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Effect of Diterpene Phytol on Pentylenetetrazol and Maximal Electroshock Seizure Models: Possible Role of GABAergic Mechanism

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ABSTRACT

Background: Recently, diterpene phytol was characterized as a potent anticonvulsant in a pilocarpine model. The present study explores the efficacy of phytol in pentylenetetrazol (PTZ) and the Maximal Electroshock Seizure (MES) models and the possible role of GABAergic mechanism. **Materials and Method:** Mice were pretreated with phytol (50, 75, 200 and 250 mg kg^{-1}, i.p.) 30 min before the convulsant or corneal electroshock, respectively. Diazepam (2, 5 mg kg^{-1}, i.p.) and flumazenil (25 mg kg^{-1}, i.p.) were used as reference drugs. Motor coordination was evaluated by rotarod test. **Results:** Phytol dose-dependently increased the latency of onset of clonic and tonic-clonic seizures in the PTZ model. However, unlike diazepam, it failed to decrease the incidence of clonic seizures. The highest dose of phytol protected the animals against the incidence of tonic-clonic seizures and death while the antagonist of benzodiazepine receptors blocked the anticonvulsant effect of this diterpene. Furthermore, although not as efficient as diazepam, the highest dose of phytol prevented the tonic extension and showed a tendency to alleviate the mortality in MES test. Flumazenil failed to block the anticonvulsant effect of phytol in the MES test. Motor impairment, evaluated with the rotarod test, was minimal at the highest dose of phytol. **Conclusion:** The results suggest that although the hightest dose of 250 mg kg^{-1} phytol suppressed the seizure activity it appeared less potent than diazepam in both the PTZ and MES test. The possible GABAergic mechanism involved in the diterpene activity is discussed.

Key words: Phytol, PTZ test, MES test, anticonvulsant, rotarod, mice

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INTRODUCTION

Overall prevalence of epilepsy which is one of the most frequent neurological disorder is about 1% of the population (Arnhold *et al*., 2002). Although, more than 10 new antiepileptic drugs have been introduced in clinical trials during the last decade, one-third of epileptic patients still have seizures and remain pharmacoresistant (Pati and Alexopoulos, 2010). In spite of numerous approaches implemented in control of convulsive attacks, it is necessary to apply other alternative approaches and one is to discover new drugs from natural sources with a broad spectrum of anticonvulsant activity and minimal side effects. Animal seizure models for initial *in vivo* identification and characterization of newly developed potential anticonvulsant drugs represent a significant and important step in epilepsy research. Although, *in vitro* model approaches have provided means for target based drug design and discovery of the cellular and molecular mechanisms of epilepsy, they are unable to asses bioavalability and brain accessibility, as well as to model the pharmacodynamic actions necessary for seizure protection (Castel-Branco *et al*., 2009). The two most widely used and well established models of acute seizures where nonepileptic animals are used are pentylenetetrazol (PTZ) induced seizures and Maximal Electroshock Seizure (MES) in mice (White *et al*., 1995). Considered one of the best-validated, the MES test predicts drugs protective against generalized tonic-clonic seizures and thereby identifies compounds effective to prevent spread of seizures (Swinyard and Kupferberg, 1985). The PTZ seizure test is used to identify compounds able to raise the seizure threshold (Goodman *et al*., 1953). It is accepted that drugs effective in PTZ seizure test can enhance GABAergic neurotransmission and/or affect T-type calcium (Ca^{2+}) channels. Different brain structures are involved in these acute seizure models (Miller *et al*., 1987). Recent studies

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have been involved in the identification and isolation of new therapeutic compounds of medicinal importance from higher plants for specific diseases (De Almeida *et al*., 2011). The essential oils extracted from aromatic plants possess a variety of pharmacological properties, such as analgesic, cardiovascular and anticonvulsant (Carlini, 2003). Terpenes are the primarily components of essential oils and most of the useful properties of medicinal herbs have been attributed to them. Low levels of phytol, a diterpene alcohol ($C_{20}H_{40}O$) have also been detected in essential oils. Degradation of chlorophyll by ruminal bacteria produces phytol which is readily converted to phytanic acid in ruminant tissues (Sermakkani and Thangapandian, 2012). It is readily converted to phytanic acid after absorption and is a precursor for vitamins E and K_1 (Inoue *et al*., 2005). Phytol was observed to have antibacterial activities against *Staphylococcous aureus* by causing damage to cell membranes, to block the teratogenic effects of retinol (Arnhold *et al*., 2002) and to possess antioxidant properties against free radicals generated *in vitro* (Santos *et al*., 2013). Recently, few reports have considered *in vivo* effects of exogenously applied phytol on the Central Nervous System (CNS) (Costa *et al*., 2012; Santos *et al*., 2013). This diterpene showed an antinociceptive activity in several models of nociception as well as an anticonvulsant activity in acute pilocarpine seizure test in mice. The objective of the present study, resulting from the above-mentioned two reports on the effects of phytol in the CNS, was designed to further characterize the anti-convulsant properties of phytol in MES test and PTZ seizure test in mice. In addition, possible involvement of benzodiazepine (BZD) binding site of $GABA_A$ receptors in modulating the effects of phytol on anticonvulsant activity was evaluated.

MATERIALS AND METHODS

Subjects: The experiments were carried out on male ICR mice (20-25 g) obtained from the breeding house of the Institute of Neurobiology, Bulgarian Academy of Sciences. Upon their arrival in the laboratory (at least a week before the experiments), animals were housed in transparent plastic cages (n = 5-6) in standardized laboratory conditions: 21±1°C, 40-50% humidity and 12 h light-dark cycle (lights on between 08.00 and 20.00 h). The mice had free access to food and water before experimental procedures. The experiments were performed between 10.00 am and 01.00 pm. The experimental procedures were carried out in accordance with the guidance and general recommendations of the European Communities Council Directive of 24th November 1986 (86/609/EEC) and the experimental design was approved by Local Ethics Committee of Institute of Neurobiology, Bulgarian Academy of Sciences on the use of laboratory animals.

Reagents and drugs: For this study, the following substances were used. Pentylenetetrazol (PTZ), used as a convulsant, polyoxyethylene-sorbitan monolated (Tween 80), flumazenil, diterpene phytol (purchased from Sigma Chemical Co. St. Louis, M., USA) and diazepam (DZP) (Sopharma, Bulgaria). 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^{-1}. It is liquid at room temperature, with a density of 0.8533 g cm^{-1}, colorless liquid with a boiling point of 202°C, flash point >200°F and refractive index 1.460-1.466. It is optically active in as much as it contains three asymmetric carbon atoms. The aqueous solubility of phytol is about 0.00327 mg L^{-1} (Belisto *et al*., 2010; Karunagoda, 2010; McGinty *et al*., 2010). It is widely distributed in nature as a constituent of chlorophyll molecules in green plants and red algae, of vitamin E (α-tocopherol) and other tocopherols and of vitamin K_1 (phylloquinone). Phytol may be obtained through acid hydrolysis of chlorophylls and through the action of enzyme chlorophyllase on chlorophylls. A stereospecific synthesis of phytol was effected in 1959 by British chemists. In plant cells, the compound is synthesized from mevalonic acid (Baxter *et al*., 1967). The dosage of all drugs was expressed as milligrams per kilogram of body weight. In the present study, the diterpene was emulsified in 0.05% Tween 80 dissolved in 0.9% saline and sonicated before use. PTZ was dissolved in saline solution (NaCl 0.9%). The drugs were applied at a volume of 10 mL kg^{-1}.

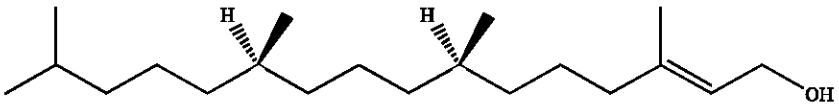

Fig. 1: Chemical structure of phytol (3, 7, 11, 15-tetramethylhexadec-2-en-1-ol)

Experimental groups

PTZ-induced clonic-tonic seizures: Mice were divided into eleven random groups (n = 8-10). The first group represented control group and received a vehicle (0.05% Tween 80 dissolved in 0.9% saline), the remaining groups received an i.p. injection of phytol at doses of 50, 75, 200 and 250 mg kg^{-1}, DZP (2 and 5 mg kg^{-1}), FLU (25 mg kg^{-1}), flumazenil+DZP (25+2) and flumazenil+phytol (25+250), respectively. Thirty minutes after treatment with vehicle/drugs, mice received PTZ (90 mg kg^{-1}, i.p.) in a volume of 0.01 mL g^{-1} b.wt. into vehicle/drug pretreated rats and were observed for the following behavioral changes: Myoclonus, defined as a whole body twitch, clonic seizures and tonic-clonic seizures consisting of Tonic Hindlimb Extension (THE) which is usually the lethal component in approximately 80% of the mice under normal conditions. The following parameters were evaluated: The latencies to the first clonic seizure and to the THE, the percentage of mice clonic seizures, THE and death.

Maximal Electroconvulsive Seizure test (MES): The same experimental groups as in the PTZ seizure test were used. The MES test involves induction of seizure with a supramaximal current. A drop of local anaesthetic was applied to the eyes of each animal, corneal electrodes were applied followed by an electric stimulus of 50 mA, 60 Hz delivered for 0.2 sec (constant current shock generator). Protection was defined as the ability to prevent hind limb tonic extensor component of the seizure (Robertson *et al*., 1988).

Motor coordination: The motor coordination following treatment with phytol was evaluated in mice using rotorod procedure. Inability of a treated mouse to maintain equilibrium for 1 min in three consecutive trials on a 25 mm diameter slowly rotating rod (6 rpm) was used as the endpoint indicating motor impairment.

Statistical analysis: Results were expressed as Mean±SEM and analyzed with one-way analysis of variance (ANOVA) and the t-Student-Neuman-Keuls test as *post hoc* test. All analysis were performed using SigmaStat® SPSS. The incidence of seizures and mortality was evaluated by Fisher's exact test. The level of statistical significance was set at 5%.

RESULTS

PTZ-induced seizures: Single intraperitoneal injection of PTZ at a dose of 90 mg kg^{-1} induced tonic-clonic convulsions as well as lethality of 100% in the control mice (Table 1). Kruskal-Wallis one way analysis of Variance showed significant dose-dependent effect of phytol on the latency of clonic seizures ($H = 21.557$, $p = 0.001$). *Post hoc* test demonstrated that administration of phytol at doses of 75, 200 and 250 mg kg^{-1}, respectively, significantly prolonged the latency of clonic seizures (*$p<0.05$) as compared to the control group. The onset of tonic-clonic seizures was also increased after pretreatment with highest dose of 250 mg kg^{-1} phytol (*$p<0.05$). Moreover, phytol was able to protect mice against incidence of tonic-clonic seizures (70%) and mortality (70%). The pretreatment with DZP (2 mg kg^{-1}, i.p.) significantly prolonged the latencies of both clonic and tonic-clonic seizures and fully protected (100%) against both clonic (5 mg kg^{-1}) and tonic-clonic seizures and mortality (2, 5 mg kg^{-1}) in mice (*$p<0.05$). Thus, DZP was more effective in the PTZ test in reducing the incidence of clonic seizure and tonic extension than the most effective dose of 250 mg kg^{-1} phytol. While flumazenil (25 mg kg^{-1}, i.p.) antagonized the increasing effect of DZP on the latency of clonic, clonic-tonic seizures and mortality, it was able only to block the effect of phytol (250 mg kg^{-1}) on the prolongation of latency to clonic seizures.

MES test: The maximal electroshock produced THE in all control mice followed by a clonus in 73 and 73%

Table 1: Effects of phytol on PTZ-induced seizures and death in mice (n = 8-10). The pretreatment time before a PTZ (90 mg kg^{-1}, i.p.) injection was 30 min

Treatments (mg kg^{-1})	Latency to onset of clonus (sec)	Latency to onset of tonic-clonic (sec)	Incidence of clonic seizure (%)	Incidence of tonic extension (%)	Death (%)
Phytol 50 (n = 8)	113.3±17.1	318.3±72.2	100	100	100
Phytol 75 (n = 8)	122.4±11.6*	336.9±70.1	100	88	63
Phytol 200 (n = 10)	173.1±47.3*	493.4±126.7	100	70	70
Phytol 250 (n = 10)	118.0±8*	481.0±85*	100	30*	30*
Vehicle	71.3±6.39	242.7±44.8	100	100	100
DZP 2 (n = 8)	236.0±46.1*	495.0±96*	100	0*	0*
DZP 5 (n = 8)	x	x	0*	0*	0*
FLU 25 (n = 8)	101.0±16.3	239.1±43.4	100	100	88
FLU 25+DZP 2 (n = 8)	110.1±31.7	222.9±35.1	100	100	100
FLU 25+Phytol 250 (n = 8)	100.4±11.1	327.9±56.8	100	100	88

Data was analyzed by one-way ANOVA followed by a Bonferroni *post hoc* test (latency to seizure) and Fisher's exact probability test (% convulsions or death).
*$p<0.05$ vs. control group

Table 2: Effects of phytol on MES-induced seizures and death in mice. Phytol, diazepam and flumazenil were administered 30 min before the MES test

Treatments (mg kg^{-1})	Incidence of tonic extension (%)	Death (%)
Phytol 50	100	63
Phytol 75	100	63
Phytol 200	100	63
Phytol 250	30*	15
Vehicle	100	73
DZP 2	50*	0*
DZP 5	13*	0*
FLU 25	100	60
FLU 25+DZP 5	100	43
FLU 25+Phytol 250	50*	30

Data (Mean±SEM, n = 8-10) was analyzed by Fisher's exact probability test (% convulsions or death). *$p<0.05$ vs. control group

Table 3: Effects of phytol in the rotarod test in mice. Phytol and diazepam were administered 30 min before the rotarod test

Treatment (mg kg^{-1})	Time of permanence
Phytol 50	51.5±5.5
Phytol 75	59.5±0.5
Phytol 200	46.1±6.1
Phytol 250	53.1±3.6
Vehicle	43.2±4.6
DZP 2	50.1±4.2
DZP 5	5.3±0.7*

Data (Mean±SEM, n = 6-8) was analyzed by one-way ANOVA followed by a Bonferroni posthoc test. *$p<0.05$ vs. control group

mortality (Table 2). The highest dose of phytol (250 mg kg^{-1}) significantly decreased the incidence of THE seizures (30%) (*$p<0.05$) and tended to alleviate the incidence of mortality (15%) ($p<0.06$). The protective effect of the highest dose of 250 mg kg^{-1} phytol was comparable to DZP at a dose of 2 mg kg^{-1} with 30% incidence of tonic extension and 50% incidence of tonic extension, respectively. Flumazenil (25 mg kg^{-1}) antagonized the effect of DZP (2 mg kg^{-1}) against the incidence of THE and protection to mortality. However, this benzodiazepine receptor antagonist failed to affect the seizure-protective effect of phytol in MES test.

Rotarod test: The effects of phytol in the rotarod test are demonstrated in Table 3. Phytol did not produce ataxia and rotorod toxicity and rotarod performance in mice was not impaired after i.p. administration phytol starting from a dose of 50 mg kg^{-1}.

DISCUSSION

The only report considering the anticonvulsant activity of phytol was published recently suggesting that this diterpene is effective in preventing tonic-clonic generalized seizures in pilocarpine model of mice (Costa *et al*., 2012). The present results did not reveal any anticonvulsant property of phytol that is superior to the used reference drug, diazepam. However, in the present study, higher doses of phytol were required to produce suppression of tonic-clonic seizures compared to the dose that has been reported for inhibition of THE in the pilocarpine model (Costa *et al*., 2012). The genesis of the seizures triggered by pilocarpine injection is associated with the agonistic effect of this convulsant on muscarinic receptors (Turski *et al*., 1983). It was suggested that the identified Antiepileptic Drugs (AEDs) which are effective in suppression of PTZ-induced clonic seizures partially overlapped with the group of AEDs effective against MES (Meldrum, 2002). In this regard, diazepam was found to be more potent against PTZ seizures than against MES test (Krall *et al*., 1978) which agree with the idea that the underlying mechanism associated with activity in PTZ test is connected to the involvement of the $GABA_A$ receptor (Huang *et al*., 2001). The mechanism involving GABAergic system activation is associated with sedation and loss of muscle tone. Indeed, a higher dose of 5 mg kg^{-1} diazepam exerted a complete protection against both PTZ-induced seizures and THE in MES test which effect was accompanied by a profound ataxia in our experiments. Although the results of this study showed that the highest dose of 250 mg kg^{-1} phytol failed to prevent the incidence of clonic seizures in PTZ seizure test its effectiveness was comparable to the standard drug DZP as concern the incidence of THE in the MES test. Therefore, although not as potent as diazepam, phytol was able to prevent the mortality in the two seizure models suggesting that this drug is worthy of further experimental study. Similar protection with lower doses of this diterpene was evident during the acute phase of the pilocarpine seizure model (Costa *et al*., 2012). The protection against the PTZ-induced THE convulsions and the ability of flumazenil to prevent the anticonvulsant effect of phytol suggest that the mechanism of action might involve GABAergic neurotransmission and activation of BZD site on the $GABA_A$-BZD receptor complex. In contrast, the presence of flumazenil failed to block the effect of the highest dose of phytol against THE in MES test, suggesting that GABA system was not involved in the anticonvulsant mechanism of phytol in this model as was reported for the pilocarpine seizure model (Costa *et al*., 2012). Literature data demonstrated that natural compounds, such as monoterpene borneol, modulate BDZ-GABA complex (Quintans-Junior *et al*., 2010) while others such as citronellol (De Sousa *et al*., 2006) and thymol (Johnston, 2005) modulate GABA receptor independently of the BZD sites. Most of the anticonvulsant chemical constituents of the essential oils are monoterpenes which protective mechanism was suggested to occur through the blockade of NMDA receptor, acetycholine mechanism or GABAergic system (De Almeida *et al*., 2011; Re *et al*., 2000).

In this study, although a significant effect of phytol on the latency to PTZ seizures was evident, an inter-animal variability in the onset of clonic as well as tonic-clonic seizures was detected which might be explained with the specific threshold brain concentration of PTZ required to induce seizures in mice (Yonekawa *et al*., 1980). In addition, by using Hierarchical Cluster Analysis for analyzing BOLD-fMRI data, Keogh *et al*. (2005) revealed that PTZ-induced seizures after i.p. injection involved multiple regions of sensitivity with heterogeneous time courses that varied markedly across animals.

In conclusion, the results of the present study suggest that phytol is less potent than diazepam in both the PTZ and MES test. The underlying mechanism of these effects probably involves GABAergic system in the PTZ test through the BDZ site. However, future experimental studies are required for the evaluation of the anticonvulsant efficacy and the neuronal mechanism of this diterpene in animal models of epilepsy.

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