Major essential oil components, antinociceptive and anti-inflammatory effects of hexane extract of *Vitexagnus- ca*stus L. fruits and possible mechanism in male mice

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ABSTRACT

Vitexagnus- castus L. [Vac.] has been used in the Iranian traditional medicine for the treatment of pain and swelling of uterus. In this study, GC and GC/MS analyses were carried out for the identification of essential oil chemical components. Formalin and Xylene-induced ear-edema were used in order to nociception and inflammation activity. Then, the possible interaction between 3drugs including Naloxone (2mg/kg), Dextrometorphane(20mg/kg), and L-NAME (10mg/kg) have been used and *Vitexagnus-castus* hexane extract was examined. 1,8-Cineole (23 %), alpha-Pinene (16 %), beta- Pinene (13 %), , Z-Caryophylene (11 %), alpha- Terpinyl acetate (9 %), E- caryophylene (9 %) and Linalool (6.5 %) were the major identified components of the essential oil of *Vitexagnus- castus* L. Hexane extract was reduced licking time as compared to the control group in the first and second phase of formalin test. In Xylene-induced ear-edema, the hexane extract of *Vitexagnus- castus* fruits was strongly inhibited inflammation as compared to the positive control group. Interaction between L-NAME and *Vitexagnus-castus* hexane extract showed significant effect. It was concluded that the anti-inflammatory and antinociceptive effects of the fruit of *Vitexagnus- ca*stus L. may be due to its very pharmacological effective essential oil components. Interaction between L-NAME and *Vitexagnus-castus* hexane extract showed that one of the possible pathways is NO pathway, but *Vitexagnus-castus* hexane extract probably acts via other pathways that need more research.

Key words:*Vitexagnus- castus* L.; Hexane extract; GC/MS; anti-inflammation ; antinociception; Male mice

INTRODUCTION

*Vitexagnus- castus*L. is a member of verbenaceae family [1] that has important medicinal properties [2]. Its fruit is used for the treatment of Premenstrual syndrome [3], hyperprolactimenia, corpus luteum insufficiency, endometriosis and menopausal symptoms [4]. Vac.'s fruit hydroalcoholic extract decreased LH and testosterone through dopaminergic system[5]. We recently reported that Vac.'s fruit has anti-gonadal activity in male mice [6].NMDA, opioid receptors and NO synthesis pathway modulate theinduction of nociception pathway.Dextromethorphan blocks NMDA receptors [7].Naloxone is an antagonist of opioid receptors[8].NO plays a crucial role in nociception and it increases after peripheral inflammation [9].Iranian traditional medicine literature affirms that Vac.'s fruit is used to relive pain and reduce swelling of uterus[10]. In previous study, Following the other researchers [11], it has been suggested that hydro alcoholic extract of Vac.'s fruit has antinociceptive and anti-inflammatory effects. These effects were attributed to essential oils and flavonoids [12],but there isn't any research about antinociceptive mechanism of Vac. extract. So, in this study, we wanted to find which fraction has antinociceptiveeffect , what pathway is effective? And weused hexane extract for the resean that, the previous studies showed that most of Vac. more active components' extract are hydrophobic

components[13] and the main components of the hexane extract is essential oil components.

MATERIAL AND METHODS

Collection and preparation of plant material and fractionations

Vac.'s fruits were collected from NW of Qom area(126 Km, .S. of Tehran, Iran), then identified and deposited at the Herbarium of faculty of Pharmacy, Tehran university of Medical Sciences under the voucher No. of 6711 –TEH.

Fruits of Vac. dried and grounded in a laboratory condition, then extracted with a hydro alcoholic solvent(80%). We applied percolation method for extraction [5] and for this propose , therefore 100gr of fruits' powder were soked in hydro alcoholic solution(80%). After 72 h, the resulting solution was removed, filtered and concentrated by rotary evaporator. The residue was dissolved in distilled water and then extracted with hexane. Finally, the hexane layer was removed and concentrated [14].

Essential oil extraction, and GC and GC/MS analysis

Essential oil extraction: The Essential oil of Vac.'s fruit was obtained by steam distillation method for 2.5 h and were dried over in anhydrous sodium sulfate and stored in tightly closed dark containers at 4°C until analyzing time [15]. GC analysis**:** GC analysis was performed by using a thermoquest gas chromatography shimadzu9A,with a flame ionization detector (FID) and carried out by using fused silica capillary DB-5 column(60×0.25 mm i.d, 0.25 µm film thickness).The operating conditions were as follows: injector and detector temperatures were 250 and 300°C ,respectively, nitrogen was used as the gas carrier at a flow rate of 1 ml /min, oven temperature programmed 60-250 °C at the rate of 5°C/ min and finally held isothermal for 10 min. Identification of compound**:** Retention indices were calculated by using retention times of n-alkenes that were injected after the oils under the same chromatographic condition. The components were identified by comparison of their mass spectra to the Wiley library or to the published mass spectra. The quantification of each compound was based on peak area method without using correction factor [16, 17].

Drugs

Drugs were purchased from different companies as follows: Dextrometorphane was purchased from Exir (Iran), Naloxone from Toolid daru(Iran), Morphine from Temad (Iran), L-NAME from Sigma (Germany), Formalin and Xylene from Romil (England) , formaldehyde from Romil (England) and Indomethacine from Exir Pharmaceutical Co. (Iran).

Animals

Male NMRI (how old were the mice ho w did the fed and what was the condition they wer kept)mice weighting 20-25gr. were used. They were kept in a controlled condition and free acces to water and pellet. In each test, mice were randomly divided into several groups(n=8) : 5 experimental groups (hexane extract was intraperitonally administered at doses of 265, 365, 465,565, 665 mg/kg) , vehicle-treated group (received solvent what was the solvent hexan or (hexan is solvent) DW) and positive control group [received Morphine (Temad, Iran) (10mg/kg, i.p.) in formalin test and Dexamethasone (15 mg/kg) in xylene test].

This research was conducted in accordance with the principles of laboratory animal use from Faculty of Pharmacy, Tehran University of Medical Sciences committee (Tehran, Iran).

Formalin test

We used method of Hunskaar, et al. (1987) with some slight modifications [18]. We injected 0.02 ml of 2.5% Formalin (40% Formaldehyde) was injected to in the sub plantar of the right hind paw. Hexane extract was intraperitonally administered at doses of 265, 365, 465,565, 665 mg/kg doses [these doses were selected to notice LD50 that was found 1650mg/kg)[5] , 30 min before formalin injection. Control group received solvent and positive control group received Morphine (10mg/kg, i.p.).

After formalin injection, the time spent for injection licking was considered as the first phase (0-5 min), and the second phase (15-30 min) [12].

Xylene-induced ear edema

The method of Atta &Alkohafi (1998) was applied with slight modifications from the original procedures[19]. Hexane extract was i.p. injected at doses of 265, 365, 465,565, 665 mg/kg doses.

After 30 min, 0.03 ml of Xylene was applied to the inner and outer of the right ear. Control group received solvent and positive control group received Dexamethasone (15 mg/kg). Two hours later, the mice were killed and both ears were removed. 7 mm in diameter sections of both treated and untreated ears were cut off and weighted. Rate of edema was measured as the weight difference between in left untreated ear and right treated ear.

The interaction of between drugs and Vac. hexane extract

To examine the mechanism of Vac. hexane extract, animals received Naloxone (2mg/kg, i.p.), Dextrometorphane(20mg/kg, i.p.) and L-NAME (10mg/kg , i.p.) 30 min before the treatment of Vac. significant dose of Vac. and Vac. hexane extract was administrated 30 min before of the Formalin test.

Statistical analysis

All data was expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey test. The difference was considered to be significant at $P< 0.05$.

RESULTS

The identified components of the Vac. essential oil were: alpha-Pinene (16 %), beta-Pinene (13 %), Limonene (1 %), 1,8-Cineole (23 %), Linalool (6.5 %), alpha- Terpineol (2 %), cis-Terpinyl acetate (1 %), alpha- Terpinyl acetate (9 %), Z- Caryophylene (11 %) and E- caryophylene (9 %)(Table 1) .

In the Formalin test, Vac. hexane extract reduced licking time significantly in both phase in compared to the control group ($P<0.001$) (Table 2) .This reduction was observed at 465 and 665 mg/kg doses in the first phase and 265 and 665 mg/kg doses in the second phase. There wasn't a significant difference between Vac. extract and positive control group (morphine-administered group 10mg/kg) in the second phase of formalin test.

Table 1: Chemical composition of the essential oil of *Vitex agnus-castus* L. fruit

No.	Compounds	KI	RRI	GC %
1	Pinene<alpha->	939	928	16
2	Pinene<beta->	979	938	13
3	Limonene	1029	1023	1
4	Cineole<1,8->	1039	1036	23
5	Linalool	1097	1081	6.5
6	Terpineol<alpha->	1189	1196	2
7	Terpinyl acetat <cis->	1318	1309	1
8	Terpinyl acetat <alpha->	1349	1337	9
9	Caryophyllen<(Z)->	1409	1393	11
10	Caryophyllen<(E)->	1419	1425	9
				TOTAL 91.5

Table 2. The effects of *Vitexagnuscastus* fruit's hexane extract on Formalin test in mice

Licking time				
Treatment (dose)	**0-5 min**	**Inhibitation(%)**	**15- 30 min**	**Inhibitation(%)**
Control	69.8 ± 17.6	-	51.2±13.6	-
Morphine (10mg/kg)	13.6± 9.7	58.1***	8.6± 3.5	83.2***
Hexane extract (265mg/kg)	66.0± 17.2	5.4	31.8± 7.6	37.9***
Hexane extract (365mg/kg)	59.3± 9.9	15.0	54.6± 6.8	-6.6
Hexane extract (465mg/kg)	38.7± 8.2	44.6***	49.8± 7.2	2.7
Hexane extract (565mg/kg)	58.7± 12.0	15.9	42.7± 10.2	16.6
Hexane extract (665 mg/kg)	44.7± 7.3	36**	29.6± 4.3	42.2***

Values are the mean ± SEM: ** P<0.01, *** P<0.001

Table 3. The effects of *Vitexagnuscastus* fruit's hexane extract on Xylene-induced ear edema in mice.

Treatment (dose)	**Ear swelling (mg)**	**Inhibition (%)**
Control	57.6± 16.3	-
Dexamethasone (15mg/kg)	19.2±7.0	66.7***
Hexane extract (265mg/kg)	47.8±7.3	17.0
Hexane extract (365mg/kg)	69.1±13.8	-20
Hexane extract (465mg/kg)	37.5±8.7	34.9**
Hexane extract (565mg/kg)	18.6±4.6	67.7***
Hexane extract (665 mg/kg)	45.1±8.8	21.7

Values are the mean ± SEM: ** P<0.01, *** P<0.001

Table4. Role of the Glutamate system in antinociceptive effects of Vac. in formalin test. Animals recieved normal saline, normal saline and Vac. (0.665), Dextrometorphane(20mg/kg) or Vac. and Dext.

Licking time				
Treatment (dose)	**0-5 min**	**Inhibitation(%)**	**15-30min**	**Inhibitation(%)**
Normal saline + Normal saline	147± 2	-	83±2.5	-
Normal saline + Vac. (665mg/kg)	61± 2.7	58.5***###	27± 1.3	67.5***###
Dext.	141±1.3	4.08	79±2.3	4.8
Dext.+ Vac.	57.8± 1	60.8***###	24± 1.2	71.1***###

**** P<0.0001 significant difference from Sham group; ###P<0.001 from Dext. Group, n=8.

Table 5. Role of the Opioid system in antinociceptive effects of Vac. in formalin test: Animals recieved normal saline , normal saline and Vac. (0.665), Naloxane (2mg/kg) or both of Vac. and Nal.

Licking time				
Treatment (dose)	**0-5 min**	**Inhibitation(%)**	**15-30min**	**Inhibitation(%)**
Normal saline + Normal saline	147± 2	-	83±2.5	-
Normal saline + Vac. (665mg/kg)	61± 2.7	58.5***###	27± 1.3	67.5***###
Nal.	140±1.9	4.76	77±2.3	7.2
Nal.+ Vac.	63± 1.8	57.1***###	30± 1.1	63.9***###

*** P<0.001 significant difference from Sham group, ###P<0.001, from Nal. Group, n=8.

Table 6. Role of the NO system in antinociceptive effects of Vac. in Formalin test.Animalsrecieved normal saline , normal saline and Vac. (0.665), L-NAME (10mg/kg) or both of Vac. and L-NAME .

Licking time				
Treatment (dose)	**0-5 min**	**Inhibitation(%)**	**15-30min**	**Inhibitation(%)**
Normal saline + Normal saline	147± 2	-	83±2.5	-
Normal saline + Vac. (665mg/kg)	61± 2.7	58.5***###	27± 1.3	67.5***###
L-NAME	139±1.8	5.4	78±0.92	6.02
L-NAME + Vac.	39± 1.4	73.5***###+	18.5± 0.62	77.7***###+

*** P<0.001 significant difference from Sham group; ###P<0.001 from L-NAME group and + P<0.05 from Vac. group, n=8.

The activities of the hexane extract on xylene-induced ear edema are illustrated in Table 3. The hexane extract caused a significant inhibition of ear edema ($P<0.001$ or $P< 0.01$) at doses of 465 and 565 mg/kg doses. In this research inhibition of ear edema paralleled to the Dexamethasone-treated group (15mg/kg). This group was as positive control group.

In Interaction groups, Vac. and Dext. group didn't show significant difference to Vac. group alone. But it has meanwhile there is a meaningful difference with Dext. group ($P<0.001$). Vac and Nal.group didn't also show any significant difference with Vac. group,but it showed meaningful difference to Nal. group($P<0.001$). Vac. and L-Name had significant difference to L-Name group($P<0.001$) and Vac. group ($P<0.05$).

DISCUSSION

In the present study, we investigated antinociceptive and anti-inflammatory properties of *Vitexagnuscastus*L.fruit's hexane extract. Hexane extract attenuated significantly licking time in both first and second phases of Formalin test as compared to control group (Table 2). The anti-inflammatory response was induced by hexane extract of Vac. fruit and the percentage of inhibition, didn't show significant differences in between extract and Dexamethasone group. In this research, Naloxone was used to inhibition of Opioid system, Dextrometorphane for inhibition of Glutamate system and L-NAME for inhibition of NO syntheses. Naloxone and Dextrometorphane didn't affect on antinociception of Vac's fruit extract (Table 4,5). L-NAME increased antinociceptive effect of Vac. hexane extract (Table 6).

In addition, We evaluated the essential oil components of Vac. extract. The essential oil of Vac. has the very effective components such as terpineol, 1,8-Cineole, Pinene and Limonene (Table 1).The essential oils have analgesia and anti-inflammatory activities [20]. Limonene and alpha- terpineol are probably casued antinociceptive properties of *Dracocephalumkotschyi*[21].

In another study, essential oil from *Rosmarinusofficinalis* aerial part produced a dose dependent antinociceptive effect. One of the principal compounds in this essential oil was pinene[22].1,8-Cineole from the essential oil of *Eucalyptus camaldulensis* leaves showed antinociceptive effect comparable to the morphine, but naloxone couldn't inhibit the effect of Cineole[23,24].These experiments indicate the existence of a non opioids mechanism for the Cineol's antinociceptive activity [25]. Essential oil of *Eremanthus erythropappus* inhibited the first and the second phase of licking time in Formalin test. Furthermore, it reduced carrageenan-induced paw oedema and leucocyte mobilization. One of the major essential oil from *Eremanthus erythropappus* was pinene[25]. Limonene had antinoceceptive activity in the second phase of Formalin test while Naloxone didn't antagonize this activity. This property may cause in order to peripheral analgesia, but not via opioid pathways[26]. P-hydroxybenzoic acid, methyl 3,4-dihydroxybenzoate and 3,4-dihydoxybenzoic acid from Vac. have anti-inflammatory activity with inhibition of lipoxygenase in a cell based on contemporary measurement[11].Lipoxygenase produces nociception metabolites. Lipoxygenase exists in macrophages and mast cells and synthesis leukotrienes. Leukotrienes mediate inflammation [27]. Data from Choudhary(2009) shows anti-inflammatory activities of Vac. that is in agreement with our research[11].

This Results from our research showed that Vac. hexane extract doesn't act from opioid pathways .Pretreatment with the Glutamate antagonist Dextrometorphane doesn't reverse antinociception effect of Vac. hexane extract. It may depends on NO Synthase system. NO is involved in the nociceptive effect in the supraspinal and peripheral tissues [28].

According to Kawabata (1993) NO has dual role in the nociception: if NO activates Opioid pathway, it will cause an antinociceptive effect, but if it affects on NO-cGMP mechanism, it can cause hyperalgesia [29]. In this research, L-NAME doesn't affect on Formalin test in by itself the first and second phase of formalin test,but while when it was administered before Vac. hexane extract, L-NAME influenced on the analgesia effect of Vac. and increased its effect. Our results agreed with Zakaria (2005) , he reported that aqueous supernatant of haruan (*Channastriatus*)

induced antinociception in abdominal constriction test[30]. L-NAME can enhance vascular permeability [31] and Vac. antinociceptive activity. So, L-NAME has antinociception effects especially in chemical model of nociception like Formalin test when it administrated before Vac. extract.

CONCLUSION

In conclusion, on the basis of our research and data from review literature, this study can confirm traditional usage of *Vitexagnus-castus* fruits for treating of pain and uterus swelling. It suggests thatVac. acts as NO possible system .In addition to NO synthase, other pathways may be involved in antinociceptive and anti-inflammatory effect of Vac. that which need more research.

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