Phytochemical Study of Flavonoid "Casticin" Present in the Fruits of *Vitex agnus-castus* L. Cultivated in Iraq

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Abstract

This study detects the presence of an important flavonoid "Casticin" in the fruits of *Vitex agnus-castus* L. grown in Iraq. The pharmaceutical importance of Casticin arise from its consideration as anti-tumor substance and have cytotoxic effects, and the absence of any study concerning Casticin content of this medicinal plant in Iraq, gave this study its importance. This study concerned with the extraction, identification, isolation and purification of Casticin from the fruits of *Vitex agnus-castus* L. The extraction of this compound was carried out using two methods. Identification of this compound was done by Thin Layer Chromatography (TLC) in which three different solvent system has been tried. This identification was further augmented by using High Performance Liquid Chromatography (HPLC) and then this compound was isolated and purified. Identification of the isolated Casticin was carried out using melting point (M.P.), Thin Layer Chromatography (TLC), and Infrared spectroscopy (IR). This study confirms the presence of Casticin in the fruits of *Vitex agnus-castus* L. grown in Iraq.

Key words : *Vitex agnus – castus* , Flavanoid , Casticin

دراسة كيموأحيائية للفلافينويد (الكاستيسين) الموجود في ثمار نبات شجرة ابراهيم المزروع في العراق

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الخلاصة

هذه الدراسة تختص بالكشف عن نوع من الفلافينويد وهو مادة الكاستيسين في ثمار نبات شجرة إبراهيم التي تنمو في العراق . ان الاهمية العلاجية لهذه المادة والناشئة من كونها مادة مضادة للاورام السرطانية وتملكها تاثيرات حيوية ضد السموم ولعدم وجود أي دراسة سابقة في العراق تتناول محتوى هذا النبات الطبي من هذه المادة هو الذي يعطي اهمية لهذه الدراسة . هذه الدراسة تناولت استخلاص وكشف وفصل وتنقية هذه المادة من ثمار هذا النبات .حيث تم الاستخلاص باستخدام طريقتين مختلفتين ، اما الكشف فقد تم باستخدام تقنية كروماتوغرافيا الطبقة الرقيقة، باستخدام مذيبات مختلفة كوسيط ناقل والكشف عنها وكذلك تقنية كروماتوغرافيا الأداء العالي السائلة وبعدها تمت عملية الفصل والتنقية. وكذلك استخدمت مجموعة من التقنيات للتحقق من نوعية المركب المفصول و درجة نقاوته والتي شملت : درجة الانصهار و تقنية كروماتوغرافيا الطبقة الرقيقة و كذلك مطياف الأشعة تحت الحمراء . هذه الدراسة تؤكد وجود مادة الكاستيسين في ثمار نبات شجرة إبراهيم التي تنمو في العراق.

الكلمات المفتاحية : نبات شجرة ابراهيم، الفلافينويد ،الكاستيسين.

Introduction

Vitex agnus-castus L. (family: *Verbanceae*), Figure (1), is an ornamental shrub or small tree small tree, approximately 1 to 6 m height, with aromatic odour, it is native to European, Mediterranean and Asian regions[1,2,3]. In Iraq it is found in north areas (Erbil, Sulaimaniya, Kirkuk, mosul and Khanaqin), and it is also found in central areas as in Baghdad [4]. The fruits as well as the leaves of *Vitex agnus-castus* L. are known to contain the lipophilic flavonoids , mostly Casticin , (synonym: Vitexicarpin) the fruit is rich in Casticin[5,6] and it is considered as finger print in HPLC of *Vitex agnus-castus* L[7]. The fruit extract of *Vitex agnus-castus* L. has been shown to be effective for the treatment of premenstrual syndrome (PMS), including premenstrual dysphoric disorder (PMDD) , that is a more severe form of (PMS) [8,9] and it is used to normalize menstruation in women with shortened , lengthened or infrequent menstruation, particularly when low progesterone and luteal phase defects [10].

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It is also used to enhance fertility in women with progesterone deficiency [11]. The cytotxic activity of *Vitex agnus-castus* L. fruit extract have been examined well and it was found that the extract inhibits the growth of breast, ovarian, cervical, gastric, colon and lung cancer cells[12], Casticin which found in the fruits of this plant showed inhibitory activity of both T- lymphocyte and B-lymphocyte proliferation in-vitro and it may be a potential lead compound for treating inflammatory and immuno-related diseases or lymphomas in vivo[13]. Also it has antifungal activity of against the plant pathogenic fungus and it has found that Casticin has an inhibitory action against Bacillus spp. and Staphylococcus spp.[14]

Figure 1: *Vitex agnus-castus* L.

Material and Methods

Plant materials

The plant materials (fruits) of *Vitex agnus-castus* L. were collected from private garden, during the months of September and October (2010), they were cleaned and dried in oven at a temperature between (60-70)°C for (4-5) hours, then these plant materials were coarsely powdered by mechanical grinder and weighted. A 100 g of dried powdered plant materials were extracted using two methods.

Extraction

Extraction method NO. 1[7]

A quantity of 100 g of dried powdered fruit of *Vitex agnus-castus* L. was macerated for 3 days at room temperature in aqueous ethanol (1 Liter) 70% (V/V) for 3 days at room temperature and the resulting extract was filtered. The residue was extracted twice again as above. The obtained filtrates were combined and concentrated using rotary evaporator until we get dry extract, For the isolation of flavonoids, the extract was partitioned successively between an equal volumes of water and n-Hexane, this step called (Defatting step) then the residual water phase was extracted with an equal volume of ethyl acetate to draw all the flavonoids containing compounds in to the organic phase and the ethyl acetate phase was concentrated by means of rotary evaporator, then the dry extract was weighted and subjected for identification of flavonoids. Figure (2) represents the general scheme for extraction of flavonoids from the fruits of *Vitex agnus-castus* L.

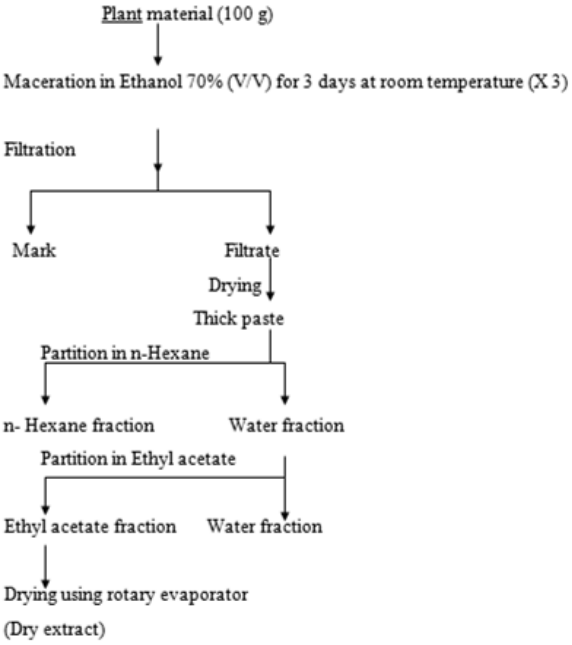


Figure 2: General scheme for method (No. 1) for extraction of Flavonoids from *Vitex agnus-castus* L.

Extraction method NO. 2[15]

A quantity of 100 g of dried powdered fruit of *Vitex agnus-castus* L. was macerated for 3 days at room temperature in 1 L of 90% methanol for 3 days at room temperature and the resulting extract was filtered. The residue was extracted twice again as mentioned above. The obtained filtrates were combined and concentrated using rotary evaporator until we get a dry extract, this step followed by step of successive partitioning of the dry extract between equal volumes of petroleum ether and water, this step called defatting step, then the water fraction was partitioned with equal volume with chloroform followed by partitioning with an equal volume with ethyl acetate to will draw all the falvonoids. The ethyl acetate phase was concentrated under reduced pressure till we get dry extract. The dry extract was weighted and subjected for identification and purifications of flavonoids. Figure (3) represents the general

scheme for extraction of flavonoids from the fruits of *Vitex agnus-castus* L.

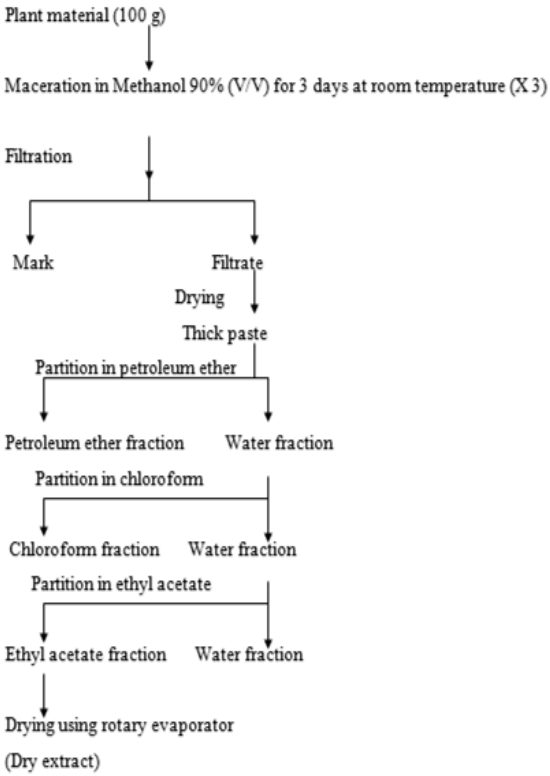


Figure 3: General scheme for method (No. 2) for extraction of Flavonoids from *Vitex agnus-castus* L.

Identification of the Flavonoid

C .1: Thin layer chromatography (TLC)

Using Thin Layer Chromatography (TLC) technique for qualitative identification of Casticin. In this identification:- using a readymade aluminum plates of silica gel GF-254. and a comparison was made with three different developing solvent systems that were[16].

Solvent 1 (S1) : Toluene: Chloroform: Acetone (8:5:7)

Solvent 2 (S2) : Chloroform: Acetone Formic acid (75:16.5:8.5)

Solvent 3 (S3) : Toluene: Ethyl methyl Ketone: Formic acid (18:5:1)

Freshly prepared dimethyl formamide (DMF) solutions of the standard reference, Casticin, and DMF solutions of the dried fruits of *Vitex agnus-castus* L. extract were applied to TLC plates manually, and then developed by the ascending technique. The solvent migration limits was 10-12 cm from the base line. The above three developing systems were tried, and placed in a glass tank (22.5cm x 22cm x 7cm), and covered with a glass lid and allowed to stand for 45 minutes before use for saturation. After development, the plates were allowed to dry at room temperature and were detected using UV-light. Two wave lengths were used 254 nm and 366 nm.

C.2: High Performance Liquid Chromatography (HPLC)

Qualitative identification of Casticin in extracts obtained from extraction methods above was authenticated by HPLC, this identification was made by comparison of the retention time obtained at identical chromatographic conditions .The analyzed sample and the authentic standard conditions were:

1- Mobile phase: Isocratic: Acetonitrile / Water (90% / 10%) + 0.1 N acetic acid

2- Column: C18 5 mm x 150 mm

3- Flow rate: 1 ml /min.

4- Detection: UV. Detector at λ 258 nm.

5- Injection volume: 5 µL

6- Injection concentration: 1 mg /ml

Isolation and purification of the Casticin

After locating of Casticin of the extract, preparative Thin Layer Chromatography was done to isolate and purified Casticin.

The preparative TLC was performed by using a readymade glass plates 20x20 cm, which were coated with silica gel; layer thickness 1 mm; made by MERCK, Germany, these plates were activated at 110°C for one hour before use.

The dried *Vitex agnus-castus* L. fruits extract was dissolved in DMF, then applied as a concentrated solution in a raw of spots using capillary tubes, the mobile phase for Casticin was S2=Chloroform: Acetone Formic acid (75:16.5:8.5), the standard sample was applied in one side of the plate, the separated compound appeared as a band identified using UV-light detection method. The band was then assigned and the assigned silica gel was scrapped out and collected a beaker and mixed with hot DMF and then filtered. The silica gel on the filter paper was washed again with hot DMF. The DMF solutions were evaporated to dryness under reduced pressure to give the corresponding precipitates. These precipitates then recrystallized using aqueous DMF and maintained for TLC , HPLC , FT-IR and measuring melting point.

Identification and characterization of the isolated Casticin

For further identification of the isolated Flavonoid, Casticin, from the dried fruits of *Vitex agnus-castus* L., the following methods were used:-

1 .TLC **:** Analytical TLC was performed by using a readymade plates of 20x20 cm, which are coated with silica gel (MERCK) layer of 0.25 mm thickness.

The isolated Flavonoid, Casticin, obtained by preparative TLC was applied on silica gel plates as one spot by using a capillary tube along with the standard, the mobile phase was S2.

2**.** Melting point **:** Melting point was estimated by using Electro-Thermal melting point apparatus for the isolated compound and compared with that of the available standard of Casticin.

3**.** IR **:** Infrared spectra were recorded using KBr disk.

4. HPLC analysis (conditions as in C.2)

Results and Discussion

Extraction method

Results showed that extraction method NO. 2 was better, because the percentage of yield of crude extract was higher than that of obtained from method NO.1. In addition, quantitative estimation by HPLC showed that flavonoids obtained in method NO. 2 contain higher percentage of Casticin than that obtained from method NO. 1. As shown in table (1).

Table 1: Percentages of crude extracts and Casticin obtained from the fruits of *Vitex. agnus –castus.*

Extraction method	% Yield of crude extract	% Yield of Casticin
Method NO. 1	12.1	6.8
Method NO. 2	13.7	7.2

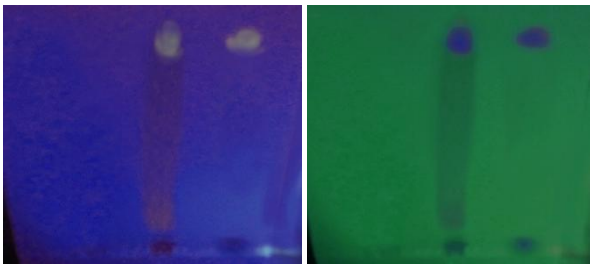
Identification of Casticin by TLC

The TLC of the extracts obtained from the fruits of *Vitex agnus-castus* L by using the extraction method No1 and No2 , confirms the presence of Casticin in these extracts in comparision with the standard by using different solvents . Result showed the solvent No2 was the best solvent. As presented in table (2) and figure (4).

Table 2: R_f values of flavonoid (casticin) as compared with the standard reference in different developing systems in TLC.

Solvent system	S1	S2	S3
R_f of the standard Casticin	0.4	0,45	0.77
R_f of Casticin of extract	0.39	0.44	0.75

S1 : Toluene: Chloroform: Acetone (8:5:7)
S2 : Chloroform: Acetone Formic acid (75:16.5:8.5)
S3 : Toluene: Ethyl methyl Ketone: Formic acid (18:5:1)



UV 254 **UV 366**
F C **F C**

Figure 4: TLC of the fruit extract of *Vitex agnus-castus* L. obtained by extraction NO. 2 using silica gel GF 60 as adsorbent and (S2) as a mobile phase. (F= fruit extract, C= Casticin standard)

Charactrization of isolated Casticin

TLC

Isolated compound (Casticin) appeared as a single spot having the Rf value as that of reference standard.

IR

For further characterization of the Casticin isolated from the dried fruit of *Vitex agnus-castus*, FT-IR spectroscopy analysis was performed using Casticin standard as a reference. The IR spectra of the isolated Casticin compared with the authentic Casticin standard gave us an identical results indicating that the isolated compound from *Vitex agnus-castus* L. was casticin[17] as shown in figure (5).

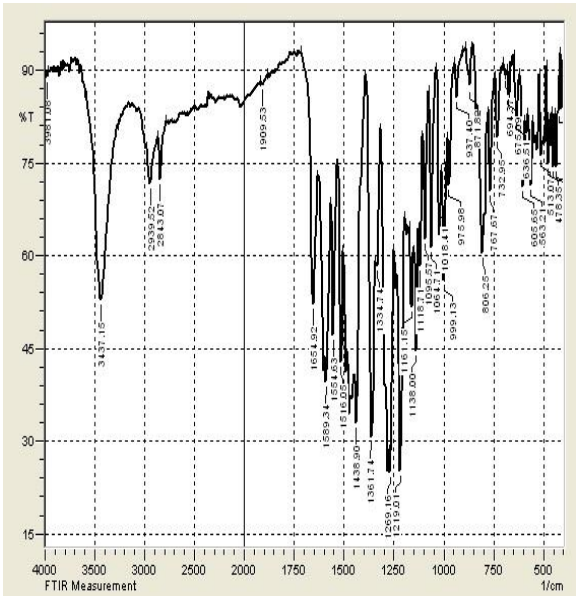


Figure 5: IR spectra of the isolated Casticin

Melting Point

The isolated compound was identified as Casticin from its sharp melting point .

Since the compound showed a melting point of (184 – 186) °C compared to Casticin standard (185 –187)°C.

HPLC analysis

The HPLC analysis of both of the authentic Casticin and the isolated compound showed an identical retention time, which is considered as a conclusive evidence that the isolated compound was Casticin.As shown in Figures (6 and 7)

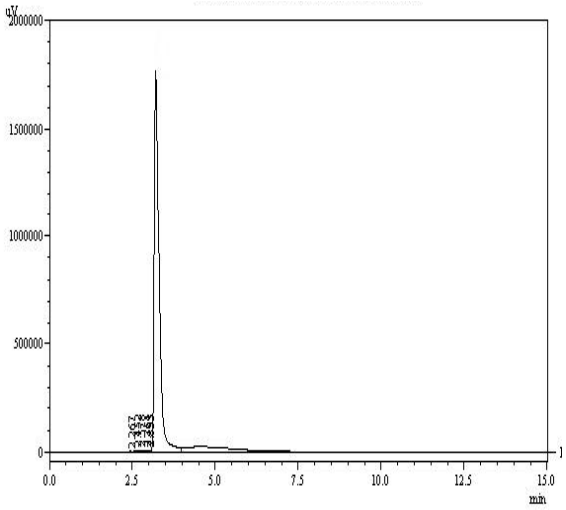


Figure 6: HPLC analysis of Casticin standard

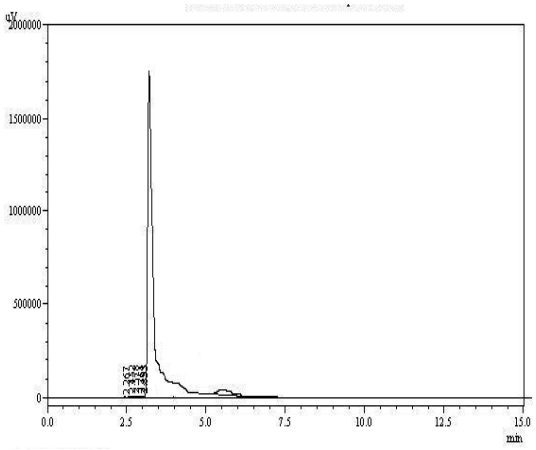


Figure 7: HPLC analysis of Casticin isolated from *Vitex agnus-castus* L.

Conclusion

The phytochemical investigation of *Vitex agnus-castus* L. fruits, grown in Iraq revealed the presence of important medicinal natural product "Casticin", which is a flavonoid. The extracted Casticin was identified using TLC and HPLC methods then it was purified and isolated from the dry crude extract by using a preparative TLC technique. The identification of the isolated compound, Casticin, was made using TLC, HPLC, melting point and IR spectroscopy.

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