-rod, light–dark, marble-burying and pentobarbital sleeping time tests were utilized. In open field test, phytol (25, 50 and 75 mg/kg) [$p<0.01$] increased the number of crossings and rearings. However, the number of groomings [$p<0.01$] was reduced. Likewise, the number of entries and the time spent in light space were increased [$p<0.01$] while the number of marble-burying was decreased [$p<0.001$], in elevated-plus-maze, light–dark and marble-burying tests, respectively. In motor activity test, phytol (75 mg/kg) impaired the rota-rod performance of mice [$p<0.01$]. In pentobarbital sleeping time test, phytol 75 mg/kg decreased for latency of sleeping and phytol (25, 50 and 75 mg/kg) increased the sleep time when compared to negative control [$p<0.05$]. All these effects were reversed by pre-treatment with flumazenil (2.5 mg/kg, i.p.), similarly to those observed with diazepam (2 mg/kg, i.p.; positive control) suggesting that the phytol presents mechanism of action by interaction with the GABAergic system. These findings suggest that acute administration of phytol exerts an anxiolytic-*like* effect on mice. Furthermore, suppose that phytol interacts with $GABA_A$ receptor, probably at the receptor subtypes that mediate benzodiazepines effects, to produce sedative and anxiolytic activities.

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1. Introduction

Anxiety is generally a normal reaction to stress and there will always be situations that create stress and discomfort (Higgins and George, 2010; Mackenzie et al., 2011). There are several different types of anxiety disorders. Benzodiazepines (BDZ) are the first-line pharmacological anxiolytics drugs, and more psychoactive medications are being developed in the last 45 year. However, despite the clinical efficacy, most drugs in this class have many problems, including sedation, muscle relaxation, anterograde amnesia and risk of accidents (Mitte et al., 2005; Cunningham et al., 2010). In addition, chronic drug use can lead to psychomotor effects, paradoxical reactions, tolerance, teratologic risk and dependence (Dell'osso and Lader, 2013).

Although conventional anxiolytics have been used for treatment of anxiety disorders, recent clinical evidences have shown that selective serotonin reuptake inhibitors (SSRIs) are also effective on various anxiety disorders. They act by preventing the reuptake of 5-hydroxytryptamine (5-HT), thereby increasing 5-HT levels within the synaptic cleft and modulating neurochemical signaling (Nishikawa et al., 2007; Tsapakis et al., 2012). However, paradoxically they may increase symptoms of anxiety when treatment is initiated and despite extensive research over the past 30 years focused on SSRIs treatment, the precise mechanisms by which SSRIs exert these opposing acute and chronic effects on anxiety remain unknown (Burghardt and Bauer, 2013). Some clinical studies have reported adverse outcomes, such as premature birth, neonatal cardiovascular abnormalities, reduced bone mineral density and an increased risk of bone fracture (Haney et al., 2010; Olivier et al., 2011).

Given the side effects of BDZ and reuptake inhibitors, numerous research groups are looking for new forms of pharmacological treatment for anxiety that can replace the conventional ones. In addition, the search for new compounds which are more effective and safer, with less possibility of adverse reactions is extremely necessary as a large number of users become dependent on chemical and physical conditions. Thus, numerous scientific researchers have been exploring for example several species of medicinal plants both in terms of chemical and pharmacological compounds in search for new anxiolytics (Brito et al., 2012 Oyemitan et al., 2013).

Several studies have shown that many plants containing essential oil possess medicinal properties. Recent research shows that the vast majority of biological activities are derived from terpenes, which are the main chemical components in these oils essential (Souto-Maior et al., 2011; Melo et al., 2013; Russo et al., 2013). Previous studies have shown that terpenes can affect the Central Nervous System (Guimarães et al., 2010; Sousa, 2011; Machado et al., 2013).

The diterpene phytol (3,7,11,15-tetrametilhexadec-2-en-1-ol) (Fig. 1) is a member of branched-chain unsaturated alcohols whose common characteristic structural elements are one hydroxyl group per molecule and a twenty-one double bond carbon atoms ($C_{20}H_{40}O$), molecular weight 296.54 mol/L. It is liquid at room temperature, with a density of 0.8533 g/cm^3, colorless with a boiling point of 202 °C, flash point >200 °C and refractive index 1.460–1.466. It is optically active inasmuch as it contains three asymmetric carbon atoms. The aqueous solubility of phytol is about 0.00327 mg/L (Belsito et al., 2010; McGinty et al., 2010; Karunagoda, 2010). This compound is particularly interesting because it is a component of the chlorophyll molecule, present in green leaves of various medicinal plants, hence it is present in nature in abundance (Rontani and Volkman, 2003). However, phytol has not yet been submitted to neuropharmacological evaluation.

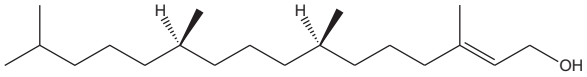

Fig. 1 – Chemical structure of phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol).

Thus, to pursuit the development and introduction of new drugs with greater efficacy and safety is essential to enhance treatment of anxiety. Importantly, the search for alternatives with less toxicity has resulted in decreased introduction of synthetic substances, which underscores the importance of pharmacological studies of natural products such as phytol. In this perspective, in view of its abundance and few data in neuropharmacological literature, the aim of study was evaluate the anxiolytic-*like* effects of phytol in animal models to clarify their possible action mechanism.

2. Results

2.1. *Open-field test*

Phytol (PHY), at doses 25, 50 and 75 mg/kg, (i.p.) showed sedative effects as assessed by open-field test in mice. Significant effects were detected with doses tested of PHY, which produced similar percentages of increased number of crossings (71%, 70% and 69.5%) and rearings (70%, 70% and 71%) when compared with to positive control [$p<0.01$]. The number of groomings (41%, 43% and 48% respectively) [$p<0.01$] were reduced with PHY 25, 50 and 75 mg/kg, whose results were similar to those observed with diazepam (2 mg/kg, i.p.), used as a positive controls [$p<0.01$]. Flumazenil was used for evaluating the possible mechanism of action of the sedative effect of phytol. The group receiving flumazenil (2.5 mg/kg, i.p.) and PHY (75 mg/kg, i.p.) exhibited similar behaviors to those from the positive control [$p<0.01$], indicating that phytol produced a benzodiazepine-type sedative effect (Table 1).

2.2. *Motor coordination test (rota-rod test)*

The rota-rod test was used for evaluating motor coordination and presence of any muscle relaxation effect. Results showed that there was no change after PHY administration (25 and 50 mg/kg, i.p.), when compared with to positive control [$p<0.01$]. PHY (75 mg/kg, i.p.) and diazepam significantly impaired the rota-rod performance of mice. PHY 75 mg/kg and diazepam 2 mg/kg reduced the take-time to fall-down (63% and 66%, respectively) [$p<0.01$]. Flumazenil was used for evaluating the possible mechanism of action of muscle

Table 1 – Effects and action mechanism of phytol in open field test.

Groups	Number of squares crossed	Number of grooming	Number of rearing
Vehicle	49.13±2.56	2.67±0.44	35.17±1.85
DZP 2	28.13±2.36[a]	4.44±0.41[a]	13.13±0.93[a]
PHY 25	48.09±1.65[b]	2.75±0.42[b]	34.70±0.69[b]
PHY 50	49.18±1.81[b]	2.62±0.16[b]	35.35±1.92[b]
PHY 75	50.25±1.88[b]	2.42±0.26[b]	36.03±1.69[b]
FLU 2.5+DZP 2	49.97±1.20[b]	2.57±0.88[b]	36.09±1.41[b]
FLU 2.5	50.75±0.71	2.27±0.52	34.78±1.09
FLU 2.5+PHY 75	49.20±2.75	2.80±0.37	38.40±1.11

[a] $p<0.01$, significantly different from control group (vehicle).
[b] $p<0.01$, significantly different from DZP group (ANOVA followed by *t*-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; and FLU: flumazenil; values are the mean±S.E.M. ($n=7$).

Table 2 – Effects and action mechanism of phytol in the rota-rod test.

Groups	Number of falls	Time of permanence (s)
Vehicle	1.40±0.25	178.7±0.56
DZP 2	2.89±0.47[a]	165.0±1.38[a]
PHY 25	1.45±0.47[b]	178.9±1.62[b]
PHY 50	1.43±0.63[b]	178.6±1.80[b]
PHY 75	1.47±0.33[b]	179.1±1.72[b]
FLU 2.5+DZP 2	1.45±0.33	178.3±0.67
FLU 2.5	1.48±0.16	178.5±0.65
FLU 2.5+PHY 75	1.44±0.51	178.5±1.20

[a] $p<0.01$, significantly different from control group (vehicle).
[b] $p<0.01$, significantly different from DZP group (ANOVA followed by *t*-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; and FLU: flumazenil; values are the mean±S.E.M. ($n=7$).

Table 3 – Effects and action mechanism of phytol on the elevated-plus-maze test.

Groups	NEOA	TPOA
Vehicle	10.67±0.74	122.5±6.37
DZP 2	15.71±0.28[a]	208.4±3.57[a]
PHY 25	15.67±0.65[a]	203.3±7.58[a]
PHY 50	15.77±0.74[a]	209.3±2.00[a]
PHY 75	17.97±0.47[a,b,c,d]	280.4±2.05[a,b,c,d]
FLU 2.5+DZP 2	10.71±0.82[b]	126.1±1.31[b]
FLU 2.5	10.72±0.43	122.6±1.28
FLU 2.5+PHY 75	10.63±0.66[e]	121.8±4.67[e]

[a] $p<0.01$, significantly different from control group (vehicle).
[b] $p<0.01$, significantly different from DZP group.
[c] $p<0.01$, significantly different from PHY 25 group.
[d] $p<0.01$, significantly different from PHY 50 group.
[e] $p<0.01$, significantly different from PHY 75 group (ANOVA followed by *t*-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; FLU: flumazenil; NEOA: number of entries in the open arms; and TPOA: time of permanence in the open arms; values are the mean±S.E.M. ($n=7$).

relaxation effect of phytol. The group receiving flumazenil (2.5 mg/kg, i.p.) and PHY (75 mg/kg, i.p.) exhibited similar behaviors relative to the controls, indicating that phytol involves benzodiazepine receptors in muscle relaxant effect (Table 2).

2.3. *Elevated-plus-maze test (EPM)*

In elevated-plus-maze apparatus, used for evaluating anxiolytic-*like* effects, there was no change after PHY administration (25 and 50 mg/kg, i.p.), when compared with negative control [$p<0.01$]. Intraperitoneal administration of PHY (75 mg/kg) significantly increased the number of entries in open arms (78%), the percentage of entries into open arms (99%), the percentage of time spent in open arms (97%) and in the time of permanence in open arms (80%) compared with the negative control [$p<0.01$]. These effects were similar to those of diazepam 2 mg/kg [NEOA, $p<0.01$; TPOA, $p<0.01$;]. On the other hand, in order to determine whether the anxiolytic-*like* effects of PHY are exerted via GABAergic systems, PHY treated mice were subjected to a co-treatment with flumazenil, a benzodiazepine receptor antagonist. The anxiolytic-*like* effect of PHY (75 mg/kg) was reversed by flumazenil (2.5 mg/kg) (Table 3).

2.4. *Light–dark box test*

The administration of diazepam increased [$p<0.01$] the time spent by mice in the illuminated space. However, no changes were found in number of entries into this place after treatment with PHY at doses 25 and 50 mg/kg (i.p.) [$p>0.01$]. In this model, the administration of PHY (25, 50 and 75 mg/kg) [$p<0.01$] increased the time spent by mice in the illuminated compartment (7%, 11% and 66%, respectively), when compared with to negative control [$p<0.01$]. This effect was dose dependent and statistically different when compared with to negative control [$p<0.01$] (Table 4). The effects of PHY (75 mg/kg) are more potent that diazepam 2 mg/kg [$p<0.01$]. On the other hand, in order to determine whether the effects of PHY on time spent light space are exerted via GABAergic systems, PHY (75 mg/kg) as a co-treatment with flumazenil (2.5 mg/kg). The effect of PHY on time spent light space was abolished by flumazenil (Table 4).

2.5. *Marble-burying test (MBT)*

In order to further expand the evaluation of the PHY anxiolytic-*like* properties, this model was also added. The group

Table 4 – Effects and action mechanism of phytol on the light–dark test.

Groups	Time spent light space (s)
Vehicle	104.2±1.48
DZP 2	158.1±2.06[a]
PHY 25	156.2±2.12[a]
PHY 50	156.6±1.26[a]
PHY 75	179.8±1.31[a,b,c,d]
FLU 2.5+DZP 2	103.3±1.01[b]
FLU 2.5	106.5±0.65
FLU 2.5+PHY 75	105.8±1.51[e]

[a] $p<0.01$, significantly different from control group (vehicle).
[b] $p<0.01$, significantly different from DZP group.
[c] $p<0.01$, significantly different from PHY 25 group.
[d] $p<0.01$, significantly different from PHY 50 group.
[e] $p<0.01$, significantly different from PHY 75 group (ANOVA followed by t-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; and FLU: flumazenil; values are the mean±S.E.M. ($n=7$).

treated with DZP 2 mg/kg (i.p.) reduced the number of marbles-buried (NMB) by 67% when compared with the negative control [$p<0.001$]. In this protocol, the administration of PHY 25, 50, 75 (i.p.) reduced NMB respectively by 71%, 73%, 80% when compared to negative control [$p<0.001$], which suggests a more potent anxiolytic-*like* effect by phytol rather than diazepam 2 mg/kg (Fig. 2). In addition, the PHY 75 mg/kg reduced NMB by 39% when compared to DZP 2 mg/kg [$p<0.001$]. In the second protocol, in order to determine whether the anxiolytic-*like* effects of PHY are exerted via GABAergic systems, PHY (75 mg/kg) was used as a co-treatment with flumazenil (2.5 mg/kg). FLU 2.5+DZP 2 decreased by 181% the effect of DZP 2 mg/kg on NMB [$p<0.001$] and FLU 2.5+PHY 75 reduced by 396% the effect of PHY 75 mg/kg on NMB [$p<0.001$] (Fig. 2).

2.6. *Pentobarbital sleeping time*

In pentobarbital sleeping time test, the administration of DZP 2 mg/kg and PHY 75 mg/kg (i.p.) decreased for latency of sleeping respectively by 40% and 31%, when compared to negative control [$p<0.05$]. In order to determine whether the anxiolytic-*like* effects of PHY are exerted via GABAergic systems, PHY (75 mg/kg) was used as a co-treatment with flumazenil (2.5 mg/kg). FLU 2.5+DZP 2 reduced by 65% the effect of DZP 2 mg/kg on latency of sleeping [$p<0.05$] and FLU 2.5+PHY 75 reduced by 48% the effect of PHY 75 mg/kg on latency of sleeping [$p<0.05$] (Table 5). In addition, DZP 2 mg/kg and PHY (25, 50 and 75 mg/kg, respectively) increased the sleep time in 44, 7, 10 and 19%, respectively when compared to negative control [$p<0.05$], suggesting an enhanced pentobarbital effect. FLU 2.5+DZP 2 decreased by 30% the effect of DZP 2 mg/kg on latency of sleeping [$p<0.05$] and FLU 2.5+PHY 75 reduced by 15% the effect of PHY 75 mg/kg on latency of sleeping [$p<0.05$] (Table 5).

3. Discussion

Before starting the discussion of present study it is important to consider that phytol was evaluated as their safety in mice.

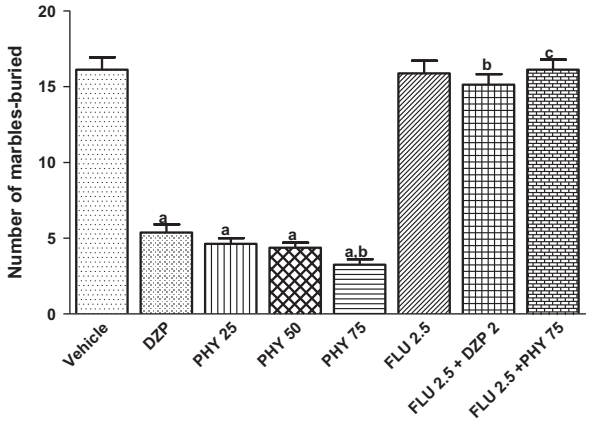

Fig. 2 – Number of marbles-buried (NMB) in the marble-burying test (MBT). [a]$p<0.001$, significantly different from control group (vehicle); [b]$p<0.001$, significantly different from DZP; [c]$p<0.001$, significantly different from PHY 75 group (ANOVA followed by t-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; and FLU: flumazenil; values are the mean±S.E.M. ($n=7$).

Table 5 – Effects and action mechanism of phytol on pentobarbital sleeping time test in mice.

Groups	Latency of sleeping (s)	Sleeping time (min)
Vehicle	261.13±19.43	47.13±4.56
DZP 2	157.69±12.43[a]	68.03±3.91[a]
PHY 25	259.32±11.78	50.56±4.45[a]
PHY 50	252.11±13.46	51.89±2.91[a]
PHY 75	178.27±18.54[a]	56.23±3.24[a]
FLU 2.5+DZP 2	260.89±16.75[b]	47.89±4.67[b]
FLU 2.5	262.674±13.78	46.99±3.67
FLU 2.5+PHY 75	263.76±11.64[c]	48.01±5.12[c]

[a] $p<0.05$, significantly different from negative control group (vehicle).
[b] $p<0.05$, significantly different from DZP group.
[c] $p<0.05$, significantly different from PHY 75 group (ANOVA followed by t-Student–Neuman–Keuls test as *post-hoc* test). PHY: phytol; DZP: diazepam; and FLU: flumazenil; values are the mean±S.E.M ($n=7$).

And according to these studies phytol can be considered moderately toxic to rodents, which emphasizes the need for this study, since this compound is present in a large amount of medicinal plants it is used by the population due to their anxiolytic properties (OECD, 2001; Costa et al., 2012a).

In addition, some studies have shown that this diterpene has pharmacological properties such as antioxidant, antinociceptive and anticonvulsant (Costa et al., 2012b; Santos et al., in press).

In the present research, the sedative and anxiolytic effects of phytol were examined in animal models using tests such as open-feld, elevated-plus-maze, rota-rod, light–dark, marble-burying and pentobarbital sleeping time, which are classic models for screening central nervous system activity and providing information about anxiety, sedation and myorelaxant

activity (Baretta et al., 2012; Chioca et al., 2013). Our results suggest that PHY reduces locomotor activity and has sedative and anxiolytic effects on mice. Therefore, these activities were investigated in order to provide information to determine the mechanism of action of this diterpene and the possible adverse effects arising from its use in anxiety therapy.

Phytol was firstly evaluated on open-field test, which gives a good indication of exploratory activity of animal. Diazepam has been used as a standard anxiolytic and also has been frequently employed in behavioral pharmacology as a reference compound for potentially anxiolytic-acting substances (Gomes et al., 2010; Mizushige et al., 2013). In relation to phytol in any doses, it produced muscle relaxation and sedative effects similarly to diazepam as these parameters were not decreased in comparison control. On the other hand, when compared to diazepam increase on number of crossings and rearings, the outcomes suggest fewer adverse reactions. Probably, it seems that anxiolytic effect of phytol is unrelated to a loss of motor coordination or to neuromuscular blockade; instead, it may be caused by central depressant activity.

In the present work, a clear anxiolytic-*like* activity of phytol has been observed. The elevated-plus-maze has been frequently used to detect and evaluate anxiolytic/anxiogenic-*like* properties of drugs. The frequency and time spent in open arms is the major index of anxiety in plus-maze model, given the fact that rodents are extremely aversive to an open area (Almeida et al., 2012). Diazepam increases the number of entries into the open arms and increase the time spent in the open arms, demonstrating the characteristic anxiolytic effect of BDZ compounds. In this test, phytol at all doses tested increased the entries into and time spent in the open arms, indicating an anxiolytic-like effect without motor impairment. These results indicate that anxiolytic activity is comparable with that produced by diazepam. In order to elucidate the mechanism of the anxiolytic effect of phytol, flumazenil was used, an antagonist of benzodiazepine drugs. The results showed that the effect of phytol was reversed by the administration of flumazenil, indicating the involvement of benzodiazepine receptors.

In the light–dark box test, anxiety is generated by the conflict between the desire to explore and to retreat from an unknown and well-illuminated space (Costa et al., 2011; Lima et al., 2013). In case of light–dark test, this effect showed a dose-dependent behavior, because it increased the residence time of the animal in box clear, confirming the anxiolytic effect of the compound. However, only at dose of 75 mg/kg phytol's effects are greater than diazepam's as it increases the parameters in control, diazepam and other doses of phytol. A phytol blocking promoted by flumazenil was also observed, suggesting that phytol can interact with $GABA_A$ receptor subunits responsible to the anxiolytic action similarly to DZP, since flumazenil is a $GABA_A$ receptor antagonist in benzodiazepine binding site that possesses the ability to induce anxiety (Ishola et al., 2012).

Despite the good predictive validity of the elevated-plus-maze and light–dark tests for anxiolytic-*like* drugs, the use of more than one animal model of anxiety is recommended to avoid false-positive results. The marble-burying was used to confirm the anxiolytic-*like* effect observed in the elevated-plus-maze and light–dark tests. Marble-burying is used as an assay to infer compulsive, anxietylike behavior and is widely used as a model of obsessive–compulsive disorder (Cryan and Sweeney, 2011; Kinsey et al., 2011). Phytol dose-dependently reduced number of marbles-buried at all doses. producing anxiolytic effects better than the positive control group, corroborating the anxiolytic-*like* behavior previously observed in the other tests. Similarly, in the group pre-treated with flumazenil, it was found a phytol blocking mediated by flumazenil, suggesting that phytol can interact with GABA receptor subunits, which is responsible for anxiolytic actions of diazepam.

At the same time, however, typical GABA/benzodiazepine receptor agonists, such as diazepam, have side effects including muscle relaxation and depressive mood (Hertz et al., 2006). Contrary to diazepam, that reduced the residence time on rotating bar and increases the number of falls in rota-rod test, indicating a muscle relaxant effect and increased incoordination characteristic of benzodiazepine compounds, PHY showed anxiolytic-*like* effects without affecting locomotor activity at lower doses, probably because they do not interact with the GABA receptor subunits responsible for these adverse reactions of BDZ (Wang et al., 2008).

Sedative and anxiolytic drugs, such as BDZ, facilitate the action of γ-amino-butyric acid (GABA) upon the $GABA_A$ receptor (Sieghart and Sperk, 2002). The pentobarbital-induced sleep test evaluated a possible sedative effect of the compound. In this test, CNS depressant and sedative drugs classically decrease the sleep latency and increase the sleeping time (Carlini and Burgos, 1979; Galdino et al., 2012). The present results showed that phytol at all doses tested increased the sleep time and only the dose of 75 mg/kg decreased for latency of sleeping, suggesting a possible sedative effect. However, this effect was not corroborated in the open-field test in a significant way, suggesting that phytol possibly was able to potentiate the pentobarbital effects showing its depressive potential, but it may not be able to induce sedative effect itself in the doses tested.

In summary, our results suggest that acute administration of phytol exerts an anxiolytic-*like* effect on mice. Furthermore, they support the idea that PHY interacts with the $GABA_A$ receptor, probably at the receptor subtypes that mediate benzodiazepines effects, to produce sedative and anxiolytic activities. Additional studies with subcronic and chronic administration, however, are needed to prove this activity and fully clarify the mechanism of anxiolytic effect of PHY. In this way, PHY could manifest these effects at doses not showing either sedative activity, being thus potentially useful in clinical practice.

4. Experimental procedure

4.1. *Animals*

Swiss (*Mus musculus*) albino mice (25–30 g) of 2 months old purchased from Central Animal House of the Federal University of Piaui were used for the present study. The animals were allowed free access to water and food (Purina® pellets) *ad libitum* and were kept under controlled lighting (12 h dark/light cycle) and temperature (26 ± 1 °C). Behavioral experiments were

conducted from 08:00 to 10:00 am; and the animals were monitored for one more hour after behavioral experiments. Experimental protocols and procedures were approved by Animal Experimentation Ethics Committee at the Federal University of Piaui (CEEA/UFPI #013/11).

4.2. Drug treatments

Phytol (PHY), natural compound isolated, was emulsified with 0.05% Tween 80 (Sigma-USA) dissolved in 0.9% saline solution. 30 min before the experiments animals were intraperitoneally treated with phytol at dosages of 25, 50 and 75 mg/kg. Negative control group was received vehicle (0.05% Tween 80 in 0.9% saline) at the same volume (10 ml/kg) administered by same route as treated groups. Sodium pentobarbital (Cristália – Brazil)) was dissolved in 0.9% saline solution. Diazepam (DZP) 2 mg/kg (Sigma Chem. Co., St. Louis, MO, USA) and Flumazenil (FLU) 2.5 mg/kg (Sigma Chem. Co., St. Louis, MO, USA) were intraperitoneally injected into mice after dissolution in distilled water, and used as standards.

4.3. Experimental protocol

The animals were tested during light period and observed in a closed room with constant temperature (26±1 °C) and illuminated with 15 V weak red light. All tests were performed in different days with distinct groups of animals.

4.4. Open-field test

The open-field area was made of acrylic glass (transparent walls and black floor; $30 \times 30 \times 15$ cm^3), and was divided into nine squares of equal area. This apparatus was used to evaluate the exploratory activity of animal during 5 min according to the model described (Archer, 1973; Almeida et al., 2012). The animals (male albino mice) were divided into five groups of seven animals per group. The first group was treated with 0.05% Tween 80 in 0.9% saline (i.p.); second group was treated with diazepam (2 mg/kg, i.p.); third, fourth and fifth groups were treated with phytol (25, 50 and 75 mg/kg, i.p.), respectively. After 30 min of treatment, the following parameters were monitored during 5 min; number of squares crossed with the four paws (spontaneous locomotor activity), number of grooming behavior (grooming) and number of surveys (rearing), not lean against the wall. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried.

To evaluate if anxiolytic of phytol were mediated by GABA/benzodiazepine receptors on locomotor activity, three groups of seven male mice were pretreated with flumazenil (2.5 mg/kg, i.p.), a recognized competitive antagonist on central benzodiazepine receptor, 15 min after above treatments, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to the groups of sixth and eighth respectively. 30 min later of the treatment, motor activity of animals was verified similar to the protocol performed previously.

4.5. Motor coordination test (rota-rod test)

A rota-rod tread mill device was used for evaluation of motor coordination and muscle relaxation produced by drugs in animals (Shiotsuki et al., 2010). Male albino mice were divided into five groups of seven mice per group. The first and second groups were treated with 0.05% Tween 80 in 0.9% saline and dizepam (2 mg/kg, i.p.) respectively, third, fourth and fifth groups were treated with phytol at dosages of 25, 50 and 75 mg/kg (i.p.). Thirty minutes after administration of treatments, mice were placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor with a rotation of 17 rpm for a period of 3 min for each animal. The time of permanence on the bar and the number of falls were recorded with triplicates at most. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried.

To evaluate if the effects of phytol were mediated by GABA/benzodiazepine receptors on muscle relaxation three groups of seven male mice in each were pretreated with flumazenil (2.5 mg/kg, i.p.), a recognized competitive antagonist at the central benzodiazepine receptor. 15 min after flumazenil treatment, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to groups of sixth and eighth respectively. 30 min later, motor coordination and muscle relaxation of animals were verified similar to the protocol performed previously.

4.6. Elevated-plus-maze test (EPM)

The elevated-plus-maze test consists of two perpendicular open arms (30×5 cm^2) and two closed arms ($30 \times 5 \times 25$ cm^3) in perpendicular position (Lister, 1987; Campelo et al., 2011). The open and closed arms were connected by a central platform (5×5 cm^2). For this test five groups of ten animals in each group were used. The first group was treated with vehicle (0.05% Tween 80 in 0.9% saline), the second group with diazepam (2 mg/kg, i.p.) and the third, fourth and fifth groups were treated with phytol at doses of 25, 50, 75 mg/kg (i.p.). Thirty minutes after administration of treatments, the animal was placed at the center of the plus maze with its nose in the direction of one of the closed arms, and observed for 5 min to determine the following parameters – number of entries in the open arms (NEOA), percentages of entries into open arms (PEOA), time of permanence in open arms (TPOA) and percentage of time of permanence in the open arms (PTOA). After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried.

To evaluate if phytol effects were mediated by GABA/benzodiazepine receptors three groups of seven male mice per group were pretreated with flumazenil (2.5 mg/kg, i.p.). 15 min after the above treatment, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to the groups of sixth and eighth respectively. 30 min later of the treatment, the parameters described of animals were verified similar to protocol performed previously.

4.7. Ligh-dark box test

The light–dark test consisted of five groups of seven mice per group. Treatment of mice was identical to the above tests. 30 min after treatment, the animals were placed in equipment one at a time as described below. The apparatus used is made of acrylic glass divided into two compartments (light

box and dark box) that was communicated through a small door (Crawley, 1981; Marques et al., 2013). The dark box (black acrylic; $27 \times 18 \times 29$ cm^3) is weakly lit. The clear box (transparent acrylic; $27 \times 18 \times 29$ cm^3) is illuminated by ambient light. The first and second groups were treated with 0.05% Tween 80 in 0.9% saline and dizepam (2 mg/kg, i.p.) respectively, third, fourth and fifth groups were treated with phytol at dosages of 25, 50 and 75 mg/kg (i.p.). Thirty minutes after administration of treatments, the activities of each animal were recorded for 5 min. The parameter used was the time spent in light box in seconds. A compartment entry was considered valid when the animal's four paws were inside that chamber. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried.

To evaluate if phytol effects were mediated by GABA/benzodiazepine receptors three groups of seven male mice per group were pretreated with flumazenil (2.5 mg/kg, i.p.). 15 min after above treatment, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to the groups of sixth and eighth respectively. 30 min later of the treatment, time spent in light box of animals was recorded similar to the protocol performed previously.

4.8. *Marble-burying test (MBT)*

The marble-burying procedure is based on a defensive burying behavior that can be elicited in rodents in response to aversive stimuli such as a shock prod, noxious food or an effective unconditioned stimulus such as glass marbles (Poling et al., 1981; Pires et al., 2013). The administration of a substance likely to have anxiolytic effects in rodents tend to reduce the number of marbles buried (NMB) in this test. The first group was treated with 0.05% Tween 80 (i.p.); second group was treated with diazepam (2 mg/kg, i.p.); third, fourth and fifth groups were treated with PHY (25, 50 and 75 mg/kg, i.p.), respectively. Thirty minutes after treatments the animals were placed individually in cages (27 long × 16 wide × 13 cm high) with 25 glass marbles uniformly distributed on a 5 cm layer of sawdust. The NMB in the sawdust was reported. A marble was considered as hidden when it was at least two-thirds covered by sawdust. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried.

To evaluate if phytol effects were mediated by GABA/benzodiazepine receptors three groups of seven male mice per group were pretreated with flumazenil (2.5 mg/kg, i.p.). 15 min after the above treatment, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to the groups of sixth and eighth respectively. 30 min later of the treatment, number of marbles buried of animals was verified similar to protocol performed previously.

4.9. *Pentobarbital sleeping time*

Pentobarbital sodium was the test compound in evaluation of pentobarbital induced sleeping time. The first and second groups were treated with 0.05% Tween 80 in 0.9% saline and diazepam (2 mg/kg, i.p.) respectively, third, fourth and fifth groups were treated with PHY at dosages of 25, 50 and 75 mg/kg (i.p.). Sixty minutes after the administration of treatments the animals received sodium pentobarbital (40 mg/kg, i.p.), and the time elapsed between the administration of pentobarbital until the loss of the righting reflex was recorded as the sleep latency, and the time elapsed between the loss and voluntary recovery of the righting reflex was recorded as the sleep time (Carlini and Burgos, 1979).

To evaluate if phytol effects were mediated by GABA/benzodiazepine receptors three groups of seven male mice per group were pretreated with flumazenil (2.5 mg/kg, i.p.). 15 min after above treatment, diazepam (2 mg/kg) and phytol (75 mg/kg) were administrated to the groups of sixth and eighth respectively. 30 min later of the treatment, the sleep time of animals was verified similar to protocol performed previously.

4.10. *Statistical analysis*

All results are presented as mean ± S.E.M. (standard error of mean) values. The data were analyzed by means of analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls's as *post-hoc* test. Data were analyzed using the Graph Pad Prism software (version 5.0) and experimental groups were compared with the control positive The levels statistical significance ranged with $p<0.05$, $p<0.01$ and $p<0.001$.

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