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Diuretic activity of lime *Citrus limetta* Risso aqueous extract in Wistar rats.

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ABSTRACT

Many of human kind diseases or illness have their solution in natural products. Plants have been use by healers and herbalists as pharmaceutics and scientists to make remedies and medicines. The Persian lime or *Citrus limetta* Risso, belongs to citric fruits family like orange, lemon and tangerine. It is a variety with more benefits to health. With respect other lemons and limes, their flavor is slightly sweet so it is nice to consume. This fruit has minerals like calcium, phosphorus, potassium, iron, and vitamins C, B_1 and B_2 and low caloric content. Also has fiber, niacin and citric acid. In this study it was evaluated the diuretic activity of aqueous extract of *Citrus limetta* lime applied to Wistar rats. Three control groups were formed: a) negative control with sodium chloride solution (0.9%), b) positive control with furosemide (20mg kg^{-1} live weight) and c) positive control with hydrochlorothiazide (10 mg kg^{-1} live weight), and two experimental groups: d) lime extract (600 mg kg^{-1} at 6 mL volume) and e) lime extract (1,500 mg kg^{-1} at 6 mL volume). The sodium and potassium concentrations were determined in accumulated urine and toxicity tests. It was observe that aqueous lime extract possess a hypo tense activity and diuretic effect at two experimental used concentrations. The diuretic activity was high with hydrochlorothiazide reference. The toxicity tests showed that lime extract do not show toxicity signs. We believe that diuretic activity can be responsible of hypo tense values in rats, so it would be interesting to continue this study to know their action mechanism.

Key words: Lime, *Citrus limetta*, diuretics, hypertension, toxicity.

RESUMEN

Muchas de las enfermedades y dolencias del hombre tienen la solución en la naturaleza. Las plantas han sido utilizadas tanto por curanderos y herbolarios como por farmacéuticos y científicos en la creación de remedios y medicinas. La lima de Persia o *Citrus limetta* Risso, pertenece a la familia de las frutas cítricas como la naranja, el limón y la mandarina, es una variedad con grandes beneficios para la salud. A diferencia del resto de limones y limas, **su sabor es ligeramente dulce,** por lo que es agradable para su consumo. Posee **minerales como el calcio, el fósforo, el potasio o el hierro, y vitaminas B1, B2 y C** y bajo contenido en calorías**. También contiene fibra, niacina y ácido cítrico. En este estudio se evaluó la** actividad diurética del extracto acuoso de la lim**a** *Citrus limetta* aplicado en rata Wistar. Se formaron tres grupos control: un control negativo con solución de Cloruro de sodio 0.9% y dos positivos, uno con furosemida a una dosis de 20 mg kg^{-1} de peso vivo y otro con hidroclorotiazida a una dosis de 10 mg kg^{-1} de peso vivo y dos grupos experimentales utilizando extracto de lima, a una dosis de 600 mg kg^{-1} de peso vivo y otra de 1,500 mg kg^{-1} de peso vivo en un volumen de 6 mL. Se determinó la concentración de sodio y potasio de la orina acumulada y se hicieron pruebas de toxicidad. Se observó que el extracto acuoso de lima posee actividad hipotensora y que posee un efecto diurético en las dos concentraciones utilizadas. Que la actividad diurética es alta, tomando como referencia a la hidroclorotiazida. Las pruebas de toxicidad mostraron que el extracto no presenta signos de toxicidad. Se cree que la actividad diurética podría ser la responsable de la hipotensión, por lo que sería interesante continuar el estudio para conocer su mecanismo de acción.

Palabras clave: Lima, *Citrus limetta*, diuréticos, hipertensión, toxicidad.

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INTRODUCTION

Plants have used for traditional medicine as remedies to treat different pathologies because they possess healing properties. Aktar et al. (2013) mentioned in a video distributed by World Health organization (WHO) that a medicinal plant is the one that contains substances that can used for therapeutic purposes or as a precursor for a drug synthesis helpful to cure a specific disease. According this definition, the *Citrus limetta* Risso lime must considered medicinal plant for their anti-hyper tense activity, anti-microbial and antioxidants, among others.

The *C. limetta* lime is a fruit of Rutaceae family, originally from Asia. It lives in warm, semi warm and temperate weather, between 200 to 2,000 meters from sea level. It is cultivated in Mexican Republic at different States like Guanajuato, Jalisco, Michoacan, Puebla and Hidalgo, among others. It is swon in homegardens in extensive production way for their commercialization.

The lime tree can measure eight meters high, their branches have very sharp spines. Have little white flowers. The leaves are lightly wavelike between 8 to 20 mm lengths with winged petiole. The fruits are round green-yellow, with a little protuberance at cicatrix region of what was once the ovary (Fig. 1). Measure up to 6 cm diameter and their pulp is abundant (Argueta-Villamar et al. 1994).

The lime *C. limetta* has been amply study and the results have been publish in scientific literature. Studies have made about pulp, pericarp or vessel seed, fruit juice and leaves; most abundant isolated compounds were flavonoids (Pérez et al. 2010; Aktar et al. 2013; Pérez-Nájera et al. 2013; Rodríguez-Rivera et al. 2014).

The flavonoids are low molecular weight organic molecules, present in plants. They are not nitrogen vegetables pigments classified like polyphenols; count oneself that these compounds confer to plants protection to ultraviolet radiation. In citrus fruit peel, seed were detected proximally 85 different molecules of these polyphenols like O-glucosides, flavone-C-glucosides, flavonol-O-glucosides, limonoid-O-glucosides, derivatives of abscisic acid, cinnamic acid and dehydro-cinnamic-glucosides (limocitrol and limocitrin derivatives), some of them were first time detected in lime (Rodriguez-Rivera et al. 2014). Meanwhile, Pérez-Nájera et al. (2013) isolated phenols content in methanolic extract and determined the quercetin, hesperidin and naringin, these last two in high concentration.

Fig. 1: *Citrus limetta* (Risso) from Vigia, State of Morelos.

From juice lime it have been isolated flavonoids like vicenin-2, lucenin-2-4'-methyl ether, eriocitrin, scoparin, orientin-4-methyl ether, rhoifolin, diosmin y hespiridin (Barreca et al. 2011), in addition to vitamin C (0.2 mg mL^{-1}), total sugars (0.566 mg mL^{-1}) and citric acid (Juárez-Consuelo et al. 2005).

Other researches have dedicated to study the biological activity of lime *C. limetta* compounds and they found that essential oils have antimicrobial and antioxidants properties (Javed et al. 2013) and the ethanolic extract citrus peel has cytotoxic effect that was proved in vivo with *Artemia salina* model (Aktar et al. 2013). Kundusen et al. (2011) studied the methanolic extract of vessel seed over diabetic rat model induced with streptozotocin, obtaining a significant decrease in blood glucose levels. The lime

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leaves have antihypertensive activity, throughout angiotensin II inhibition (Pérez et al. 2010, Talha et al. 2011, Esquivel-Gutiérrez et al. 2012). The juice show antioxidant activity (Barreca 2011) like vessel seed too (Pérez-Nájera et al. 2013). Otherwise, Szent-Gyorgy (1938) was one of the first researches to propose that flavonoids have effect over capillaries fragility, making them more resistant due to presence of called vitamin "p".

Today it can founded in scientific literature a considerable amount of works related to flavonoids biological activity like: antioxidants, anti-inflammatory, anticancer, anti-immune, antimicrobial, antihypertensive and anti-atherosclerotic.

The hypertension count oneself an organism's homeostasis failure, which can generate cardio vascular diseases. According to literature a 90% of all cases with this pathology presents in primary way (idiopathic origin) and remaining 10% as secondary type to a disease, which can endocrine or cardiac type. The hypertension was consider like risk factor of atherosclerosis because pressure increase in arteries, damage the endothelial vessels cover and stimulate the formation of atherosclerotic plaques, besides increase the heart tension afterload.

Many hypertension treatments were supporter in cardiovascular physiology. There are drugs denominated calcium channels blockers; the beta blockers acting over β_1 receptors decrease stimulant activity of catecholamine over blood volume; The enzyme inhibitors of angiotensin converter (IECA), antagonist of angiotensin II (ARA II) receptors and low doses which reduce blood volume and decrease systolic and diastolic blood pressure (5-10 mmHg) (Silverhorn-Unglaub et al. 2009; Esquivel-Gutiérrez et al. 2012).

With this background and the observed in Morelos State, for tradition, people take infusion of lime peel to decrease blood pressure, was decide to begin a study to know the diuretic activity of aqueous extract of *C. limetta* peel in Wistar rats, considering that this compound can be the way to explain the decrease of blood pressure.

MATERIAL AND METHODS

Plant

The *C. limetta* lime was collect in Vigia town, 10 km from San Felipe Neri, Morelos State region, during months of January to July of 2015.

Elaboration of aqueous extract of C. limetta

C. limetta peel was dried and minced previously to take 52.3 g and was introduce in a beaker of 1 L capacity. Boiling tap water was add to cover minced lime peel and let stand during 20 minutes. Then, the solution was filter by gravity with conical funnel and filter paper and placed in 500 mL beaker. This step was made triplicate. The extract (35 mL) was placed in 100 mL vials and settled in a freezer (- 70° C). Until samples were freeze, samples were place in LABCONCO (Freeze Dry System, Freezone 12) equipment, at -53°C and level 2 vacuum. The remaining time in those, conditions were the necessary (proximately 13 days) until vials did not have ice or liquid, only aqueous extract (powder state).

Animals

Fifty males Wistar rats were used, produced in Animal Production and Experimentation Laboratory-Bioterio Unit (APEL-B) from Universidad Autónoma Metropolitana-Xochimilco from Winstar strain, with 170-180 g weight, under lighting (12:12 light;darkness) and water controlled conditions, fed with Purina® *ad libitum*, 21±2°C temperature; 55 % humidity. The organisms were random distributed in five groups with 10 rats each one.

Experimental design

Three control groups formed with 10 organisms each:

I. Negative control: Sodium chloride solution (0.9%), administrated 40 mL kg^{-1} (*in vivo* weight).

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II. Positive control: Furosemide administrated at 20 mg kg^{-1} doses (*in vivo* weight). The furosemide is loop of sulfonamide family used in edema treatment associated to cardiac congestive insufficiency, cirrhosis and renal disease, including nephrotic syndrome. Also was use in mild or moderate hypertension and as adjuvant in hypertension crisis and acute pulmonary edema. The furosemide was use for hypercalcemia treatment also. Belongs to diuretic high ceiling group. (Vademecum, 2010).

III. Positive control: Hydrochlorothiazide administered in 10 mg kg^{-1} doses (*in vivo* weight). This compound is a thiazide diuretic used for edema and hypertension treatments. In this case, used only as initial treatment or associated with other ant-hypertensives. The hydrochlorothiazide was use associated with beta-blockers, calcium antagonists, enzyme inhibitors antagonist's convertors of ECA. Also was use for insipidus diabetes and hypercalcemia, as well as the edema associated to premenstrual syndrome (Vademecum 2013).

Two experimental groups were form, maintaining same 10 organisms for each batch, and lime aqueous extract was add in different doses:

I. A 600 mg kg^{-1} (in vivo lime extract) was add in 6 mL volume.

II. A 1,500 mg kg^{-1} doses (in vivo lime extract) was add in 6 mL volume.

The organisms were place in cages and maintained during 10 days previously experimental tests for their adaptation. They were deprive of their food during 18 hours and drinking trough one hour before start experimentation (Pérez-Machin et al. 2011).
After organisms were treated, were place in metabolic cage and allow drink water during experimentation. Urine volume was registered 1, 2, 3, 4, 5, 6 and 24 hours after extract and controls were applied. Concluded experiment, euthanasia was apply to organisms using a CO_2 chamber.

Sodium and potassium concentrations were register from all five experimental groups collected from urine during 24 hours. Registered was made in Clinical Analysis Laboratory from Veterinary Faculty in Pathology Department from Universidad Nacional Autónoma de México.

Toxicity tests

Acute toxicity of extract was determined according guidelines for chemical testing (OECD, 2008): test No. 420 (Acute toxicity test). For this test it were used 20 males mice, with 25-28 g weight, CD-1 strain, produced in Animal Production and Experimentation Laboratory-Bioterio Unit (APEL-B) from Universidad Autónoma Metropolitana-Xochimilco.

The organisms were distributed in four groups (n= 5). The aqueous extract was applied oral via with a metallic cannula. The doses were 5, 50, 300 y 2 000 mg kg^{-1} (*in vivo* weight). The organisms were in observation during 14 days, where mortality and behavior were registered. The organisms were weight at day 7 and 14 after aqueous extract was apply. At end of experiment, necropsies done to organisms slaughtered in gas chamber.

Evaluation of extract diuretic activity

The urine excretion, diuretic action and activity were calculate with equations described in literature (Pérez-Machin et al. 2011).

Formulas used for calculus of variables related to diuresis:

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$$\text{Urine excretion} = \frac{\text{Produced Urine}}{\text{Physiological solution administered}} \text{ x } 100$$

$$\text{Diuretic action} = \frac{\text{Urine excretion of treated group}}{\text{Urine excretion of control group}} \text{ x } 100$$

$$\text{Diuretic activity (DA)} = \frac{\text{Diuretic action}}{\text{Diuretic action drug}}$$

Scale = High = DA≥ 0.90, Moderate =DA (0.89-0.70), Low= DA (0.69-0.50), None= DA ≤ 0.50

Statistical analysis

The data was processes with All pairs test, Tukey HSD with statistical program JMP v.8.

RESULTS AND DISCUSSION

Produced urine volumes by rats of the five experimental groups increased gradually (Fig. 2 and 3), being higher with furosemide, hydrochlorothiazide and with lime peel extracts that those that received physiological solution (0.9%) of NaCl (negative control). Comparing all urine volumes of all groups, the highest values was positive control with furosemide. The diuretic effect was very irregular during first two hours and third urine excretion was uniform. The difference of diuretic activity initiation of these substances can related with gastrointestinal absorption characteristics of the active ingredients; however, it was observe a faster response than controls treatments. Urine volumes of extract and hydrochlorothiazide groups tend to be similar from third hour until sixth. At 24 hours, we observed that 1,500 mg km^{-1} weight group tend to have high diuresis above the 600 mg kg^{-1} weight and hydrochlorothiazide groups (Lahlou et al. 2007).

Compared with the two doses of lime peel extract, 1,500 mg kg-1 weight dose show a highest urine volume without following a linear relation, compared with 600 mg kg^{-1} concentration.

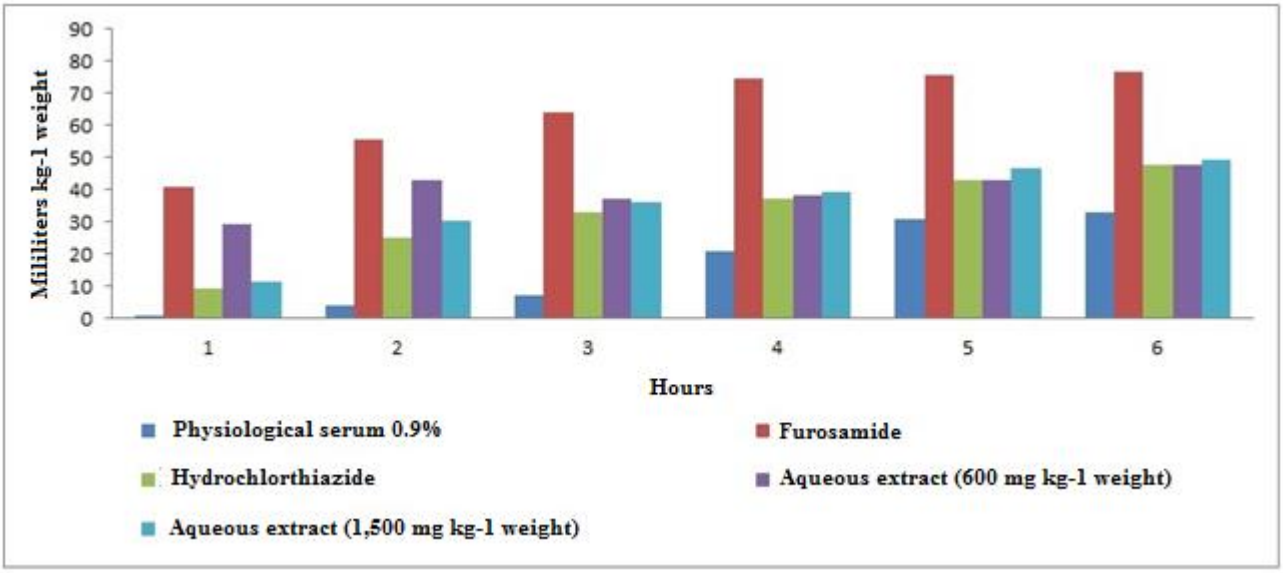

Fig.2: Accumulate urine volume at 6 hours (mL kg^{1}).-

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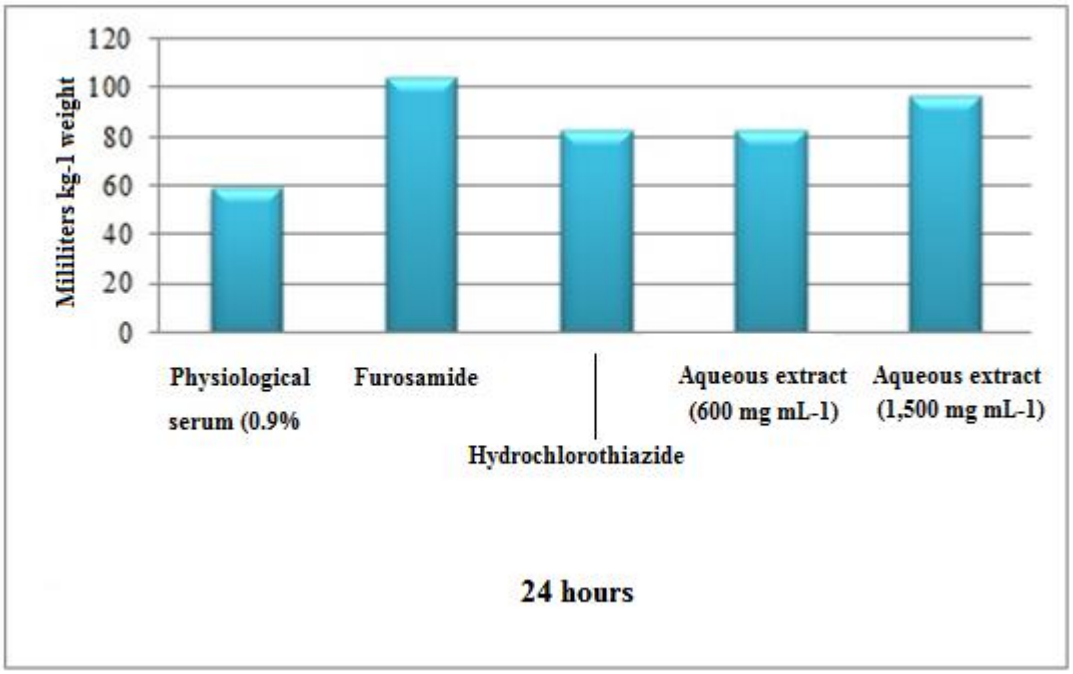

Fig.3: Urine volumes of experimental groups.

With respect to statistical tests applied to diuresis results, between treated groups with lime peel extract (600 and 1,500 mg kg^{-1} weight) and furosemide and physiological serum controls, we observed that after six hours it shows significant differences (Table 1). As can inferred that extracts respond equally to positive control with hydrochlorothiazide.

On the other hand, there are significate differences ($P<0.05$) between groups lime peel 1,500 mg kg^{-1} weight with respect to hydrochlorothiazide and physiological serum at 24 hours.

Diuresis has two components: a) increase of urine volume (water excretion) and b) Solutes loss (electrolytes) in urine (Lahlou et al. 2007). These process were produced by water tubular renal suppression and electrolytes circulation, The furosemide acts from ascendant section of intestinal lumen (loop of Henle), blocking in selective form the cotransporter $Na^+/K^+/2Cl^-$ and therefore, water reabsorption and electrolytes, produce an increase of Na^+ (25 %) and Cl^- elimination, accompanied of K^+

Table 1: Effect of aqueous extract of *C. limetta* over diuresis in white rat Winstar strain.

Treatment	6 hours	24 hours
Physiological serum (0.9 %)	32.95 ± 0.6^c	59.09 ± 0.8b
Hydrochlorothiazide	47.73 ± 1.8^b	82.38 ± 1.8b
Furosemide	76.7 ± 1.8a	104.55 ± 4.3a
Lime 600 mg kg^{-1} weight	47.73 ± 1.6b	82.28 ± 3.4ab
Extract 1,500 mg kg^{-1} weight	49.43 ± 2.2b	96.59 ± 4.9a

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elimination and increase of aldosterone activity. The diuretics thiazide (hydrochlorothiazide) act at initial portion of distal convoluted tubule where inhibit the cotransporter Na^+ Cl^- and increase the renal excretion of both ions. The result is a moderate excretion of Na^+ (5 – 10%), Cl^- and water; increasing the elimination of K^+ (Morales-Olivas 2013) (Table 2).

To evaluate diuretic activity of aqueous extract of lime peel results shown in Table 3. The reference values were positive control with furosemide and hydrochlorothiazide. To check values, scale was show. It was observe that with two doses and at sixth and 24 hours, positive results obtained. The 1,500 mg kg-1 weight concentration has highest effect at 24 hours compared with furosemide treatment (0.92). On the other hand, when hydrochlorothiazide treatment was take as reference, two doses at sixth and 24 hours presented high levels, because values are bigger than one. Therefore, diuretic activity from lime peel is equal at two positive controls that suggested that this plant has diuretic activity, need to know at what level of kidney it acts, so it is important to continue its study.

The positive controls were select based on different action mechanisms that two diuretic types have and used to know diuretic activity from any plant. These results were similar to those founded in plant studies like: *Tropaeolum maju* (*G*asporotto et al. 2009), *Polyporus umbellatus* (Zhang. 2010), *Carum carvi* and *Tanacetum vulgare* (Lahlou), *Smilax canariensis* (Abdala et al. 2008) considering the diuresis increase.

Phytochemical studies of polar extracts (aqueous and methanol) lime peel have been made and were found flavonoids. Most reported were hesperidin and naringin, both at 90%, unlike no polar

Table 2: Aqueous extract effect of *C. limetta* over urine excretions of Wistar strain rat.

Treatment	Urine excretion	Diuretic action	Urine excretion	Diuretic action
Physiological serum (0.9 %)	96.6		173	
Lime extract (600 mg kg^{-1})	140	1.44	242	1.4
Lime extract (1500 mg kg^{-1})	144	1.49	283	1.64
Furosemide 20 mg kg^{-1}	225	2.33	306	1.77
Hydrochlorothiazide 10 mg kg^{-1}	137	1.42	187	1.08

Table 3: Diuretic activity evaluation of *Citrus limetta.* Scale: High: AD≥ 0.90, Moderate: AD (0.89 - 0.70), Low: AD (0.69 - 0.50), None: AD <0.50.

	Lime extract lima (6 h)		Lime extract lima (24 h)	
Diuretic activity	(600 mg kg^{-1})	(1500 mg kg^{-1})	(600 mg kg^{-1})	(1500 mg kg^{-1}
Furosemide	0.62	0.64	0.78	0.92
Hydrochlorothiazide	1.02	1.05	1.29	1.51

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extracts like hexane, where these type of molecules were not find (Pérez-Nájera et al. 2013). In this research it was used the aqueous extract, that according to mentioned in literature, it contain flavonoids and these were reported in plants with diuretic activity (Trease y Evans 1987, Abdala et al. 2008). These types of compounds increase the renal circulation, also glomerular filtration rate, which promote urine formation (Abdala. 2008). As it can inferred that diuretic effect of aqueous extract was promote by these molecules.

By measuring the sodium concentration in rat's urine after 24 hours (Table 4), it was find that there are non-significant differences between experimental groups and controls. This is important because furosemide considered as potent diuretic that why can used like a very efficient antihypertensive because it has rapid absorption and action when it is oral applied and appears in plasma between 0.5 to two hours.

The diuretic hydrochlorothiazide is absorbed orally. This action decrease in people who have heart failure, their effect began after one hour and appear in plasma between 1.5 to four hours. For their action mechanism, it was eliminate more potassium than sodium and bicarbonate, decreasing calcium elimination. This type of diuretic has an action range medium to prolonged effect, so it can produce hypopotassemia, also hyponatremia, hypochloremia and hyperglycemia (Esparza y Diez 1990).

Table 4 show that lime extract in both concentrations has a natriuretic effect. This means that sodium was excrete in high concentration than potassium in urine. This was observed when ratio between Na/K^+ was calculated in 24 hours urine of experimental rats. The ratio was higher at 1,500 mg kg^{-1} concentration. With respect to potassium concentration in urine, significant differences ($P<0.05$) exists between saline solution group and 600 mg kg^{-1} weight group. At 1,500 mg kg^{-1} weight concentration, the results show likeness to those obtained with two positive controls, they behave like diuretic thiazide and furosemide.

On the other hand, in toxicity studies after necropsy to experimental organisms, it was observed that lime peel extract at proved doses did not produced tissue damage in rats and therefore is not toxic, so it can be ingested, in traditional medicine without problems.

A clinic study made with aqueous extract of *Citrus limetta* leaves, to prove antihypertensive effect in a 140 people and founded that after it was apply during six weeks, the blood pressure decrease in 90% of experimental group. The hydrochlorothiazide used as positive control (Cano 2011).

Therefore, take lime peel infusion was an option to control hypertension in places where people cannot buy their medicines so they do not start or leave their pharmaceutical treatment. Moreover, another important activity founded in lime peel was to avoid platelet aggregation through inhibition of cyclooxygenase enzyme and thus prevents thrombosis (Perez et al. 2010).

CONCLUSIONS

- The aqueous extract of lime has a diuretic effect in both used concentrations.

Table 4: Sodium and potassium concentration (mM L^{-1}) of purine after 24 hours from each experimental groups.

Treatment	Sodium	Potassium	Na K^{-1} media
Physiological serum (0.9 %)	153.33±11.5	86.67±3.51^{b}	1.77
Hydrochlorothiazide	139.67±2.52^{a}	126.67±0.53^{ab}	1.1
Furosemide	131.33±16.5^{a}	123.67±9.5^{ab}	1.06
Lime 600 mg kg^{-1}	131.33±16.5^{a}	135.4±12^{a}	1.07
Lime 1,500 mg kg^{-1}	131.33±16.5^{a}	102.6±14^{ab}	1.53

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- The diuretic activity was high, with hydrochlorothiazide as reference.
- The flavonoids like hesperidin and naringin founded in lime peel can be the responsible molecules of diuretic activity (Tripoli et al. 2007).
- Separation with chromatographic studies could isolate molecule or molecules responsible for this activity.
- Studies have been published (Argueta et al. 1994, Pérez et al. 2010; Cano 2011; Bidham and Remadevi 2014) where it reported hypotensive activity of lime peel extracts and according with obtained results in this study where reported that diuretic activity must be responsible of this effect, so it would be interesting to continue the study to know mechanisms action.
- Something very important that was find in this study is that aqueous extract of *C. limetta* is not toxic, according evaluation tests results.

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