PHYTOCHEMICAL INVESTIGATION OF SYNEPHRINE IN THE FRUIT PEELS OF IRAQI SOUR ORANGE.


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ABSTRACT

Citrus aurantium (sour orange) of the Rutaceae family is growing widely in Iraq. Literature survey revealed that there was no phytochemical study concerning the alkaloids of Citrus aurantium fruits in Iraq. Literature data also revealed that citrus genus is a good source of synephrine. Synephrine from citrus genus has become of particular importance due to its broad spectrum of therapeutic activities, including antifungal, antibacterial and antiviral activities in addition to its use for weight reduction; therefore a research on Iraqi Citrus aurantium fruit peels will be of important value. This study is concerned with the extraction, identification, isolation, purification and quantitative estimation of synephrine in Iraqi Citrus aurantium fruit peels. Extraction was carried out by Soxhlet apparatus. The isolated compound was identified by melting point (M.P.) measurement, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), Fourier transforms infrared spectroscopy (FT-IR), Elemental microanalysis (CHN) and nuclear magnetic resonance spectroscopy (NMR) analysis. This study confirms the presence of synephrine in the fruit peels of Iraqi Citrus aurantium.

KEYWORDS: Citrus aurantium, Synephrine, TLC, HPLC.

INTRODUCTION

Citrus aurantium (bitter orange, also known as sour orange) is a widely grown Citrus species in Iraq; it is a small tree, about five meters tall;¹¹ it is not widely used as an edible fruit due to
its sour and bitter taste. The fruit peels are used in the production of jam and the juice is used in salads for sour taste instead of lemon juice.[2] Most of sour orange uses are medicinal rather than culinary. In today’s market, one of the primary interest constituent of *Citrus aurantium* peels is synephrine, due to its broad spectrum of medicinal and therapeutic activities including antifungal, antibacterial, antiviral activities and the most important is its recent use for weight reduction. Synephrine is a trace endogenous bioamine widely occurring in plants, bacteria, invertebrates and vertebrates, including humans. Synephrine is a proto alkaloid (figure 1) occurring naturally in Citrus species and can exist as one of two enantiomers, d- and l-synephrine, that do not have identical pharmacological activities; however, literature survey revealed that synephrine is found as the l-isomer in *Citrus aurantium*, which is the more potent enantiomer.[3] Synephrine is related to a large group of drugs including ephedrine and phenylephrine whose structures are based on the phenethylamine skeleton. The main differences between synephrine and ephedrine are the hydroxy-substitution on the aromatic ring. Synephrine is a direct sympathomimetic drug while ephedrine is both direct and indirect sympathomimetic. One of the main reasons for these differential effects is the increased polarity of the hydroxy-substituted phenyl ethyl amines which renders them less able to penetrate the blood-brain barrier.[4] Ephedra containing weight-loss products have been rapidly replaced by Ephedra-free products. Most of these new products contain *Citrus aurantium*; in which synephrine has adrenergic effects and activates beta-3 (but not beta -1 or beta -2) adrenoreceptors resulting in lipolysis and appetite suppression. The functions of endogenous synephrine have not been fully understood; synephrine may be true neurotransmitters and may affect platelet-mediated signaling events, and may contribute to the pathophysiology of migraine and other types of headaches.[5] To the best of our knowledge, There is no data on the composition of Iraqi *Citrus aurantium* fruit peels, Therefore, the aim of this research is to investigate synephrine in Iraqi *Citrus aurantium* fruit peels, so it can provides insight into how to explore further the benefits of Iraqi *Citrus aurantium* fruits for human health.

Figure (1): Synephrine structure.
MATERIALS AND METHODS

Plant material
The fruits of Iraqi *Citrus aurantium* were collected in January (2015) from Baghdad in Iraq. The fruits were peeled and the peels were air dried in the shade for two weeks (figure 2); then the dried peels were pulverized by mechanical mills and weighed. The plant material (*Citrus aurantium* fruit) was identified in the department of pharmacognosy/College of pharmacy/University of Baghdad and was authenticated by National Iraqi Herbarium.

![Figure (2): Plant material.](image)

Extraction of peels
One hundred gm. of dried powdered fruit peels of Iraqi *Citrus aurantium* were defatted with n-hexane for 24 hours; and allowed to dry at room temperature. The defatted plant material was packed in a thimble and placed in soxhlet extractor. Five hundred ml of 80% methanol was used as a solvent and placed in a 1 liter round bottom flask fitted with a soxhlet extractor. The extraction was continued for 12 hours. The extract was filtered and concentrated under reduced pressure to dryness using rotary evaporator at a temperature not exceeding 40°C; then the dry extract was weighed and dissolved in 2N hydrochloric acid on a water bath, shaken and filtered; then the obtained filtrate was shacked with chloroform to remove undesirable matters. The acidic aqueous layer was adjusted to alkaline pH with ammonia to liberate alkaloidal bases then the aqueous layer was concentrated under vacuum and subjected to identification, isolation and purification procedures.

Identification of plant constituents by TLC
The extract was examined by TLC, using readymade plates of silica gel GF254 (20×20cm) of 0.25mm thickness (MERCK). Detection was done by using 2% ninhydrin in n-butanol spraying reagent. Syneprhine standard was purchased from Chengdu Biopurify Phytochemicals.
Developing solvent systems

One hundred ml of solvent system was placed in a glass tank (22.5gm×22cm×7cm) and covered with a glass lid and allowed to stand for 45 minutes before use. A small amount of extract (1 mg dissolved in 1 ml solvent) was applied with standard sample (1mg/ml) to TLC plates manually, using capillary tubes. Five different developing solvent systems (S1, S2, S3, S4 and S5) were used for the detection of synephrine in peels extract:

S1=Ether: Methanol: 58% NH4OH (17:2:1).
S2= Chloroform: Ethanol: NH4OH (24:6:0.23).
S3= n-Butanol: acetic acid: Water (4:1:2.2).
S4= Chloroform: n-Butanol: conc. Ammonia (50:50:2.5).
S5= Ethyl acetate: Methanol: 58% NH4OH (17:2:1).

Isolation of the active constituent

Isolation of synephrine was done by preparative layer chromatography, using glass plates of 20cm x 20cm which were coated with slurry of silica gel GF 254 (2mm thickness) using Jobling laboratory division TLC coater. The peels extract was applied as a concentrated solution in a row of spots using capillary tube. Reference standard of synephrine was applied at the right side of the baseline. The application of the extract was repeated four times in each plate, one should wait after each application until all the solvent is evaporated. The detection was done by using 2% ninhydrin in n-butanol spraying reagent; a small part of the plate corresponding to Synephrine standard at the right side was left for spraying with 2% ninhydrin reagent while the rest of plate was protected from spraying by a glass plate (figure 3). The bands corresponding to synephrine standard were scrapped out and collected in a beaker and eluted with gentle heating and filtered; then the filtrate was evaporated to dryness under vacuum to give white precipitate. Recrystallization was done to get pure compound. The mobile phase used was S3= n-Butanol: acetic acid: Water (4:1:2.2).
Figure (3): Preparative layer chromatography of synephrine.

Qualitative and quantitative estimation of synephrine by HPLC

HPLC was used for qualitative and quantitative estimation of synephrine. HPLC analysis was carried out using (Knauer/Germany). Identifications were made by Comparism of retention times obtained at identical chromatographic conditions of analyzed samples and authentic standards. The mobile phase used was **acetonitrile: water: trifluoroacetic acid** (5:95:0.01) and the column used was C18 (150mm × 4.6mm/5um); flow rate was 0.6 ml/ min and detection by UV. Detector at λ 220 nm.[6]

RESULTS AND DISCUSSION

TLC confirms the presence of synephrine in the extract of Iraqi *Citrus aurantium* fruit peels; synephrine appeared as a single round compact spot having the same color and Rf values as that of reference standard on TLC plates in five different developing solvent system (figures 4a, 4b). Although synephrine was separated in TLC when S1, S2 and S4 were used as mobile phases; however synephrine moved only short distance from the base line due to high percentage of nonpolar solvents (ether or chloroform) in these mobile phases and since synephrine is freely soluble in water and alcohol and less soluble in chloroform and ether; so the migration was improved by increasing the polarity of the mobile phase as in **S3= n-Butanol: acetic acid: Water** (4:1:2.2) which was found to be the best developing solvent system for the separation of synephrine as indicated by Rf values (table 1). The presence of synephrine in Iraqi *Citrus aurantium* fruit peels was further augmented by HPLC; where a peak observed in the chromatogram of Iraqi *Citrus aurantium* peels extract which has the same retention time (3.20 minutes) as that of synephrine reference standard, indicating that the peak is likely to be synephrine (figures 5a,5c). HPLC was also used for the identification of the isolated compound which showed the same retention time as that of synephrine reference standard (figure 5b); the identification of the isolated compound was further confirmed by M.P. measurement, FT-IR, CHN analysis and NMR analysis.
Figure (4a): TLC of peels extract (left) and synephrine standard (right) using S3 as a mobile phase.

Figure (4b): TLC of peels extract (right) and synephrine standard (left) using S5 as a mobile phase.

Table (1): R_f values in different developing solvent systems.

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synephrine Standard</td>
<td>0.033</td>
<td>0.052</td>
<td>0.50</td>
<td>0.075</td>
<td>0.123</td>
</tr>
<tr>
<td>Isolated Compound</td>
<td>0.033</td>
<td>0.053</td>
<td>0.50</td>
<td>0.075</td>
<td>0.122</td>
</tr>
<tr>
<td>Synephrine In peels extract</td>
<td>0.032</td>
<td>0.053</td>
<td>0.49</td>
<td>0.075</td>
<td>0.122</td>
</tr>
</tbody>
</table>
Figure (5a): HPLC chromatogram of synephrine standard.

Figure (5b): HPLC chromatogram of the isolated compound.

Figure (5c): HPLC chromatogram of *citrus aurantium* fruit peels extract.
Measuring melting point
The isolated compound was identified from its sharp melting point using electro-thermal melting point apparatus (Stuart/UK); it showed a melting point of (162-165°C) (with decomposition) compared to synephrine standard melting point (162-164°C).

CHN analysis
Elemental microanalysis was performed (using EuroEA Elemental analyzer/Italy) for the isolated compound to confirm the chemical structure and purity (table 2).

Table (2): Elemental Microanalysis of the isolated compound.

<table>
<thead>
<tr>
<th>Isolated Compound</th>
<th>C%</th>
<th>H%</th>
<th>N%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>66.702</td>
<td>7.959</td>
<td>8.562</td>
</tr>
<tr>
<td>Calculated</td>
<td>64.65</td>
<td>7.84</td>
<td>8.38</td>
</tr>
</tbody>
</table>

FT.IR.
The identification of the isolated compound was further confirmed by FT-IR spectroscopy using Shimadzu FT-IR-8400S Infrared Spectrometer (figure 6 and table 3) indicating that the compound is likely to be synephrine.\(^{[7]}\)

![FT-IR spectrum of the isolated compound.](image)

Table (3): Characteristic FT-IR Absorption Bands (in cm\(^{-1}\)) of the isolated compound.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Group frequency wave number (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H</td>
<td>3298.38</td>
<td>N-H stretching</td>
</tr>
<tr>
<td>C-H</td>
<td>2872.10</td>
<td>C-H stretching (aliphatic)</td>
</tr>
<tr>
<td></td>
<td>1602.90</td>
<td>Aromatic C=C stretching</td>
</tr>
<tr>
<td>C=O</td>
<td>1539.25</td>
<td>N-H bending</td>
</tr>
<tr>
<td></td>
<td>1442.80</td>
<td>O-H bending</td>
</tr>
<tr>
<td>N-H</td>
<td>1462.09,1338.64</td>
<td>C-H bending (aliphatic)</td>
</tr>
<tr>
<td></td>
<td>835.21,800.49</td>
<td>C-H &amp; C=C out of plane bending</td>
</tr>
</tbody>
</table>
NMR analysis

The NMR data displayed the six carbon atoms of the aromatic ring, methylene group side chain, carbon bearing OH group and the methylamino group, were all diagnostic for phenyl ethyl amine moiety, containing a hydroxyl group at the para position of the aromatic ring. The aromatic protons, displayed, each as a doublet at $\delta=7.11$ and 6.69 ppm, the methylene protons ($\text{CH}_2$-NH), shown as a multiplet in the range of $\delta=2.58-2.45$ ppm and also three protons appeared as a singlet at $\delta=2.28$ ppm correlated to carbon signal at $\delta=35.9$ ppm (NH-$\text{CH}_3$). All the carbon signals of the aromatic ring and the side chain are diagnostic for the proposed structure of synephrine. These data are closely similar to that reported in the literature,[8] and the compound is identified as synephrine. $^1$HNMR for the isolated compound (300 MHz, DMSO$_{d6}$) $\delta$ [ppm]: 7.11(2H,d,H2,H6); 6.69 (2H,d,H3,H5); 4.51(1H,t,H7); 2.28 (3H,s,N-CH$_3$); 2.58-2.45 (m,2H,CH2-N); (figure 7a). $^{13}$CNMR for the isolated compound $\delta$ [ppm]: 156.1 (C1); 134.8 (C4); 126.9 (C2,C6); 114.7 (C3,C5); 70.8 (C7); 59.9 (C8); 35.9 (N-CH$_3$); (figure 7b).

![Figure (7a): $^1$H-NMR analysis of the isolated compound.](image1)

![Figure (7b): $^{13}$C-NMR analysis of the isolated compound.](image2)
This study confirms the presence of synephrine in the fruit peels of Iraqi *Citrus aurantium* and the percentage of synephrine in the peels was found to be 0.45%, indicating that Iraqi *Citrus aurantium* fruit peels are an important dietary source of synephrine. Different synephrine concentrations in *Citrus aurantium* fruits grown in other countries were published; however, these differences can be attributed to several factors including location or environmental factors that affect plant nutrients including nitrogen; where a positive correlation between soil nitrogen concentration and synephrine percentage in citrus fruits was reported. Other factors that may cause variations in synephrine concentrations include temperature, light, moisture content and aeration which create big differences in the active constituents even in the same country; also the genetic origin, harvesting time, and the part used of the plant.

**CONCLUSION**

*Citrus aurantium* is easily available and widely grown in Iraq; and the fruit peels were found to be rich in synephrine which has important medicinal and therapeutic applications particularly its increasing use in fighting obesity. So it is important to give more research attention for this herbal drug; such research will provide insight into how to explore further the benefits of Iraqi *Citrus aurantium* fruits for human health. *Citrus aurantium* fruit byproducts could be interesting not only due to their important fiber content but also due to their higher levels of synephrine as confirmed in this study. The results revealed that Iraqi *Citrus aurantium* is a good source of synephrine alkaloid. This study provides methods for the extraction, identification and isolation of synephrine; the most suitable mobile phase for the separation and isolation of synephrine by TLC was determined in this study.

**REFERENCES**


6. M.D. Arbo et al. Concentrations of p-synephrine in fruits and leaves of Citrus species (Rutaceae) and the acute toxicity testing of Citrus aurantium extract and p-synephrine. Food and Chemical Toxicology, 2008; 46: 2770–2775.


