ANTINOCICEPTIVE EFFECTS OF HYDROALCOHOLIC EXTRACT OF *THYMUS VULGARIS*

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ABSTRACT

Previous investigation has shown that *Thymus Vulgaris* (*TV*) modulates pain. The aim of this work was to examine the role of *TV* on acute and chronic pain and compares its effect with dexamethasone (DEX) and stress (ST) by using Hot plate, Tail flick and Formalin tests in mice. In this study male albino mice (25-30 g.) in 21 groups (n=147) were used. *TV* (100, 500 and 1000mg/kg), DEX (0.5, 1 and 2 mg/kg) and vehicle (VEH) were injected 30 minutes before pain assessment tests. Stress was applied by 1 min swimming in cold water (18 – 22°). Acute and chronic pain was assessed by Hot plate, Tail flick and Formalin tests. For assessment of the role of opioid receptors in antinoceception of *TV* extract, Naloxon (NAL, 2mg/kg, ip) as opioid receptor antagonist was injected before the injection of the more effective dose (500mg/kg) of *TV* extract.

Results indicated that *TV*, DEX and ST have analgesic effects in all tests ($P<0.01$ in comparison with control group).

Above findings showed that *TV* extract, DEX and ST have modulatory effects on acute and chronic pain. Further research is required to determine the mechanisms by which *TV* extract has an inhibitory effect on pain sensation.

Keywords: Acute and chronic pain, formalin test, hot plate, tail flick, *Thymus vulgaris*

INTRODUCTION

During the last decade, numerous studies have been carried out to explore the effects of Drug on the different methods of antiniociception tests. A number of behavioral methods have been developed to study nociception in animals. Out of these, the tail flick test, and the hot plate test, measured the response to brief noxious stimuli. The animal's response in these tests is integrated at rather low levels in the central nervous system, probably giving information mainly about the pain threshold. The formalin test, on the other hand, measures the response to a long-lasting nociceptive stimulus and thus may have a closer resemblance to clinical pain. In this test, two types of pain were postulated: a short lasting pain caused by a direct effect on nociceptors and followed by a long lasting pain due to inflammation.

Thymus vulgaris (*TV*) fig. 1 is a member of family of Lamiaceae which are strongly aromatic and consist of approximately 38 species that are distributed in subtropical countries (Azaz, *et al*., 2004). Main components of *TV* are the phenols, thymol (40%) and carvacrol (15%) fig. 2. During the winter, *T. Vulgaris* contains less amounts of phenol. Furthermore, components in the essential oil are thymol methyl ether (2%), cineol, cymen, pinene, borneol and esters of the latter two substances (Azaz, *et al*., 2004). In traditional medicinal reported *TV* have many beneficial effects in the asthma, bronchitis and other respiratory diseases (Vigo *et al*., 2004). Other effects of *TV* are smooth muscle relaxing effect (Briseid and Dyrud, 1962), anti fungal activity (Segvic *et al*., 2006), Anti-Candida albicans Activity (Abe *et al*., 2003), Inhibition of Aeromonas caviae (Abu-Ghazaleh, 2000), Antibacterial activity (Burt and Reinders, 2003), antioxidant potential (Lee *et al*.2002; Youdim and Denis, 1999), platelet aggregation inhibitors (Okazaki *et al*., 2002). Effect on different mycotoxigenic fungi (Soliman and Badeaa, 2002), Spasmolytic activity

Fig. 1: *Thymus vulgaris*

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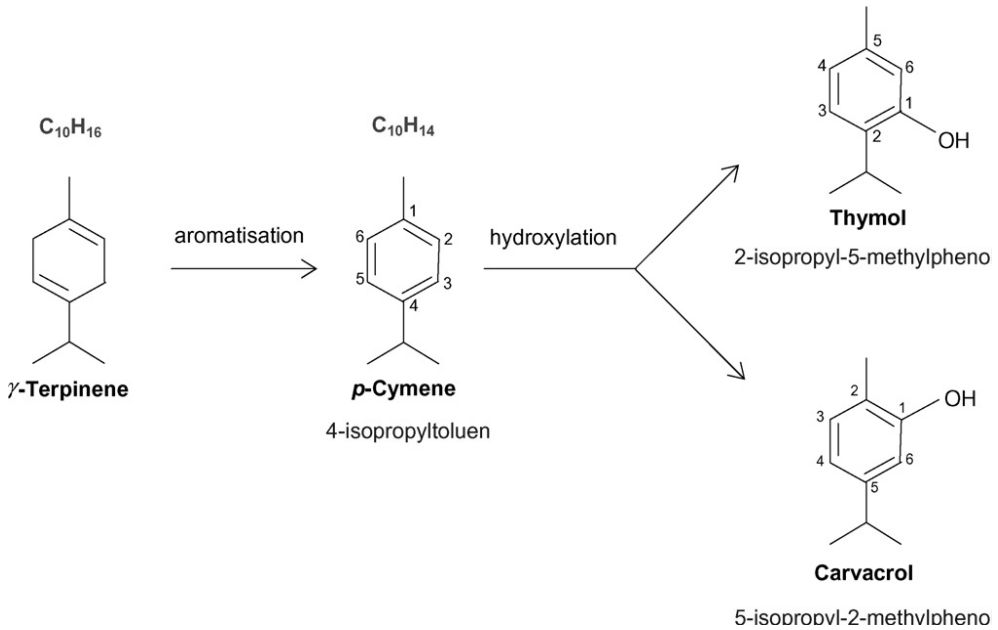

Fig. 2: Pathway for the biosynthesis of thymol and carvacrol from terpinene and *p*-cymene.

(Van Den and Broucke, 1983; Astudilo *et al*., 2004) antibacterial effects against Gram-positive and Gram-negative bacteria (Essavi and Srour, 2000; Panizzi *et al*., 1993), antispasmodic activity on the isolated guinea-pig trachea (Meister *et al*., 1999), antispasmodic activity on the isolated guinea-pig ileum (Babaei *et al*., 2008), inhibition of the growth of M. tuberculosis (Lall and Meyer, 1999). Effect on the spontaneous contractile activity of the smooth muscles (Berra *et al*., 2007). Although the specific biochemical mechanisms responsible for the therapeutic efficacy of *Thymus Vulgaris* are unknown, this drug affects a variety of physiological processes. The results of previous study indicated that glucocorticoids and stress have antinoceceptive effects (Mousa, 1983; Mousa, 1981). The objective of present study was carried out to examine the antinociceptive effects of hydro alcoholic extract of *TV* on acute and chronic pain in Hot plate, Tail flick and Formalin tests and compares its effects with dexamethasone and stress in mice. For determine the role of opioid system in the antinociceptive effect of *TV*, we used naloxone (as non-selective opioid antagonist).

MATERIAL AND METHODS

Animals

After obtaining approval from the Animal Care and Use Committee at the University, Young adult male albino mice weighing 25-30g were obtained from the animal house of physiology research center of Semnan University of Medical Sciences (Semnan, Iran) used in this study. Animals were housed seven per cage in standard clear plastic cages in a temperature-controlled room (22 ± 2^oC) on a 12-hr light-dark cycle (6 A.M. lights on), with food and water provided ad libitum. All the investigations were conducted in the light phase, on a natural light cycle, between 10 a.m. and 2 p.m. and the animals were used only once in each test. All procedures for care were executed in accordance with national and international guidelines for the care and use of laboratory animals.

Drugs

Plant material and preparation of extracts

The leaves and flowers of *TV* were collected, identified, classified and extracted by Dr. Etemadi in June 2006, in the Applied Scientific Educational Center of Jihad-e Agriculture of Semnan. The voucher specimen (No.83-21) was preserved and deposited in herbal library of School of Practical and Agricultural Sciences. For extraction and isolation, forty-five grams of air-dried leaves and flowers were dried, grinded and then extracted with 350mL 80% methanol and 650ml water for 24 hrs in a continuous extraction by soxhlet apparatus. The methanol and water of extract were evaporated below temperature (45^oC) in incubator. Finally the dried material was pulverized in Pestle and Mortar and the powder of *TV* extract diluted by saline and used in (100, 500 and 1000mg/kg) intraperitoneal injection by 26-gauge syringe 30 min before tests.

Dexamethasone

Dexamethasone (DEX, Synopharm, Italy) as a specific GR agonist (0.5, 1 and 2 mg/kg) or vehicle (VEH) were injected interaperitonealy same as TV. DEX was dissolved initially in 100% ethanol and diluted to a final

concentration of 2% ethanol in 0.9% saline. A 2% ethanol solution in saline was used for vehicle control injection.

Naloxone

To investigate the participation of opioid system in the antinociceptive effect of the *TV* extracts, animals were pretreated with naloxone (NAL, Sigma Co., 2 mg/kg IP) as a non-selective opioid antagonist 15 min before administration of the best dose of *TV* extract (500mg/kg) according the method indicated in (Abdelfattah *et al*., 2000) NAL was dissolved initially in SAL. All drugs and solutions were freshly prepared before each experiment. The drugs doses were mainly derived from our pilot study and also a survey of other studies using these drugs (Golshani *et al*., 2004; Eihabazi *et al*., 2006).

Antinociceptive tests

The hot plate, tail flick, and formalin test are three popular models of nociception. The hot plate test uses heat as the noxious stimuli to model acute pain. In this model a mouse or rat is placed on a hot plate. The dependent variable is measured by the time it takes for the rodent to respond to the stimuli, by either licking its paw or by jumping up from the plate. The tail flick test also uses heat as the noxious stimulus. With this test, the stimulus causes a simple nociceptive reflux response in which the rat or mouse flicks its tail away from the heat source. Unlike the hot plate and tail flick test that used heat as the noxious stimulus, the formalin test uses a formalin solution as a chemical noxious stimulus. By injecting the formalin solution into the paw of a rat or a mouse, a model of persistent pain caused by peripheral tissue injuries and inflammation is created.

Hot plate

For conventional hot plate testing, the method of Woolfe and Mc Donald (1994) was used. The mice were placed in a plexiglas cylinder (diameter = 19 cm, height = 25 cm) on 52 ± 0.5^{o}C Hot plate and latency for the animal to lick its hindpaw (or jump) was measured. To avoid tissue damage, ("cut-off" time) was set at 30sec (Maleki-Dizaji *et al*., 2007; Wesolowska *et al*., 2006).

Tail flick

In this test we used The Tail-flick apparatus were described by D'Amour and Smith, 1941. The tail is placed on a level surface, a radiant heat is applied to the tail and the latency of the mouse to remove its tail from the heat is recorded. In our studies, the maximum time of heat exposure ("cut-off" time) to avoid tissue damage was 13 sec. (Le Barse *et al*., 2001).

Formalin test

The formalin test is an important animal model in the study of acute long-lasting pain (Francesca *et al*., 2004). The method used in this study was determined using the formalin test as described by Dubuission & Dennis (1977). Each mouse received 20µl of formalin (2%) subcutaneously in to the dorsal surface of the right hind paw by using a micro syringe with a 26-gauge needle. Immediately after formalin injection, animals were placed individually in glass cylinder (20 cm wide 25 cm length) on a flat glass floor and a mirror was arranged in a 45^{o} angle under the cylinder to allow clear observation of the paws of the animals. Throughout 5 min. prior to this procedure, each mouse was allowed to adapt the testing box and left freely moving and exploring (habituation). The formalin test in rodent consists of two successive phases (Hunskaar and Hole, 1987; Tjolsen *et al*., 1992). This behavior is seen in three phase: initial phase that occurs about 3 minute after injection and produces due to direct stimulation of nociceptors (neurogenic pain), quiescent phase during which the animal does not show licking behavior and third phase that occurs between 20th and 30th minutes due to inflammatory process (inflammatory pain) (Le Barse *et al*., 2001). The total time (seconds) spent licking and biting the injected paw were measured as an indicator of pain.

Acute toxicity (Lethal dose)

After injection of extract to each group, the number of death was counted during a 48 hours period and LD values were calculated.

STATISTICAL ANALYSIS

Data were analyzed by one-way and two-way analysis of variance (ANOVA), followed by Tukey test for multiple comparisons. Values of $P<0.05$ were considered significant.

Experiments

Experiment 1

The aim of this experiment was to determine the effects of *TV* extract, Dexamethasone and stress on acute pain sensation in tail flick model. Mice were randomly divided into ten groups (n=7 in each group): sham (no any injection, n=7), *TV* extract (n=21 in 3 groups), DEX (n=21 in 3 groups), ST (n=7), VEH (n=7, at volume equal to extract). All injections were done 30 min before tail flick test. NAL (n=7) injected 15 min before use of the more effective dose (500mg/kg) of *TV* extract.

Experiment 2

The aim of this experiment was to determine the effects of *TV* Extract, Dexamethasone and stress on acute pain sensation in hot plat model. Mice were randomly divided into ten groups (n=7 in each group): sham (no any injection, n=7), *TV* extract (n=21 in 3 groups), DEX (n=21 in 3 groups), ST (n=7), VEH (n=7, at volume equal to extract). All injections were done 30 min before tail flick test. NAL (n=7) injected 15 min before use of the more effective dose (500mg/kg) of *TV* extract.

Experiment 3

The aim of third experiment was to determine the effects of *TV* extract Dexamethasone and stress on neurogenic and inflammatory pain. Mice randomly divided into ten groups (n=7 in each group): sham (no any injection, n=7), *TV* extract (n=21 in 3 groups), DEX (n=21 in 3 groups), ST (n=7), VEH (n=7, at volume equal to extract). All injections were done 30 min before tail flick test. NAL (n=7) injected 15 min before use of the more effective dose (500mg/kg) of *TV* extract.

RESULTS

LD

Because no animal was died during 48hrs after extract injection, the LD value was zero in this experiment.

Experiment 1

The results of Expt. 1 are shown in fig. 3. Analysis of data in the control and treated groups indicated that different doses of *TV*, DEX and ST, significantly (P<0.01) increased reaction time in comparison to vehicle-treated and control animals in tail flick. In addition animals that received 500mg/kg of *TV* extract have best reaction time in comparison to other groups. Pretreatment of NAL at best dose of extract of *TV* (500mg/kg) attenuated the reaction time in tail flick test. Thus, NAL attenuates antinociceptive effects of *TV* extract (fig. 3).

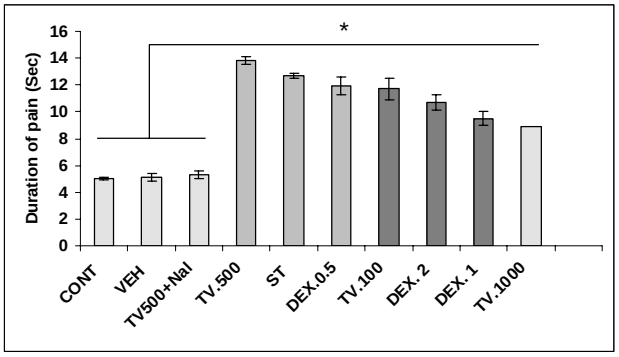

Fig. 3: Antinociceptive effects of hydroalcoholic extract of *T. vulgaris* in Hot Plate model. Administration of hydroalcoholic extract of *T. vulgaris* (100 and 500 and 1000mg/kg I.P) 30 minutes before Hot plate test increased reaction time (P<0.01). NAL reversed this effect.

Experiment 2

The results of Expt. 2 are shown in fig. 4. Analysis of data in the control and treatments groups indicated that different doses of *TV*, DEX and ST, significantly (P<0.01) increased reaction time in comparison to vehicle- treated and control animals in hot plate model. In addition animals that received 500 mg/kg of *TV* extract have best reaction time in comparison to other groups. Pretreatment of NAL at best dose of extract of *TV* (500mg/kg) attenuated the reaction time in hot plate test. Thus, NAL attenuates antinociceptive effects of *TV* extract (fig. 4).

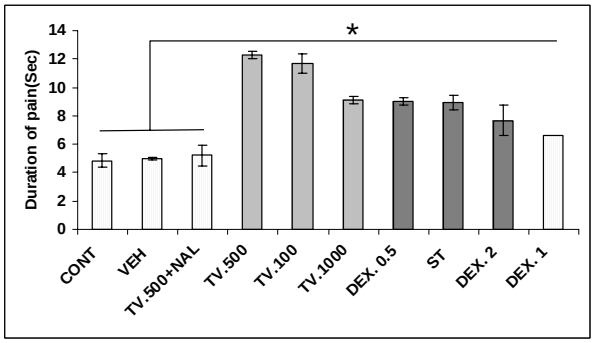

Fig. 4: Antinociceptive effects of hydroalcoholic extract of *T. vulgaris* in Tail flick model. Administration of hydroalcoholic extract of *T. vulgaris* (100 and 500 and 1000mg/kg I.P) 30 minutes before tail flick test increased reaction time (P<0.01).

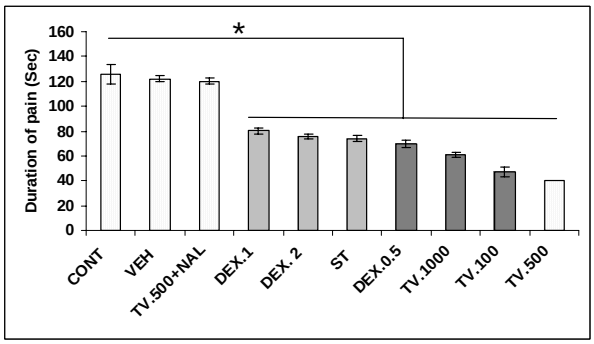

Fig. 5A: antinociceptive effects of hydroalcoholic extract of *T.vulgaris* on the initial phase of formalin test. Administration of hydroalcoholic extract of *T.vulgaris* (100 and 500 and 1000mg/kg I.P) 30 minutes before formalin test decreased licking behavior time in the initial phase (P<0.01).

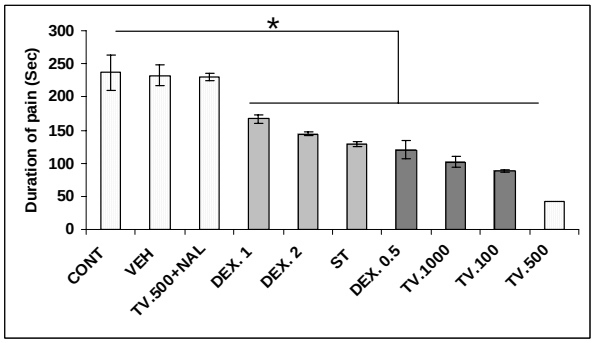

Fig. 5B: Antinociceptive effects of hydroalcoholic extract of *T. vulgaris* on the late phase of formalin test. Administration of hydroalcoholic extract of *T. vulgaris* (100 and 500 and 1000mg/kg I.P) 30 minutes before formalin test decreased licking behavior time in the late phase (P<0.01).

Experiment 3

The results of Expt. 3 are shown in fig. 5 (A and B). Analysis of data in the control and treatment groups

indicated that different doses of *TV*, DEX and ST, significantly ($P<0.01$) decreased reaction time in comparison to vehicle-treated and control animals in the early (A) and late (B) phases of formalin test. In addition animals that received 500mg/kg of *TV* extract have best reaction time in comparison to other groups. Pretreatment of NAL at best dose of extract of *TV* (500mg/kg) increased the reaction time in early phase and late phase of formalin test. Thus, NAL attenuates antinociceptive effects of *TV* extract.

DISCUSSION

The results of the present study demonstrates that the hydroalchoholic extract of *thymus vulgaris*, have a significant effect against pain in the three current antiniociceptive models in mice: formalin, tail flick and hot plate tests. These results also showed that the analgesic effects of hydroalchoholic extract was in the range of 100 to 1000mg/kg. But the dose of (500mg/kg) was the most effective and more potent than that of the other two analgesic agents (DEX and ST). The previous investigations confirmed that dexamethasone and stress have modulatory effect on pain (Mousa, 1983 and 1981). There is always a question that which of the active components of whole extract of a medicinal plant is more important. There are many reports about the beneficial effects of *TV* and their compounds. In *TV* it has been reported that thymol and carvacrol are consisting the most important components of the extract (Azaz, *et al*., 2004). Carvacrol is a phenolic compound and has shown antiseptic, antibacterial, antifungal as well as anti-nociceptive and anti-inflammatory properties (Kurk *et al*., 2001; USDA., 2005; Hajhashemi *et al*., 2002).

In our previous study extract of *TV* produced an inhibition of the contractions induced by acetylacholine. Through acetylcholine agonistic effect on Muscarinic receptors since atropine could block these contractions. It is possible that pharmacological mechanism of the antispasmodic action of *T. vulgaris* extract may be due to the anticholinergic effect and block of serotoninergic activities (Babaei *et al*., 2008]. The causative role for thymol in the antispasmodic effects observed in some studies, however, the possibility for carvacrol to be involved in such effect is not clear (Broucke and Lemli,1982; Aydin and Seker.,2005). Nevertheless it can not be simply ruled out that other components of the *TV* may be involved in the observed effects.

The formalin test which is sensitive for various classes of analgesic drugs have two distinct phases, reflecting different types of pain. The early phase (initial pain) reflects a direct effect of formalin on nociceptors (neurogenic pain) whereas the late phase reflects tissue injury or inflammatory pain (Hunskar and Hole 1987;Elisabetsky *et al*., 1995).

In the formalin test, several mediators such as histamine, kinin, serotonin and prostaglandins are released from damaged cells which take part in the inflammatory response and are able to stimulate nociceptors and induction of pain (Rang *et al*., 1998). We don't found any interactive effects of *TV* extract with these mediators in reported articles. In this test, the centrally acting drugs such as narcotics inhibited both phases equally, while peripherally acting drugs only inhibited the second phase (Shibata *et al*., 1989). It is also well known that the formalin model may involve sensorial C-fibers (Heapy *et al*., 1987) in early phase and a combined process generated by peripheral inflammatory tissue and functional changes in the dorsal horn in late phase (Dickenson and Sullivian, 1987; Dalal *et al*., 1999). Our results showed that the time spent in licking and biting of the injected paw was significantly reduced by intraperitoneal administration of the hydroalchoholic extract of *TV*, in both phases. In fact, the effect of the extract on both phases showed that they contain active analgesic principles acting both centrally and peripherally.

On the other hand, our result showed that naloxone reversed the antinociceptive effect of extract in both phase in the formalin test. This finding indicated that opioid system at least partially involves in antinociception action. Intraplantary injection of formalin may be activated the endogenous micro-opioid system and exerts a tonic inhibitory effect on the pain behaviors (Zaho *et al*., 2003). This probably suggests or indicates that the extract exerts its analgesic effect through both peripheral inhibitory actions or released prostaglandins (inflammatory pain) and central activity relates to antagonistic action of the nociceptors (neurogenic pain) (Goodman Gilman, 1996). In the hot plate test, a central model that has a selectivity for opioid-derived analgesics (Abbott and Melzack, 1982), intraperitoneal treatment with hydroalcoholic extract showed a potent antinociceptive effect on the acute noxious thermal stimulation and confirming the central activity of this extract. In this test, pre-treatment with naloxone reversed this antinociceptive effect confirming that this effect is produced by activation of the opioid system. The central analgesic effect of extract may be supported by the results recorded in the tail flick test which is a selective method which is able to screen centrally acting opiate analgesic drugs. This test is very sensitive to centrally acting drugs (Carlisson and Jurna, 1987). The flavonoids are known for their antinociceptive and /or anti-inflamatory activity (Pathak *et al*., 1991; Meyer-Silva *et al*., 1999; Bittar *et al*., 2000). In our study the extract have inhibitory effects in reaction time in nociceptive stimulus. This effect may be due to components such as flavonoids in *TV* extract.

Scince the flavonoids are known for their antinociceptive and/or anti-inflammatory activity (Pathak *et al*., 1991;

Meyer-Silva *et al*., 1999; Bittar *et al*., 2000) may be flavonoids in *TV* extract induced modulatory effect on peripheral antinociceptive effect of *Thymus* extract.

In conclusion, this study demonstrates analgesic activity of *TV* extract which parallels as a traditional use of this extract as an analgesic and anti inflammatory medicine. The extract (500mg/kg) was most effective and more potent than the DEX and ST. The mechanism(s) of action of *TV* probably may be due to components such as thymol, carvacrol, and flavonoids, and acts partly through an opioid mediated mechanism. It seems opioid, serotonergic and cholinergic receptors involves in antinociceptive effects of hydroalcoholic extract of *TV*. The mechanism(s) of action of *TV* to be elucidated by further study.

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