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Preliminary phytochemical study and TLC analysis of the fruit, leaves and flowers of *Citrus limetta* Risso

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Abstract
Citrus limetta Risso specie belongs to Rutaceae family, commonly known as sweet lime in Mexico. It has traditionally been used for several medicinal purposes. Phytochemical screening and TLC study of fruit, leaves and flowers were made to identify the qualitative presence of main bioactive compounds. Phytochemical analysis was carried out using solvents at different polarity as n-butane, ethanol, methanol, and water. As a result, it was found a high diversity of metabolites such as alkaloids, flavonoids, quinones, steroids, cardiac glycosides, sesquiterpene lactones, tannins, saponins and leucoanthocyanins. At last, a Thin Layer Chromatography (TLC) was used to confirm the presence of alkaloids and flavonoids giving different R_f values. The result showed the presence of alkaloids and flavonoids in fruits, leaves and flowers of *Citrus limetta* Risso, which can be correlated as a possible potential as anxiolytic, sleep regulator, and antioxidant.

Keywords: *Citrus limetta* Risso, phytochemical, alkaloids, flavonoids, Thin Layer Chromatography

1. Introduction
The use of plants as medicine is an ancient practice common in Mexico. Mexican flora has over 3000 species that have been documented as therapeutic plants used in folk medicine, these include Citrus species [1-3]. One of these species is *Citrus limetta* Risso, known as sweet lime. It presents a large source of biologically active compounds with different properties using the flowers, fruit juice and leaves that are used in abundance in Mexican folk medicine [2, 4]. For instance, traditionally, the fruit juice has been used to counteract eye and scalp infection, as antioxidant and as fever reducer [4-7], besides, the fruits are used for their antihyperglycemic and antihypertensive activity [1,8], also, the fruits and leaves infusion has widely been used as blood pressure modulator, decreasing cholesterol level and helping to reduce inflammation [2, 5, 9] ; moreover, the fruits and flowers (an aqueous extract) have been used for the treatment of sleep, anxiety disorders, and depression [2, 8-11].
Furthermore, secondary metabolites isolated from *C. limetta* Risso have recently been found. For example, flavonoids such as hesperidin and naringin have several biological activities specifically as antiinflamatory, antimicrobial, antiglucemic, antitumor, antiviral and others [9, 12-14]. According to Padilla-Camberos *et al* 2014, the aqueous extract of peel fruit showed a potent inhibition of the enzymatic activity of α-glucosidase and α-amylase that were related to the polyphenol content [15]. For the tongue cancer cell line, the acetone and methanol extracts of *C. limetta* Risso showed inhibitory activity [16]. Finally, Perez *et al* 2010, report that leaves extract antagonizes the hypertensive effect of angiotensin II, suggesting an important bioactive capability [17]. Nevertheless, there is not a complete phytochemical study of this specie. Therefore, the aim of this study is to investigate the qualitative presence of phytoconstituents on fruit, peel fruit, leaves and flowers of *C. limetta* Risso specie.

2. Materials and methods
2.1. Plant collection
Fresh fruits, leaves and flowers of *C. limetta* Risso were collected from February to May 2016 in the Mujica Hacienda at Lagos de Moreno, Jalisco; Mexico. The fruits were harvested after the flowering period. Fruits were green-coloured, with a 5-7 cm of diameter.

2.2. Preparation of plant extracts
The fruits, leaves and flowers from *C. limetta* Risso were washed with water to remove all undesirable material. After that, fresh fruit peels of *C. limetta* Risso were manually removed from the fruits, taking care of excluding the membranous matter and pith.

The peels, leaves, and flowers were shade-dried at room temperature of 25 °C. A total of 30 g fresh and dried sample of each plant material of fruits, leaves and flowers was weighed and extracted using 140 mL of each different solvent such as n-hexane, 95% ethanol, 95% methanol and water for 48 hr. At the end of the extraction, each extract was filtered using Whatman No 1 filter paper. The extract was subjected to preliminary phytochemical screening.

2.3. Phytochemical screening

The preliminary phytochemical screening was carried out using standard procedures for determining the presence of alkaloids such as Dragendorff´s, Mayer´s and Wagner´s tests; flavonoids by Shinoda, sodium hydroxide and Pew´s test; quinones using Borntrager´s test; sterols by Liebermann-Burchard and Salkowski´s tests; cardiac glycosides using Kedde´s test; sesquiterpene lactones by Baljet´s test; tannins using Benedict´s test; saponins by foam test, and leucoanthocyanins test [18, 19]. All laboratory assays were performed by triplicate.

2.4. Thin Layer Chromatography analysis

In order to verify the presence of alkaloids and flavonoids, the extracts in fresh and dry samples from fruit peels, leaves and flowers were analyzed by Thin Layer Chromatography (TLC). Three different solvents such as ethanol, methanol, and water were used in each extract that were divided into two fractions, named fraction A and B. The fraction A was used for the determination of alkaloids and fraction B was used for the determination of flavonoids. The TLC studies were made using pre-coated silica gel GF254 plates Merck; and the separated components were visualized using UV lamp fluorescent at 254 nm. Different spots were observed and its corresponding R_f values were calculated [20, 21].

2.4.1. TLC study of alkaloids

Each extract was alkalinized with 20% NaOH solution. Then, 25 mL each extract was individually placed in a glass separatory funnel and 25 mL of dichloromethane was added to each. Mixtures were allowed to settle down for ten minutes until two fractions were observed. From fraction A, 12 µL were taken with a capillary glass tube and placed into TLC plate. Then, the mobile phase was added using a solvent mixture butanol, acetic acid and water (10:2:1) until TLC plate was completely dried. After that, spraying Dragendorff´s reagent aimed to record R_f values of the separated alkaloids under ultraviolet at 254 nm. [19-21].

2.4.2. TLC study of flavonoids

Meanwhile, the bottom phase was identified as fraction B for the determination of flavonoids. A total of 20 mL of each extract was placed in water bath to get a final volume of 10 mL. Afterwards, a total of 1.5 mL of solution of each extract was placed in four tube test to add later 1.5 mL of the following solvent: ethyl ether (EE), ethyl acetate (EA), acetic acid (AA) and n-butanol (NB). From each subfraction obtained, 12 µL were taken with a capillary glass tube and placed into TLC plate. Finally, mobile phases were used on each subfraction: B1 (n-hexane-ethyl acetate, 6:4), no additives; B2 (ethyl acetate-formic acid-acetic acid-water, 10:1:1:2), no additives; B3 (chloroform-methanol, 99:1), revealed by 10% KOH solution in methanol; and B4 (chloroform-methanol, 5:5) also revealed by 10% KOH solution in methanol. R_f values were identified with an UV lamp fluorescent at 254 nm. [19-21].

3. Results and discussion

The preliminary phytochemical analysis of the fruit, fresh peels and dry peels extracts showed the presence of alkaloids, flavonoids at Shinoda and 5% NaOH tests, quinones, sterols at Salkowski test, sesquiterpene lactones, tanins and saponins. However, flavonoids at Pew´s test, sterols at Liebermann-Buchard test, cardiac glycosides and leucoanthocyanins were absent in these extracts as indicated in Table 1.

Table 1: Phytochemical constituents of the fruit extract of *C. limetta* Risso.

Phytochemical test	Fruit extract				Fresh peels extract				Dry peels extract			
	H	**E**	**M**	**W**	**H**	**E**	**M**	**W**	**H**	**E**	**M**	**W**
Alkaloids												
Dragendorff	+	+	+	-	+	+	+	-	+	+	+	-
Mayer	-	+	+	+	-	+	+	+	-	+	-	+
Wagner	-	+	+	+	-	+	+	+	-	+	+	+
Flavonoids												
Shinoda	+	-	+	-	+	+	+	+	-	-	-	-
NaOH	+	+	+	+	+	+	+	+	-	+	+	+
Pew´s	-	-	-	-	-	-	-	-	-	-	-	-
Quinones												
Borntrager	-	+	+	+	-	+	+	+	-	+	+	+
Sterols												
Liebermann-Buchard	x	-	x	x	x	-	x	x	x	-	x	x
Salkowski	+	+	+	-	+	+	+	-	+	+	-	+
Cardiac glycosides												
Kedde	x	-	x	x	x	-	x	x	x	-	x	x
Sesquiterpene lactones												
Baljet	+	-	-	+	+	-	-	+	+	+	+	+
Tannins												
Benedict	-	+	+	+	-	+	+	+	-	+	-	+
Saponins												
Foam test	-	+	-	+	-	+	+	+	-	+	-	+
Leucoanthocyanins	x	-	x	x	x	-	x	x	x	-	x	x

Key: + = present, - = absent, **x**= does not apply.
H: n-hexane extract, **E**: ethanol extract, **M**: methanol extract, **W**: water extract.

As it is shown in Table 2, the phytochemical screening on fresh and dry leaves revealed the presence of alkaloids at Dragendorff and Wagner tests, flavonoids, quinones, steroids, cardiac glycosides, sesquiterpene lactones, tannis, saponins and leucoanthocyanins. Unlike Mayer´s test in alkaloids on fresh and dry leaves that was negative.

Table 2: Phytochemical constituents of the leaves extracts of *C. limetta* Risso.

Phytochemical test	Fresh leaves extract				Dry leaves extract			
	H	E	M	W	H	E	M	W
Alkaloids								
Dragendorff	-	+	+	-	-	+	+	+
Mayer	-	-	-	-	-	-	-	-
Wagner	-	+	+	+	-	+	+	+
Flavonoids								
Shinoda	-	+	+	-	-	+	-	+
NaOH	+	+	+	+	-	-	+	+
Pew´s	-	+	+	+	-	+	+	+
Quinones								
Borntrager	+	+	+	+	-	+	+	+
Sterols								
Liebermann-Buchard	x	+	x	x	x	+	x	x
Salkowski	+	-	-	-	+	-	-	-
Cardiac glycosides								
Kedde	x	+	x	x	x	+	x	x
Sesquiterpene lactones								
Baljet	+	-	+	-	+	-	+	-
Tannins								
Benedict	-	+	+	+	-	+	+	-
Saponins								
Foam test	-	+	-	-	-	+	-	+
Leucoanthocyanins	x	+	x	x	x	+	x	x

Key: + = present, - = absent, **x**= does not apply.
H: n-hexane extract, **E**: ethanol extract, **M**: methanol extract, **W**: water extract.

As can be seen in Table 3, the phytochemical screening of the flowers showed the presence of alkaloids, flavonoids, quinones, steroids, cardiac glycosides, sesquiterpene lactones, tannis, saponins and leucoanthocyanins with the exception of Pew´s test at Flavonoids identification that was completely negative on both samples.

Table 3: Phytochemical constituents of the flowers extracts of *C. limetta* Risso.

Phytochemical test	Fresh flowers extract				Dry flowers extract			
	H	E	M	W	H	E	M	W
Alkaloids								
Dragendorff	-	+	+	+	+	+	+	+
Mayer	-	-	+	+	-	+	+	+
Wagner	-	-	+	+	-	+	+	-
Flavonoids								
Shinoda	-	+	+	+	-	+	+	+
NaOH	-	+	+	+	+	+	+	+
Pew´s	-	-	-	-	-	-	-	-
Quinones								
Borntrager	-	+	+	+	-	+	+	+
Sterols								
Liebermann-Buchard	x	+	x	x	x	+	x	x
Salkowski	-	+	+	-	-	+	+	-
Cardiac glycosides								
Kedde	x	+	x	x	x	+	x	x
Sesquiterpene lactones								
Baljet	-	+	+	+	-	+	+	+
Tannins								
Benedict	-	+	+	+	-	+	+	-
Saponins								
Foam test	-	+	-	-	-	+	+	-
Leucoanthocyanins	x	+	x	x	x	+	x	x

Key: + = present, - = absent, **x**= does not apply.
H: n-hexane extract, **E**: ethanol extract, **M**: methanol extract, **W**: water extract.

In summarize ethanol and methanol was better solvent used than the others to identify presence of some secondary metabolites in fruits, leaves and flowers. It is because of its intermediate polarity that leads to the extraction of polar and non-polar compounds. Also, water solvent showed presence of secondary metabolites; however n-hexane solvent was inefficient to extract phytochemical compounds. Regarding the TLC analysis to determine alkaloids and flavonoids using three solvents (ethanol, methanol, and water), the next results was obtained as R_f values of alkaloids and flavonoids as it is shown in table 4 and table 5, respectively.

Table 4: R_f values by TLC solvent systems from different extracts of the peel fruit, leaves and flowers extract of *C. limetta* to alkaloids identification.

Solvent	Alkaloids											
	Peel of fruit				Leaves				Flower			
	Fresh		Dry		Fresh		Dry		Fresh		Dry	
	No. of spots	Rf value	No. of spots	Rf value	No. of spots	Rf value	No. of spots	Rf value	No. of spots	Rf value	No. of spots	Rf value
Ethanol			3	0.41 0.63 0.98			1	0.85				
Methanol					1	0.66						
Water	2	0.60 0.97							3	0.32 0.74 0.97	2	0.55 0.95

Table 5: R_f values by TLC solvent systems from different extracts of the peel fruit, leaves and flowers extract of *C. limetta* to flavonoids identification.

Solvent	Mobile phase	Sub-fraction	Flavonoids											
			Peel of fruit				Leave				Flower			
			Fresh		Dry		Fresh		Dry		Fresh		Dry	
			No. of	Rf value	No. of spots	Rf value	No. of	Rf value	No. of	Rf value	No. of	Rf value	No. of	Rf value
Ethanol	B1	EE					2	0.51, 0.60						
		AE					2	0.51, 0.60						
		AA							2	0.76, 0.87				
	B2	EE			2	0.42, 0.89								
		AE			2	0.40, 0.89								
		AA			2	0.44, 0.98								
		NB			2	0.42, 0.98								
	B3	EE	2	0.80, 0.95	2	0.82, 0.93	2	0.87, 0.96						
		AE	2	0.84, 0.94	2	0.77, 0.93	2	0.84, 0.96						
		AA			2	0.78, 0.97	2	0.84, 0.96						
		NB			2	0.71, 0.98								
	B4	EE			2	0.76, 0.98								
		AE			2	0.76, 0.98					2	0.70, 0.94		
		AA			2	0.71, 0.98					2	0.70, 0.94		
		NB			2	0.71, 0.98								
Methanol	B1	EE					2	0.27, 0.58	5	0.18, 0.31, 0.44, 0.56, 0.71				
		AE					2	0.27, 0.51	4	0.18, 0.44, 0.67, 0.73				
		AA							3	0.60, 0.80, 0.86				
		NB							2	0.78, 0.87				
	B2	EE			2	0.71, 0.97	3	0.20, 0.40, 0.96	2	0.40, 0.96	3	0.31, 0.40, 0.98	3	0.68, 0.86, 0.94
		AE			2	0.46, 0.97	3	0.20, 0.40, 0.96	2	0.40, 0.96	3	0.31, 0.37, 0.98	3	0.70, 0.94, 0.96
		AA			2	0.48, 0.97	2	0.40, 0.94	2	0.46, 0.94	3	0.28, 0.40, 0.94	3	0.67, 0.89, 0.96
		NB			3	0.15, 0.47, 0.97	2	0.38, 0.94	2	0.44, 0.96	3	0.30, 0.40, 0.94	3	0.62, 0.87, 0.96
	B3	EE	2	0.86, 0.97										
		AE	2	0.82, 0.98	2	0.87, 0.96	2	0.89, 0.96						
		AA	2	0.76, 0.97	2	0.71, 0.96	3	0.76, 0.84, 0.92	2	0.90,0.98				
		NB	2	0.87, 0.98			2	0.74, 0.90	2	0.90,0.98				
	B4	EE									3	0.72, 0.78, 0.97	2	0.80, 0.98
		AE									3	0.72, 0.78, 0.92	2	0.78, 0.94
		AA					2	0.80, 0.96			3	0.27, 0.82, 0.96		
		NB			2	0.84, 0.95					3	0.78, 0.96		
Water	B1	EE											3	0.71, 0.82, 0.98
		AE											3	0.71, 0.87, 0.98
		AA									2	0.29, 0.93		
	B2	EE			2	0.82, 0.96			3	0.67, 0.78, 0.96	2	0.22, 0.94	6	0.34, 0.44, 0.60, 0.74, 0.84, 0.94
		AE			2	0.80,0.98					2	0.24, 0.94	5	0.36, 0.50, 0.66, 0.80, 0.94
		AA									3	0.11, 0.22, 0.91	4	0.40, 0.53, 0.67, 0.96
		NB									2	0.22, 0.93	6	0.36, 0.49, 0.67, 0.73, 0.82, 0.96
	B4	AE									2	0.80, 0.94		
		AA									2	0.80, 0.93	2	0.74, 0.92
		NB											2	0.76, 0.90

EE: ethyl ether, EA: ethyl acetate, AA: acetic acid, and NB: n-butanol.

In one hand, alkaloids on fresh peels in water, R_f value 0.60 and 0.97 were detected; on dry peel in ethanol extract 3 spots were obtained having of R_f values 0.41, 0.63, and 0.98. On the other hand, fresh leaves in methanol extract R_f 0.66 was visible, and dry leaves in ethanol extract only one spot was detected R_f value 0.85. Finally, on fresh flower in water extract 3 spots were obtained having of R_f 0.32, 0.74, and 0.97; on dry flower in water extract R_f values 0.55 and 0.95 were visible.

Furthermore, on flavonoids results showed in table 5, on fresh peels in ethanol extract, R_f value on mobile phase B3 at EE and AE were similar 0.80, 0.95 and 0.84, 0.94 respectively; on dry peels in ethanol extract, in each subfraction on mobile phase B2, two spots were obtained having of R_f values for EE 0.42, 0.89; AE 0.40, 0.89; AA 0.44, 0.98; NB 0.42, 0.97. Likewise, similar behavior showed using methanol extract on

mobile phase B3 fresh peels, 2 spots in each subfraction, for instance, EE 0.86, 0.97; AE 0.82, 0.98; AA 0.76, 0.97; NB 0.87, 0.98. On mobile phase B2 dry peels methanol extract R_f values were for EE 0.71, 0.97; AE 0.46, 097; AA 0.48, 0.97; NB 0.15, 0.47, 0.97. Finally using water extract in fresh peels no spots were detected at any mobile phase. On the contrary, in dry peels 2 spots only were detected on B2 in EE 0.82, 0.96; AE 0.80, 0.98.

Subsequently, fresh leaves in ethanol extract mobile phase B1 and B2, identical R_f 0.51 and 0.60 were both visible in subfraction EE and AE. Meanwhile, on dry leaves in ethanol extract only two spots were detected on B1 subfraction AA, R_f value 0.76, 0.87. Regarding using methanol extract, on fresh leaves B2 and B3 several R_f values were obtained being the most significant and equal values on B2 in EE and AE 0.20, 0.40, 0.96; AA 0.40, 0.94; NB 0.38, 0.94. On B3, R_f values in AE 0.89, 0.96; AA 0.76, 0.84, 0.92; NB 0.74, 0.90. Despite on dry leaves using methanol extract values were obtained on B2 and B3. The most significant were on mobile phase B1, in EE R_f values 0.18, 0.31, 0.44, 0.55, 0.71; AE 0.18, 0.44, 0.67, 0.73; AA 0.60, 0.80, 0.86; NB 0.78, 0.87. Finally using water extract in fresh leaves no spots were detected at any mobile phase. On the contrary, in dry leaves 3 spots only were detected on B2 in EE 0.67, 0.78, 0.96.

In the same way, on fresh and dry flowers many spots were obtained. For example, on mobile phase B4 in ethanol extract, equal R_f values were in AE and AA of 0.70 and 0.94. Nevertheless, none spots were detected on dry flowers using ethanol extract. By using methanol extract several spots were detected on B2 in each subfraction. Thus, R_f values in EE 0.31, 0.40, 0.98; AE 0.31, 0.37, 0.98; AA 0.28, 0.40, 0.94; NB 0.30, 0.40, 0.94. By the same token, on mobile phase B4, EE 0.72, 0.78, 0.97; AE 0.72, 0.78, 0.92; AA 0.27, 0.82, 0.96; NB 0.78, 0.96. On dry flowers methanol extract R_f values in mobile phase B2 were in EE 0.68, 0.86, 0.94; AE 0.70, 0.94, 0.96; AA 0.67, 0.89, 0.96; NB 0.62, 0.87, 0.96**.** Last, using water extract in fresh flowers on B2 R_f values were obtained in EE 0.22, 0.94; AE 0.24, 0.94; AA 0.11, 0.22, 0.91; NB 0.22, 0.93. Moreover, in dry flowers R_f values were detected in B1, B2 and B4 as it is follows. For example on mobile phase B1 in EE 0.71, 0.82, 0.97; AE 0.71, 0.87, 0.97; B2 in EE 0.34, 0.44, 0.60, 0.74, 0.84, 0.94; AE 0.36, 0.50, 0.66, 0.80, 0.94; AA 0.40, 0.53, 0.67, 0.96; NB 0.36, 0.49, 0.67, 0.73, 0.82, 0.96; and B4 in AA 0.74, 0.92; NB 0.76, 0.90.

It was observed some of the extracts used to identify alkaloids and flavonoids were positive by TLC method. Although there is no evidence on prior literature in secondary metabolites of *C. limetta*, R_f values represent that samples had different secondary metabolites such as alkaloids and flavonoids. In the first place for alkaloids identification, the most significant outcome is that the solvent used detected number of spots for specific samples. That is to say, ethanol extract identified in dry peel fruit and leaves. Another example is methanol extract that showed only one spot in fresh leaves. Finally, water extract showed more spots in fresh peel fruit as well as fresh and dry flower. On the contrary for flavonoids identification, there was more number of spots on all dry than fresh samples in all solvent used. As a result, the presence of secondary metabolites such as alkaloids and flavonoids are related to anxiety disorders and treatment of sleep used in Mexican folk medicine.

4. Conclusion

Based on the results on phytochemical screening as well as TLC method on peel fruit, leaves and flowers, it is concluded that *C. limetta* Risso is rich in bioactive compounds. However, further studies are needed in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds responsible for sleep and anxiety disorders in this specie.

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