PHYTOCHEMICAL ANALYSIS AND PURIFICATION OF CHIEF COMPONENTS OF ONONIS SPINOSA

N.FAYYAZI[1]*, F.NAGHIBI[2], B.KERAMATIAN[3], S.TAHERI[3]

1. Traditional medicine and materiamedica research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Deputy of research, Traditional medicine and materiamedica research center. Associate professor School of pharmacy, Shahid Beheshti University
3. Traditional medicine and material medical research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Chemistry and chemistry engineering institute research center, Associate professor School of chemistry. Tehran, Iran

Abstract: Ononis plant is a category of Fabacea family. This category includes 75 different species which are seen as weeds growing on plains and high lands. Regarding strong antibacterial features in the species like O.fruticosa, O.spinosa, O.natrix، O.ramosissima, O.tridentate, there are several reports finally leading to applying ethanol extract of O.spinosa species in some of medicinal and hygienic products manufactured by European and American companies since the year 2010. The tests required to separate active and effective compounds of O.spinosa were developed and accomplished based on two different methods. TLC test for the fractionates resulted from chromatography of the extracts used in the 1st and the 2nd methods was performed to compare the methods and it indicated that the 2nd method was better because of less compounds existing in TLC and therefore better separation of compounds. TLC test for semi-polarized to polarized compounds of the fractions resulted from the chromatography accomplished on the 2nd method chosen extract contains most spots in yellowish green at the wavelength 366 nm. In many papers, this color is attributed to Flavonol compounds. Amon the compounds separated from this plant, one may mention needle crystals purified by recrystallization in which the final purity has been proved by TLC test.

Keywords: Flavonoid, Fabacea, Extraction, Purification

1. Introduction

The name of Ononis spinosa was given from Greek word Onos meaning donkey and is a favorite food of this animal. This plant with the scientific name Ononis spinosa is of Fabacea family. The mentioned plant is found as weeds growing on plains and heights; geographically it grows in Mediterranean region, Europe to Central Asia (9).

A subspecies of this plant called Leiosperma Ononis spinosa L.Sub Sp grows in many of Iranian points including Isfahan, central part, and Kohgilouyeh. The concerning plant collected from Central Province (Arak city) has branchy trichoms containing sticky sprinkles which are reported to have Falvonoid compounds.

The tests required for separation of active and effective principles in O.spinosa have been developed and performed in two different ways described below.

Separation of these compounds (Ononis spinosa) through high quality liquid chromatography was compared to the separation of Pisum stivum, Trifolium Pratense, and glycine max plants. The result of this comparative study has been shown in fig(1).

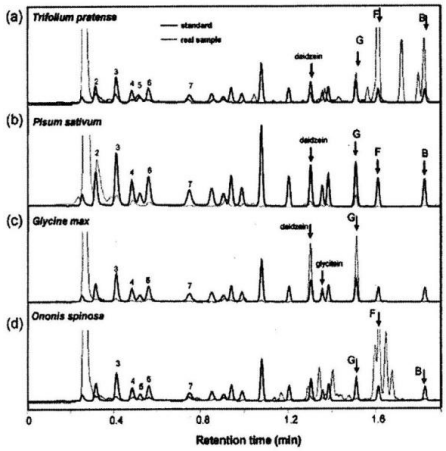

Figure 1. Real sample phenolic acids and isoflavones analysis.chromatograms of methanolic extract prepared from different plants species acquired at Zorbax CNSB chromatographic column. Abbreviations: G,genistein; F,formononetion; B, biochanin A and for others,1;challi acid, 2; protocatechuic acid, 3;p-Hydroxybenzonic acid, 4;vanillic acid, 5;caffeic acid, 6;Syringic acid, 7;p-coumaric acid.

According to performed investigations, biosynthesis of P-Coumaric acid and caffeic acid produces several derivatives. Fig 2 illustrates biosynthesis mechanism and molecular structures of the mentioned components (phytochemistry-Steck 1986)

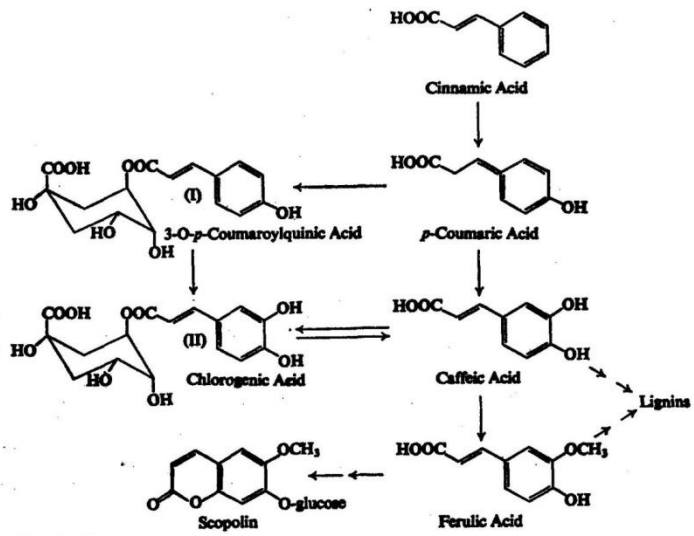

Figure 2. Biosynthesis mechanism and molecular structures of the mentioned components

The identified compound introduced as Isomer has different biological effects. Its antioxidant effect is completely as certained and its effects on fungi and bacteria have been surveyed and proven. Another positive result about this compound is reported as controlling Hepatitis B virus. Also, some reports on antibacterial effects of different extracts of Ononis spinosa on warm bacteria (+ and -) have been provided. The plant sample analyzed in Iran certifies the existence of Chlorogenic acid.

In order to specify the structure of the sample, H-NMR and C-NMR spectra were developed. The resulted compound certain to Caffeolquinic acid derivatives.

2. Material and Methods:

1**. Plant material**:

Required quantity of the plant Ononis spinosa was collected from a point of central province, 25 kilometers far from Arak, in the late Khordad (June). Upon approval by plant experts regarding the category and species of the desired plant, air top branches were cut, dried, and finally milled testing purposes. This plant identify regarding to Iranian flore.

2. Extraction: extraction for Ononis spinosa was performed by maceration and in two ways of total extraction and fractionation for investigating the rate of active principles extraction in each method.

2-1. Total extraction:
250 grams of dried and powdered Ononis spinosa was poured in a 1000 ml flask to which 500 ml of ethanol-water solvent with the ratio 50:50 was added and then it was put on the shaker in three 24-hour phases; after each phase the resulted extract was condensed and dried by rotary evoperator system. Upon completion of these three phases, the plant residuum was dried and returned into the flask. Then 500 ml of ethyl acetate-water solvent with the ratio 50:50 was added and the compound was put on the shaker in another three 24-hour phases. After each phase, the resulted extract was condensed and dried by rotary operator system. Finally, two resulted extracts were mixed with each other and total extract was obtained. All of solvents is used formMerk Company.

2-2. Extraction through fractionation:
In this extraction method, 100 grams of powdered and dried Ononis spinosa weighted previously was transferred to a 500 ml flask and 250 ml of hexane normal solvent was added to the flask. The resulted compound was put on the shaker in three 24-hour phases and after each phase the resulted extract was condensed and dried by rotary evaporator system. Upon completion of these three phases, the residuum was dried and returned to the flask and the rest of extraction procedure in the mentioned way for extraction by hexane normal solvent was continued by respectively chloroform, ethyl acetate, ethanol, methanol, water-methanol 70%, water-methanol 50%, and water 100% and in conclusion 8 extracts were obtained.

3. Purification:
Purification was performed by the extracts obtained in two ways mentioned under extraction paragraph.

3-1. Purification of Ononis Spinosa total extract:
In this method, chromatography column with 100 cm length and 5 cm diameters bearing silica gelMerck (0.04-0.063mm) steady phase and hexane normal solvent moving phase was prepared.
The resulted total extract was completely dissolved in some methanol to which a minimum amount of silicagel was added. The compound was rubbed to get a uniform powder. Then the result was injected into the chromatography column.
Fractionation of the components existing in total extract of plant was accomplished through washing chromatography column respectively by hexane normal, ethyl acetate, and ethanol solvents and based on changing the polarity of moving phase of chromatography column gradually from 10% to 100% concentration.
In this method, effective components of the plant left the column through several fractions and in order to recognize the nature of the materials existing in each fraction and in accordance with reference books we made use of TLC test through different solvents system with different ratios as moving phase of TLC tank including hexane-ethyl acetate, ethyl acetate-methanol-water, ethyl acetate-water-ethyl methyl ketone-acetic acid-formic acid, ethyl acetate-water-acetic acid-formic acid etc. and in fact changing the solvents ratios in TLC tank was performed respecting the motion rate of samples on TLC paper and Rf difference of compounds and also by making use of general and specificreagents introduced in reference books including methanolic sulphuric acid 100%, anisaldehyde sulphuric acid, vanillin sulphuric acid 10%, Natural Product reagent, and etc. applied for more accurate recognizing of the substances available in each fraction.

3-2. Purification through fractionation extraction:
In order to purify the components (compounds), chromatography column with 60 cm length, 2.5 cm diameter, silicagel steady phase and hexane normal moving phase was prepared and the ethyl acetate extract which shows the greatest amount of active principles of plant based on TLC test results injected into the chromatography column upon formation of a uniform mixture of the extract. The column was washed out by respectively hexane normal, chloroform, ethyl acetate, methanol and water in order to separate the components existing in ethyl acetate extract and regarding TLC results of ethyl acetate extract faced with this solvents system with percentages ranging from 10 to 100% and in each phase.
Upon completion of chromatography task, some crystals were observed in resulted fractions which had some impurities. Therefore, recrystallization method was applied in order to purify the crystals. Resulted crystals are highly polarized and they are dissolved completely in water. TLC test was performed on them to determine the nature of such crystals. Among tested solvents, in ethyl acetate, water, formic acid, acetic acid with the ratios respectively from right to left (100:26:11:11) and ethyl acetate, water, formic acid, acetic acid, ethyl methyl ketone with the ratios respectively from right to left (30:3:7:10:50), the crystals were on the water and its single spot nature was demonstrated in uv light (254nm wave lengths. Also, the derived

compound gives good responses to specific reagent of flavonoid compounds, i.e. Natural Product.

4. Result and discussion

Regarding extraction methodology, we came to the conclusion that extraction by fractionation of plant by different solvents bearing different polarities was more efficient and provided better results in comparison with total extraction, because there are several components in plant compounds each containing several subgroups of components. Component sets have different polarities and the groups available in each set are very close regarding their polarity status.

Hence, by applying fractionation extraction for a plant, a number of those existing components with similar polarities being in the same group are dissolved in a solvent and leave the plant; and by changing the polarity of solvent and making use of a more polarized solvent, another group of components existing in plant are dissolved in and leave the plant. Accordingly, in every extract we have some components of the plant which slightly penetrate into another extract and therefore make less noise. On the other hand, the active principles desired by the researcher which he/she intends to reach for a particular purpose, usually is found in some groups of plant compounds with bearing polarities close to each other and fractionation extraction let the researcher to reach such compounds in an easier way.

TLC test performed on the crystals resulted from the second separation method using ethyl acetate extract properly responding Natural Product indicates that this compound is closed to falvonoid structures and NMR spectrum is taken in order to make sure of the structure and nature of the compound. NMR device of the model Bruker, Tm500CRX (500 MH) is a property of Pharmacy Faculty of University of Tehran. The spectra assessed are HNMR, CNMR, HSQC, DEPT, and COSY for which the results were found as follows:

HNMR(500MHz, D_2O),δHNMR:
2.21(1H,J=13.6,3.1Hz , dd), 2.07(1H, J=13.6,4.4 Hz ,dd), 4.20(1H, J=3.1,4.4,3.1 Hz ,ddd), 3.76(1H, J=8.5,3.1 Hz ,ddd), 5.37(1H, J=9.3,4.8,8.5 Hz ,ddd), 2.11(1H, J=13.6,9.3 Hz ,dd), 2.26(1H, J=13.6,4.8 Hz ,dd), 7.0(1H, J=2.0 Hz ,dd), 6.81(1H, J=2.0 Hz), 6.98(1H, J=8.2,2.0 Hz , dd), 7.49(1H,J=15.9 Hz ,d),6.29(1H,J=15.9 Hz ,d)
13_C
NMR:36.9,37.7,69.5,70.19,70.34,70.88,114,115,116, 122,146.
MS(m/z):163(1.5%),180(3.85%),244(1.45%),288(1.3 %),320(1.5%),336(1.5%),354(89%),355(39%),378(1. 34%)
Formula: $C_{16}H_{18}O_9$

The identified structure appears to be pertinent to caffeolquinic acid compounds called chlorogenic acid with the following identified structure fig(c). This compound bears two operating groups of acid and ester and its mass number lies among visible mass spectrum.

Therefore, the compound separated from Ononis spinosa as described above in purification section appears to be from Carboxylic Phenol compounds, albeit the separated material has a very low concentration. Hence, in order to gain greater amount and higher concentration, one shall use more material from the first step of fractionation.

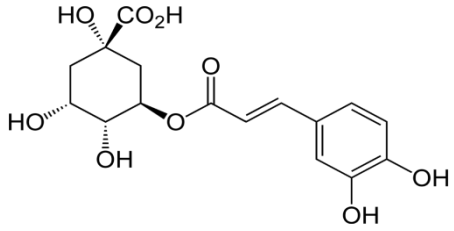

Figure 3. Chlorogenic Acid,M+=353(molecular mass)

Chlorogenic acid compound is of identified and effective medicinal compounds, medicinal plants, and natural compounds. Its medicinal effects have been reported as controlling cancer cells, increasing body security and antivirus, antibacterial, and anti-mutation antioxidant, reducing penetrability and frangibility of capillaries, and etc. The materials identified in the plant (Ononis spinosa) are some components including Onocerin, Ononin, Biochanin, fermonentin, and coumarin compounds and also p-hydroxi benzoic acid-paracoumaric acid, and caffeic acid reported by Vacek Kledius et al. in 2008 in chromatography journal. The identified compound introduced has different biological effects such as antioxidant, anti-fungi and bacteria.

References

1. EMAMI,A.,ARDAKANI, SHAMS,&MEHREGAN,A. (2004) ENCYCLOPEDIA OF MEDICINAL

PLANTS.SHAHID BEHESHTI UNIVERSITY RESEARCH CENTER.
2. GHAHREMAN,A.(1978-2002)IRANIAN FLORE (VOL.22) TEHRAN,IRAN
3. MOZAFFARIAN,V.(1996). ENCYCLOPEDIA IRANIAN PLANTS NAME. TEHRAN,IRAN
4. ALTANLAR, N., CITOGLU, G., & YILMAZ, B. (2006). ANTIBACTERIAL ACTIVITY OF SOME PLANTS USED IN FOLK MEDICINE. PHARAMACEUTICAL BIOLOGY (44), 91-94.
5. AMER, M., ABDEL-KAD, M., MAHMAOUD, Z., ABDEL SALAM, N., YANG, S. S., & MABRY, T. (1989). FLAVONOIDS OF ONONIS VAGINALIS. REV.LATINOAMER.QUIM (20), 150-153.
6. AUFFRET, G., LABAIED, M., FRAPPIER, F., RASOANAIVO, P., GRELLIER, P., & LEWIN, G. (2007). SYNTHESIS AND ANTIMALARIAL EVALUATION OF A SERIES OF PIPERAZINYL FLAVONES. PHYTHOCHEMISTRY (17), 953-963.
7. BAKERI, A. G., & AFIFI, F. U. (2007). EVALUATION OF ANTIMICROBIAL ACTIVITY OF SELECTED PLANT EXTRACTS BY RAPID XTT COLORIMETRY AND BACTERIAL ENUMERATION. JOURNAL OF MICROBIOLOGICAL METHODS, 68, 19-25.
8. BARRERO, A., HERRADOR, M., ARTEAGA, P., RODRIGUES- GARCIA, I., & GARCIA-MORENO, M. (1997). RESORCINOL DERIVATIVES AND FLAVONOIDES AND FLAVONOIDS FROM ONONIS NATRIX SUB SP.BREVIFLORA. PHYTOCHEMISTRY (48), 187-190.
9. BARRERO, A., SANCHEZ, J., BARRON, A., CARRALES, F., & RODRIGUEZ, I. (1989). RESORCINAL DERIVATIVES AND OTHER COMPONENTS OF ONONIS SPECIESE. PHYTOCHEMISTRY (28), 161-164.
10. BARRERO, A., SANCHEZ, J., REYES, F., & RODRIGUEZ, I. (1991). RESORCINOL DERIVATIVES FROM ONONIS VISCOSA. PHYTOCHEMISTRY (30), 641-643.
11. KAMEL, H. S., DOCKENDORFF, K., BWRNHARDT, M., & SEIFERT, K. (2004). A NEW TRITERPENOID SAPONIN FROM ONINIS SPINOSA AND TWO NEW FLAVONOID GLYCOSIDES FROM ONONIS VAGINALIS. (596), 124-128.
12. KHALIL, A., ASALMEH, S. M., ABDALLA, A., TOSA, S., & IINUMA, M. N. (1995). N-ARACHYDYLANTHRNUILIC ACID, A NEW DERIVATIVE FROM ONONIS NATRIX. J.NAT.PROD, 58, 760-763.
13. KLEJDUS, B., VACEK, J., LOJKOVA, L., BENESOVA, L., & V.KUBAN, L. (2008). ULTRA- PRESSURE LIQUID CHROMATOGRAPHY OF ISOFLAVONES AND PHENOLIC ACIDS ON DIFFERENCNT STATIONARY PHASES. JOURNAL OF CHROMATOGRAPHY (A, 1195), 52-59.
14. KOHLER, I., SIEMS, K. J., SIEMS, K., ANTONIO, M., AIBARRA, H. R., G.BERENDSOHN, W., ET AL. (2002). INVITRO ANTIPLASMODIAL INVESTIGATION OF MEDICINAL PLANTS. PORM EL SALVADOR NAT.PROD (57C), 277-281.
15. MAHASNEH, A., & EL - OQLAH, A. (1999). ANTIMICROBIAL ACTIVITY OF EXTRACTS OF HERBAL PLANTS USED IN THE TRADITIONAL MEDICINE OF JORDAN. JORDAN OF ETHNOPHARMACOLOGY (64), 271-276.
16. ROLLINGER, J., EWELT, J., SEGER, C., STURM, S., ELLMERER, E., & STUPPNER, H. (2005). NEW INSIGHTS IN TO THE ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF LYCOPODIUM CLAVATUM. PLANTA MEDICA (71), 1040-1043.
17. STECK, W. (1968). METABOLISM OF CINNAMIC ACID IN PLANTS CHLOROGANIC ACID FORMATION. PHYTHOCHEMISTRY (70), 1711-1717.
18. WOLLENWEBER, E., DORR, M., RIVERA, D., & JAMES, N. (2003). EXTERNALLY ACCUMULATED FLAVONOIDS IN THREE MEDITERRANEAN ONONIS SPECIES. VERLAG DER ZEITSCHRIFT FUR NATURFORSCH (58C), 771-775.