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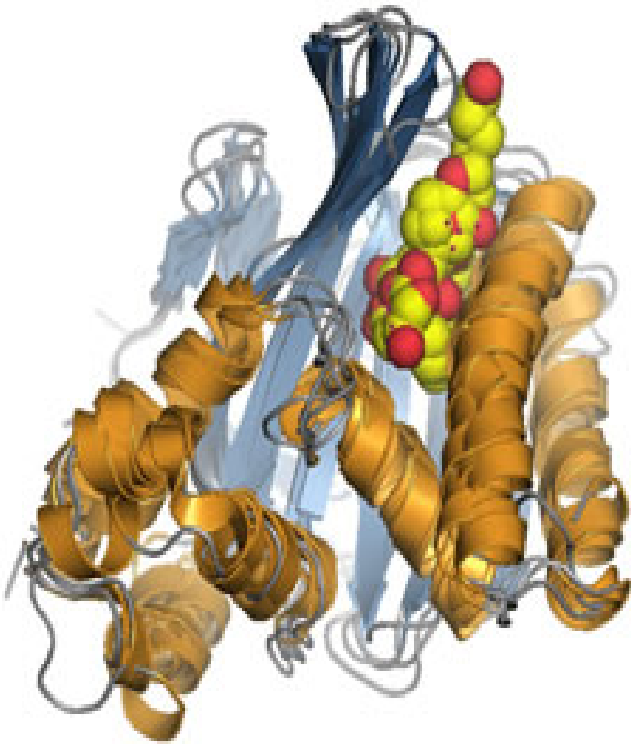

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In vitro effect of Hesperidin & Hesperitin on calcium oxalate Crystallization: The Chiral Impact

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Abstract. *Hesperidin, an abundant bioflavonoid in citrus fruits, has been reported to possess a wide range of pharmacological properties. In this study, the antilithiasic activity of hesperidin a glycoside flavanone extracted from leaves peel of three different citrus species, (C. reticulata, C. clementina, C. sinensis), this activity may be explained by interaction of two enantiomers or diastereomers of hesperidin and hesperitin.*

Key words: Hesperidin, Hesperitin, flavanone glycoside, orange peels, calcium oxalate, antilithiasic activity.

1. INTRODUCTION

Urolithiasis is a global problem affecting human beings for several centuries and calcium oxalate is one of the main constituents of kidney stones. A large number of studies have been carried out to identify substances which inhibit calcium oxalate crystallization and any plants extracts have been investigated to their anti-lithiasic effect, but no pure natural product has been studied yet.

Hesperidin is one of the bioflavonoids which is greatly found in Citrus species and is the major active constituent of tangerine *(Citrus reticulata)* and sweet orange *(citrus sinensis)* peel. Hesperidin is a flavanone glycoside comprising the flavanone hesperitin and the disaccharide rutinose. The potential activity and chiral characteristic of Hesperidin and Hesperitin give a new research promotion in the pure anti-lithiasic compounds from natural resource [1-6].

Hesperitin are obtained in good yield by Hydrolysis of Hesperidin in energetic condition.

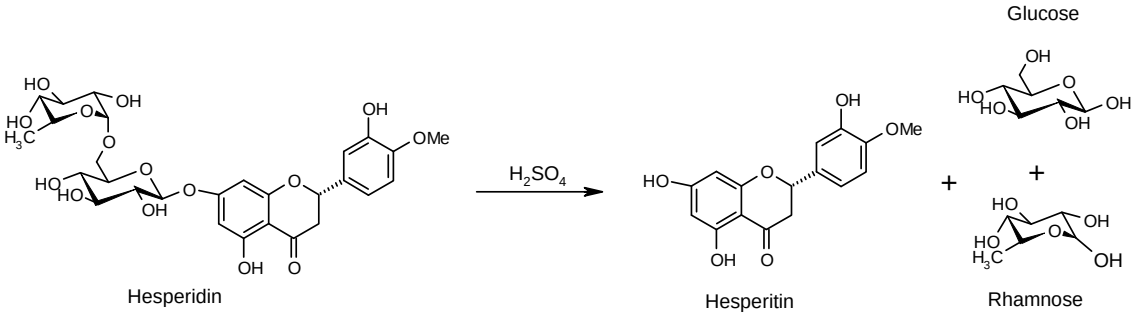


Fig. 1. Conversion of Hesperidin into Hesperitin

2. MATERIALS AND METHODS

Plant material: A peels of three citrus species has obtained from the university restaurant and from the wastes of home consumption.

Apparatus : Crystals were identified under microscope (Zeiss) equipped with a camera WINDER M 476079 , IR spectra obtained with AVATAR 320 FT-IR spectrophotometer ,UV spectra were obtained with UNICAM UV 300 spectrophotometer , and chiral analysis were obtained with HPL (Schimadzu LC20A).

Extraction and purification of Hesperidin and Hesperitin: Air dried sweet orange peels were grinded into powder (960 g) and Extracted in a reflux condenser successively by petroleum ether and Methanol. The filtrate was concentrated with distillation column, leaving a syrupy residue crystallized from dilute acetic acid (6%), and yielding orange needles (crude Hesperidin) mp 268°C.
The crude Hesperidin was added to chloroform. The white crystalline Hesperidin was then filtered through a Buchner funnel. Pure Hesperidin has mp 240-253 °C.
Hesperitin was obtained by hydrolysis of hesperidin in the methanol under energetic conditions, Hesperetin is extracted and purified by acetone , water and acetic acid, the crystal washed with water led a pure hesperitin has mp 220-221 °C. The pure compounds were identified by spectroscopic methods UV , IR , NMR.

Anti-lithiasic activity
The crystal size development was monitored after the tests in absence and in presence of plant extract. Crystals were identified with x40 magnifying lens under microscope, and analyzed by infrared spectrometer.

Study in absence of Heperidin and Hesperitin
Two solutions $CaCl_2$, $2H_2O$ (0,1 M) and $Na_2C_2O_4$ (0,01M) were prepared using sodium chloride solution (0.15 M). A volume of 50 mL of calcium chloride was transferred into the UV/Vis cell and blank reading was taken. Add 50 mL of sodium oxalate, to the previous volume, and the

measurement is immediately started for a period of 25 mn. For each experiment, 3 replicates were taken.

Study in presence of Hesperidin and Hesperitin

Mother solutions of 1g/L of hesperidin and hesperitin were prepared in distilled water. Diluted solutions of 0.5 g/L, 0.25 g/L, 0.125 g/L and 0,0625 g/L were prepared [7,8].

The same methodology was repeated with 50 mL of calcium chloride, 50 mL of sodium oxalate and 50 mL of hesperidin or hesperitin solution. The rate of inhibition using the following formula:

$$I\% = \left(1 - \frac{S_{presence}}{S_{absence}}\right) \times 100$$

I% : Rate of inhibition

$S_{presence}$**:** Slope in presence of inhibitor

$S_{absence}$**:** Slope in absence of inhibitor

3. RESULTS AND DISCUSSION

Crude hesperidin extracted from tangerine has weighing 1.75%, from Clementine and sweet orange 2.4%.

Chiral analysis

Using normal phase HPLC on Chiral pack IC, the Hesperidin was separated in two diastereomers with 2S epimer is predominant (65:35), but the chiral analysis of Hesperitin show two enantiomers in racemic mixture (50:50).this analysis showed the interaction of two equivalent of enantiomer of hesperitin with calcium oxalate ,but three equivalent of diastereomer of hesperidinin this case.

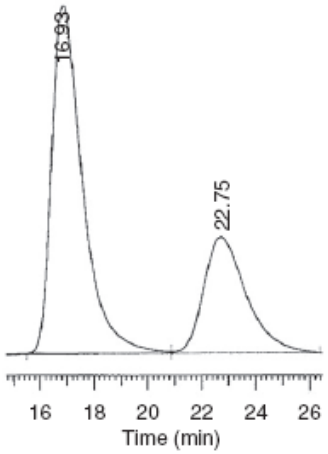
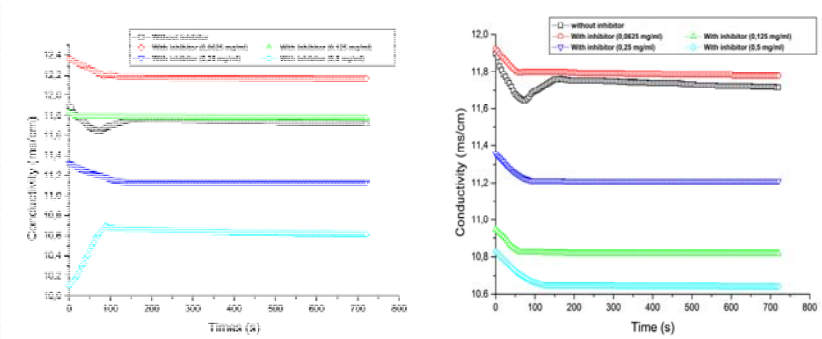


Fig 2. Chiral analysis of Hesperidin on chiral pack IC

Anti-lithiasic activity



a b

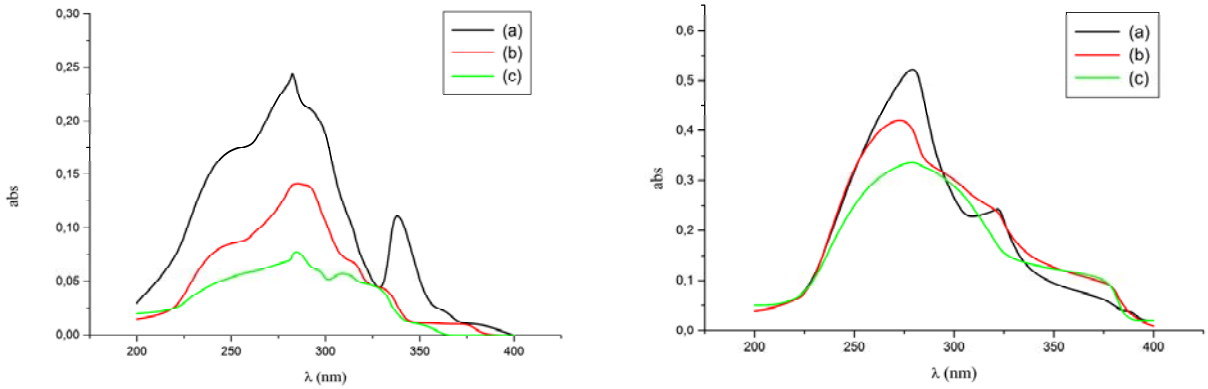
Fig.3. Conductimetric curves in absence and in presence of (a : Hesperidin , b:Hesperitin)

The calcium oxalate molecular structure present two interaction sites, wich the inhibition of this cristal achieved by the complexation of the falvanone diastereomer or enantiomer.
The chiral analysis explain the difference between the inhibition factors for example in low concentration (0.125g/L) under similar conditions (ranged at 86.89%), this result is confirmed by uses of three equivalent of diastereomer in case of hesperidin with two osidic parts (65/35%) , but in other case we used 2 equivalents of hesperitin : an aglycone flavanone (50/50%).

Table 1. Conductimetric cruve parameters in absence and in presence of hesperidin and hesperitin.

	CI (g/l)	***R***	**ΔT**	***I%***	***P***	**C*v (%)***
In absence of inhibitors	0	-0,94723	0 - 20	-	<0.0001	5,08
	0.0625	-0,99956	0-85	84,94	<0.0001	5,66
Hesperidin	0.125	-0.99555	0-25	86,89	<0.0001	3,72
	0.25	-0,98751	0-150	90,4	<0.0001	7,22
	0.5	-	-	-	-	-
	0.0625	-0,99509	0 -60	84,1	<0.0001	5,92
Hesperitin	0.125	-0,99351	0 - 65	79,6	<0.0001	6,27
	0.25	-0,98544	0 - 90	87,94	<0.0001	6,39
	0.5	-0,98365	0-125	88,91	<0.0001	7,29

CI concentration of inhibitor, ***R*** linear regression, **ΔT** variation of time,
I percentage of inhibition, ***Cv (%)*** : coefficient of variation,



(A) **(B)**

Fig.4. (A) UV-vis spectra of (a) hesperidin (0.5 mg/ml), (b) $CaCl_2$ $2H_2O$ (0,1 M) containing hesperidin (0.5 mg/ml) and (c) $CaCl_2$ $2H_2O$ (0.1 M) containing hesperidin (0.5 mg/ml) and $Na_2C_2O_4$ (0.01M). (B) UV-vis spectra of (a) hesperitin (0.5 mg/ml), (b) $CaCl_2$ $2H_2O$ (0,1 M) containing hesperitin (0.5 mg/ml) and (c) $CaCl_2$ $2H_2O$ (0.1 M) containing hesperitin (0.5 mg/ml) and $Na_2C_2O_4$ (0.01M).

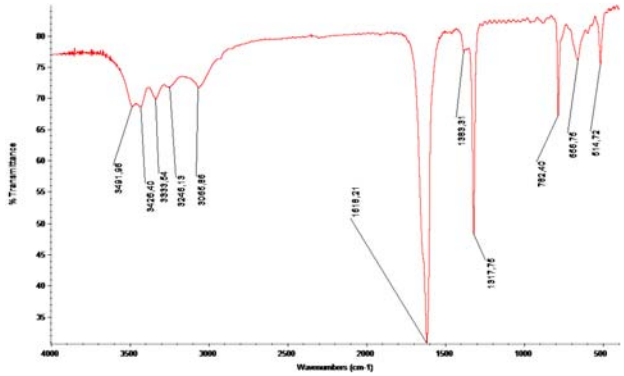


Fig.5. Infrared spectrum of pouder of $CaCl_2$ $2H_2O$ (0.1M)-$Na_2C_2O_4$ (0.01M) system.

It was observed from spectra in IRTF that the main characteristic bands of crystals without inhibitor were conformed to the characteristic peaks of COM crystals. In Fig.5, the characteristic absorptions of COM crystals were at 656 and 782 cm^{-1} and the band at 1618 cm^{-1} was the C=O valence oscillation, the hydration valence oscillation band of COM crystals split into five single

bands from 3065.85 to 3492 cm^{-1}. The band at 1318 cm−1 indicated a mixture of COM and COD crystals [9].

The peak at 1618 cm^{-1} observed in the IRTF spectrum of the calcium oxalate crystallization in absence of inhibitor disappeared on addition of hesperidin and hesperitin at different concentration, indicating the binding of $CaCl_2$ with inhibitor Fig.6,7 & 8.

When $CaCl_2$ was mixed only with hesperidin or Hesperitin the peak at 1629.14 cm^{-1} was disppeared, what confirms the coordination of Ca^{++} with the inhibitor [10,11].

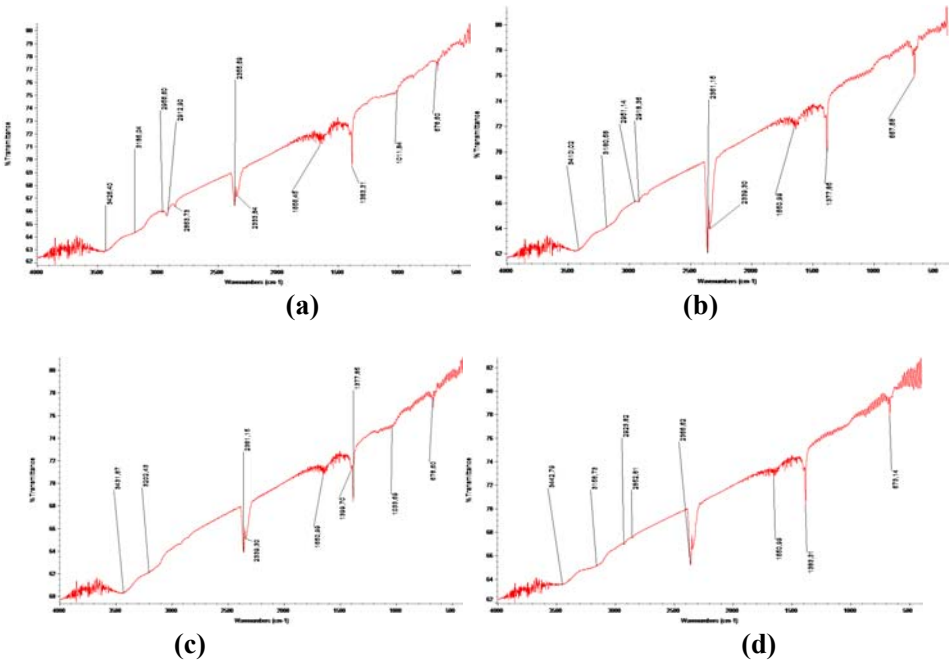
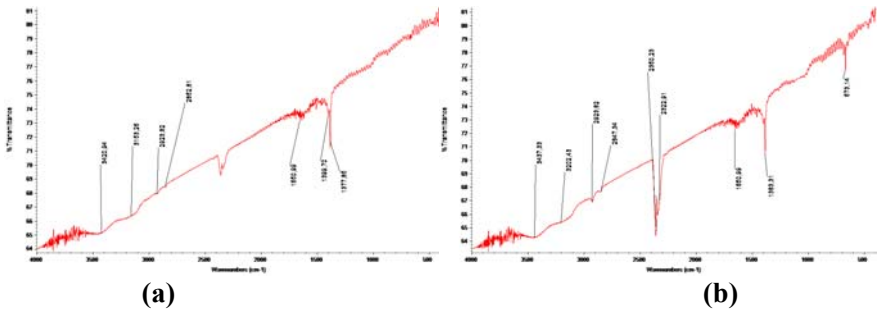
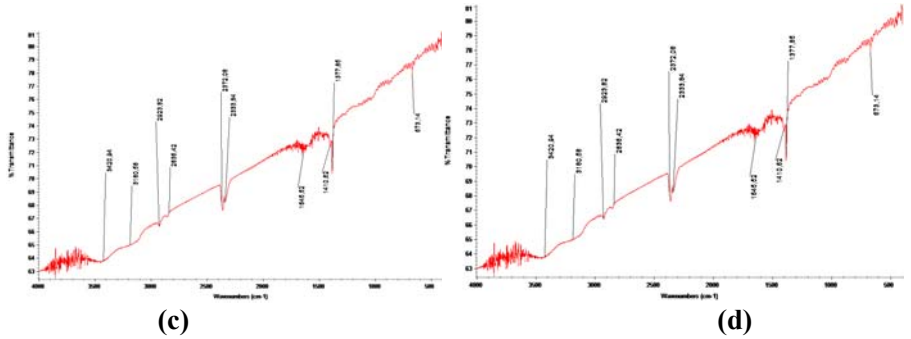


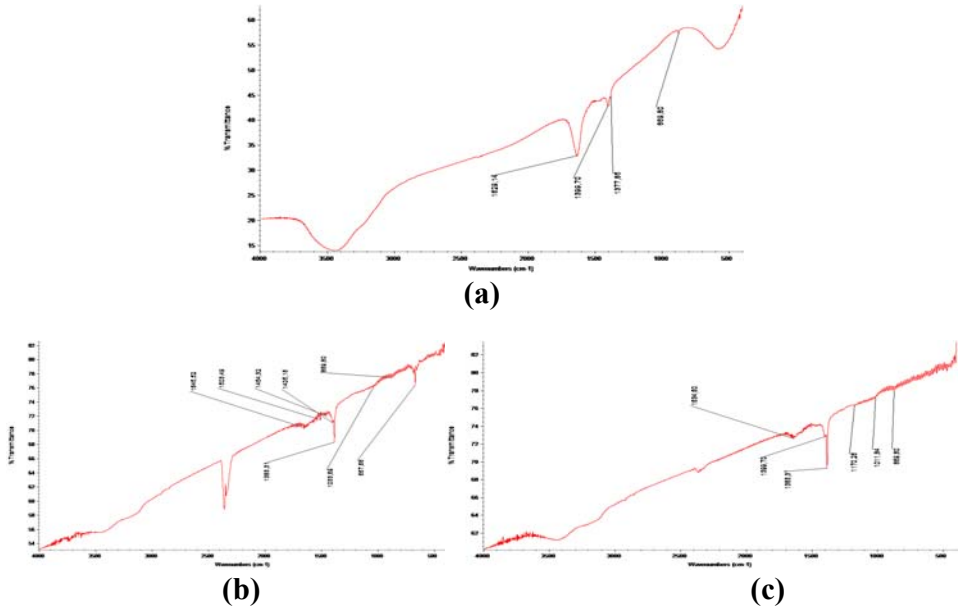
Fig.6. Infrared spectra of reaction products of $CaCl_2$ $2H_2O$ (0.1M) containing Hesperidin 0.0625 (a), 0.125 (b), 0.25 (c), 0.5 mg/ml (d)- $Na_2C_2O_4$ (0.01M).





(c) (d)

Fig.7. Infrared spectra of reaction products of $CaCl_2$ $2H_2O$ (0.1M) containing Hesperitin 0.0625 (a), 0.125 (b), 0.25 (c), 0.5 mg/ml (d)- $Na_2C_2O_4$ (0.01M).



(a)

(b) (c)

Fig.8. Infrared spectra of (a) $CaCl_2$ $2H_2O$, (b) $CaCl_2$ $2H_2O$-Hesperidin (0.5mg/ml) and (c) $CaCl_2$ $2H_2O$-Hesperitin (0.5mg/ml).

CONCLUSION

The various results obtained in our work show that hesperidin and hesperitin promoted the nucleation of calcium oxalate crystals, increasing their number but decreasing their size. It also inhibit calcium oxalate crystal aggregation. These properties of hesperidin and hesperitin might be beneficial in preventing kidney stone formation.

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