CHARACTERIZATION OF SOME BIOACTIVE COMPOUNDS FROM CITRUS LIMON (L.)
AND EVALUATING THE BIOLOGICAL EFFECTS ON THYROID HORMONE AND
CELLULAR TOXICITY

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ABSTRACT

The present study was carried out to estimate the vital effects of the water and alcohol extract of Citrus limon (L.) seeds in thyroids hormone levels secretion in adults female Albino rats. Plant Seeds was extracted with distilled water and alcohol using ethanol. Then phytochemical compound detected using classical methods and thin layer chromatography (TLC).

animal’s groups were injected with L-Thyroxine and plant extracts. Blood samples were collected from all animals after the end of the trial time of 10 days.

The results of study showed that the water and ethanol extract contain many bioactive substances, including carbohydrates, tannins, glycoside, saponin, terpenes, alkaloids and phenols. Meanwhile, Glycosides detection by (TLC) plate from water and alcohol extracts of C. limon showed 4 and 5 bands using the BAW (4:1:5) solvent system respectively, 4 and 5 bands with alkaloids, showed 4 and 5 bands of terpenoids compound, 4; 5 spots with tannins compounds were observed.

Key words: Citrus limon (L.); Cellular toxicity; Thyroid hormone

INTRODUCTION

In old years, medical plant and their secondary metabolite have been an important purpose, in several applications overlap with human and animal help to design new therapies such as pharmaceutical, drugs and natural cosmetic. The World Health Organization (WHO) reported that about 70-80% of the world populations, depend on natural products in their primary health care (Pal and Shukla, 2003; Singh, 2012). The discovery of new medicinal drugs manufacturer from plant secondary metabolite which have efficient protection and have roles against different type of diseases, including cancer, Alzheimer’s disease, antihypothyroidism and antioxidant activity (Roy et al., 2013; Jegadeswari et al., 2012, Dhongade and Chandewar, 2013). These compounds consist of alkaloids, flavonoids, saponins, tannins, phenols, pigments, enzymes, vitamin C, vitamin E, carotenoids and minerals (Madhuri and Pandey, 2009; Aberoumand and Deokule, 2010; Al-Sabary et al., 2017). C. limon plant is considered as an herb and a spice which contributed in health defense for humans. Seeds are used to as conventional treatment anti-diuretic agents had both insulin-releasing and insulin-like activity (Alison et al., 1999), cholesterol reducing and anti-inflammatory (Chithra and Leelamma, 1997), stimulant, stomachic, aphrodisiac and analgesic (Chaudhry and Tariq, 2006).

Several studies focused at the efficacy of anti hypothyroidism agents can be improved by combining them with crude plant extracts. This study investigation is to evaluate the bioactive compounds (Phytochemical) present in all extracts from C. limon seeds and studies the biological effect on the thyroid hormones levels under hyperthyroid inflammatory in female rat induced by L-Thyroxine drug.

MATERIALS AND METHODS

Citrus limon Seeds Extract Preparation: Fresh lemon fruits of C. limon were collected from local market in August 2017 and were washed well using tap water. Seeds were sprayed with ethanol (70%) to prevent degradation by the enzymatic degradation, then dried at room temperature (25°C) and were crush using electric blender, then powders of plants seeds were prepared to extract biological active compounds by ion methods according to reported method of Patil et al., (2012) and stored in the refrigerator.

Alcoholic and water seeds extraction: Twenty gram of C. limon seeds powder was extracted with 200 ml ethanol solvent (90 %) and with distilled water (Patil and Patil, 2012) respectively and placed on the plate magnetic stirrer for the purpose of mixing and left for 24 hours. The solution of each extracts was filtered through clean cloth and then filtered by use filter paper Whatman No.1 and then the filtrate centrifuged at 3000 r/min for 10 minutes for the purpose of separation of sludge and get a pure extract. Then the volume of alcoholic extract was reduced to then evaporated by rotary evaporator at 45°C to reduced volume to 40 ml then to form a paste by evaporative methods. The percentage yield of the extract was calculated and was...
placed in the refrigerator for use later (Patil and Patil, 2013).

**Preliminary Qualitative Chemical Detection:**

**Thin Layer Chromatographic (TLC):** TLC chromatography was carried out according to the method of Mritunjay et al., (2011) to separates of bioactive contents in *C. limon* seeds extracts. Silica gel 60 F254 plates were activated at 100°C for 10 minutes in an oven and cooled at room temperature. Where 2 ml from crude extract were took and concentrated as spots on silica gel plates. Then plates were placed in gar container contain the mobile phase and covered provisions, after the arrival of the solvent to approximately the end of plate, then separated spots sites was identified with the naked eye and then under the ultraviolet rays (UV) at 366 wavelengths. Relative flow (Rf) calculated from below equation: \( R_f = \frac{a}{b} \) where a is compound moves/distance solvent front (Harborne, 1984).

**Preparation of Laboratory Animals:** About 12 adult albino female sex rats weighing between 130 and 150 g and age between 8-10 weeks and kept in the animal house of the Faculty of Science / University of Kufa at temperature 23-28°C with 50 ± 5% humidity and lighting (11 hours of light and 13 hours of darkness), and have received the same diet. The animals were allowed free access to pellets and drink water.

**Groups of experimental animals:** The experimental animals were divided randomly into six groups by 3 females animals in each group and, as in the below:

- **Group (1):** were left dealing with natural food and water were considered as healthy control group.
- **Group (2):** Animals rats were given an oral dose of (20µg/kg b.w) L-thyroxine once daily for four weeks to induce hyperthyroidism, given distilled water only for 10 days and was considered control infected (hyperthyroid).
- **Group (3):** Hyperthyroid rats were received aqueous extract of the *C.limon* seeds at 200mg / kg of body weight single dose daily for 10 days.
- **Group (4):** Hyperthyroid rats were received alcoholic extract of the *C. limon* seeds at 200mg / kg of body weight single dose daily for 10 days.

**Stock solution of extracts in animal orally:** Extract sample were prepared by dissolved 2g of extracted plant material in 30ml of normal saline to prepare a stock solution, which is used to prepare concentrations 200mg /kg of both aqueous and alcoholic extract of *C. limon* seeds according to following equation

**Cellular toxicity [Lethal dose 50 (LD50)]:** Cellular toxicity of *C. limon* seeds extract was conducted according to the method of Al-Ali et al (2008). Alteration in the animal behavior, death-rate and toxicity signs and weight animals were recorded.

**Statistical analysis:** Statistical analysis were carried out using SPSS version 20, where data were expressed as the Means, Standard deviation, one – way of variance (ANOVA) was used to compare the results of all groups, P value (p<0.05) was considered statistically significant.

**RESULTS AND DISCUSSION**

Detection of the phytochemicals in *C. limon* seeds extract by (TLC): TLC Chromatography has good potential analysis for raid detection of diverse types of bioactive compounds with the possibility of multiple phytochemicals detection and uses for different extracts and solvent types.

**Alkaloids compounds detection in *C. limon* seeds:** Figure 1A and B shows the characteristics of alkaloids compounds present in of ethanolic crude extract of *C. limon* seeds revealed that contain five spots with relative flow (Rf) equal to (0.16, 0.51, 0.81, 0.86, 0.95) products were detected by UV 254 nm which gave (blue, pink, blue, brown and pink color) respectively, while these spots gave (dark purple, red, red, yellow and dark purple) respectively when seen by the eyes after spray the TLC plate with Dragendorff’s reagent. While. The water extract contains also five spots with (Rf) equal to (0.16, 0.46, 0.81, 0.86, 0.95) were detected by UV 254 nm which gave also (blue, pink, blue brown and pink color) respectively, while these compounds gave (dark purple, red, red, yellow and dark purple) respectively when seen by the eyes after spray the TLC plate with Dragendorff’s reagent. The phytochemical investigations indicated that alkaloids compounds were found in ethanol alcohol extract of *C. limon* seeds which is proved by Dragendorff’s and Mayer tests and TLC, this result agrees with the result mentioned by Tiwari et al., (2011). Study by Kumar et al., (2014) investigated Rf values for β-carotene in *Citrus limonum* and *Citrus sinensis* peels and obtained 0.91 and 0.92 Rf respectively. However, the present study agrees with study of Kumar et al., (2014), which found that Alkaloids compound are not present in Citrus limonum peels.

**Glycosides compounds detection in *C. limon*:** Figure 1A and B shows the characteristics of glycosides compounds revealed that contain three glycosides compounds with relative flow (Rf) equal to (0.22, 0.73, 0.96) products were detected by Uv 280 nm which gave (gray, blue and white color)
respectively, while these compounds gave black, red and blue respectively by visible light after spry the TLC plate with reagent. The water extract contains also three glycosides compounds with relative flow (Rf) equal to 0.21, 0.78, 0.97 were detected by UV 280 nm which gave brown, blue and white color respectively, while these compounds gave yellow, red and milky white respectively by visible light after spry the TLC plate with. Mathew et al., (2012) reported that alcoholic extract (ethanol) of C. limon containing carbohydrates, saponins, tannins, fixed oils, cardiac glycosides, steroids, phytosterols, phenols, flavonoids, amino acids and proteins.

The glycosides compounds are found in both types of extract of C. limon seeds which proved by both Bendict test and TLC, this result agrees with that result mentioned by Tiwari et al., (2011) and Kulkarni et al., (2012) who found that both types of extracts have ability to extract the glycosides compounds and may be due to the solvents regard as universal solvents and these compounds are easily dissolved in this high polarity medium (Houghton and Raman, 1998; Cowan, 1999).

**Phenolic compound detection in C. limon:** Figure 1A and B shows the characteristics of Phenolic compounds that found in crude of alcoholic extract of C. limon seeds revealed that contain six spots with relative flow (Rf) equal to 0.14, 0.26, 0.46, 0.86, 0.98 and products were detected by UV 280 nm which gave white, light blue, white, pink and light brown color respectively, while these compounds gave blue, blue, red and dark brown by visible light after spry the TLC plate with reagent. The water alcohol extract gave negative result. The phenols compounds are found only in water extract of C. limon seeds which proved by both ferric chloride (1%) test and TLC, this result agrees with the result mentioned by Tiwari et al., (2011) and Gupta et al., (2012). These results are due to the enzyme polyphenol oxidase, which degrade polyphenols in ethanolic extracts, whereas in water extract they are inactive, moreover the ethanolic solution is more efficient in cell walls and seeds degradation which have medium polar character and cause polyphenols to be released from cells (Lapornik et al., 2005).

**Terpenoids compounds detection in C. limon:** Figure 1A and B shows the characteristics of terpenoids compounds that found in crude extract of ethanol extract of C. limon seeds revealed that contain seven terpenoids compounds with relative flow (Rf) equal to 0.05, 0.08, 0.12 , 0.31, 0.40, 0.64 and 0.95 products were detected by UV 280 nm which gave light red, dark blue, yellow, light blue light blue, blue and light brown color respectively, while these compounds gave red, purple, orange, blue, blue, brown respectively by eye after spry the TLC plate with reagent. The water extract gave also two compounds with (Rf) equal to 0.31 and 0.95 products were detected by UV 280 nm which gave also light blue and blue color respectively, while these compounds gave blue, blue respectively by eye after spry the TLC plate with reagent.

Asha and Ahirwar (2015) applied TLC Chromatography to indicates of terpenoids compound from Acorus alamus Linn found that Rf values about 0.68, 0.7, 0.79 and 0.81 refers to the presence of terpenoids Terpenoids in both extracts which by precipitation test like Liebermann’s Burchard reaction and thin layer chromatography (TLC), this result agrees with that result mentioned by Tiwari et al., (2011) and Kulkarni et al., (2012).

**Essential oils compounds detection in C. limon:** Figure 1A and B shows the characteristics of essential oils compounds that found in crude extract of ethanol in C. limon seeds contain five spots with relative flow (Rf) equal to 0.21, 0.40, 0.75, 0.89, 0.96 respectively. These products were detected by UV 280 nm which gave light blue, bright blue, blue, blue and blue colors respectively, while these compounds gave blue, blue, blue, blue, eye by after spry the TLC plate with reagent. The water extract also contains five spots with relative flow (Rf) equal to 0.23, 0.37, 0.66, 0.90, 0.96 respectively. These products were detected by UV 280 nm which gave light blue, bright blue, blue, blue and blue colors respectively, while these compounds gave blue, blue, blue, blue, eye by after spry the TLC plate with reagent. Several studies showed that C. limon are rich sources of essential oils, protopine and, lactons, acyclic sesquiterpenes, hypericin and pseudohypericin compounds (Keles et al., 2001).

**Steroids compounds detection in C. limon:** Figure 1A and B shows the characteristics of steroids compounds that found in crude extract of ethanol extract of C. limon seeds revealed that contain three spots with relative flow (Rf) equal to 0.06, 0.21, 0.34 respectively. These products were detected by UV 280 nm which gave light brown, brown and light blue colors respectively, while these compounds gave orange, red, dark blue by eye after spry the TLC plate with reagent. The water extract of C. limon seeds revealed that contain four spots with relative flow (Rf) equal to 0.06, 0.21, 0.34, 0.82 respectively. These products were detected by UV 280 nm which gave light brown, brown and light blue colors respectively, while these compounds gave orange, red, blue, blue by eye after spry the TLC plate with reagent. Extr-
extract with solvent ethanol shows higher antimicrobial activity against tested microorganisms in comparison with the extracts of *C. limon* peel in other solvents like methanol and acetone.

**Figures 1:** Detection of compounds in *C. limon* seeds by TLC technique. A: *C. limon* water extract; B: *C. limon* alcoholic extract

**Effect of L-Thyroxine drug and extract of *C. limon* seeds in thyroids hormone levels:** Data in the table-1 refer to the healthy groups G1 and G2 refer to hyperthyroid rats induced by orally L-thyroxine drug (control). G3 refer to group that orally received water extract of *C. limon* plant and L-thyroxine drug and their effect on T3, T4 and TSH hormones. G4 refer to group that orally received alcoholic extract of *C. limon* plant and L-thyroxine drug and their effect on T3, T4 and TSH hormones respectively. Previous study refers to all factors and periods of oral administration (10 days) of two types of extracts (water and alcoholic extract) in compared with the control group.

Results in the same table showed that G2 control groups had significant increase (p<0.05) in T3 levels 1.92±0.015 nmol/l compared with 1.8±0.333nmol/l in healthy groups G1. G3 groups had significant decrease (p<0.05) in T4 level 1.80±0.008 compared with 1.92±0.015 nmol/l in control groups and had no significant differences (P< 0.05) in compared with healthy groups 1.8±0.333. However, G4 group had significant decrease (P < 0.05) in T4 hormone levels 1.84±0.008 nmol/l in compared with control groups and present significant differences (P < 0.05) in compared with healthy groups.

The results in the same table also showed there was a significant difference (P < 0.05) in T4 hormone levels of all experimental groups when compared the healthy group and orally received with L-thyroxine (Control) and with G3, the group that was orally received with L-thyroxine and water extract of *C. limon* plant) and G4, the group that was orally received with L-thyroxine and alcohol extract of *C. limon* plant). Results showed that was a significant increase (P< 0.05) for T3 hormone levels in G4 groups 1.84±0.008 in compared with control groups 1.92±0.015nmol/l also showed significant differences (P < 0.05) in compared with healthy groups 1.8±0.333.

Thyroxin T4, the results in the table (1) showed that there was a significant difference (P < 0.05) for all groups when compare the group G1 healthy groups and no significant difference (P < 0.05) with the control group (the group that was orally received with L-thyroxine) that has a significant increase (P < 0.05).

Results in the same table showed that G2 control groups had significant increase (p<0.05) in T4 levels 47.56±0.688 nmol/l compared with 46.9±1.285 nmol/l in healthy groups G1. Group G3 had significant decrease (p<0.05) in T4 level 44.36±0.608 compared with 47.56±0.688 nmol/l in control groups and had significant decrease (P < 0.05) in compared with healthy groups 46.9±1.285 nmol/l. However, G4 group had significant decrease (P < 0.05) in T4 hormone levels 44.36±0.384 nmol/l in compared with control groups and present significant differences (P < 0.05) in compared with healthy groups.

For the thyroid stimulating hormone TSH, the results in the same table showed that there was no significant difference (P > 0.05) in controls group G1 0.05±0.005 when comparing the group G2 0.06±0.005 (the group that was orally received with L-thyroxine and present significant difference between G3 0.07±0.003 with the control group G1 and that has no significant difference (P < 0.05) with healthy groups. However, G4 groups 0.05±
0.005 has a significant decrease (P < 0.05) with healthy groups and control groups.

This study reported that all types of experimental animal groups except G1 groups effected by all types of seeds extract of C. limon. Present significant decrease in T3 and T4 thyroids hormone levels in comparing with effect of L-thyroxine drug which increase of T4 and T3 levels and appear to parallel levels of these hormone with healthy groups. The results showed there was no significant difference (P < 0.05) T4 and T3 for when comparing the group that was orally injected with L-thyroxine and water extract of C. limon plant and showed there was significant difference (P < 0.05) when comparing the control group with groups G3 (the group that was orally received with L-thyroxine and water extract of C. limon plant) and showed there was significant difference (P < 0.05) T4 and T3 for when comparing the control group with groups G4 (the group that was orally received with L-thyroxine and alcohol extract of C. limon plant which was a significant increase (P < 0.05). Body temperature can be increased when thyroid hormone expression is too high (Silva, 2003). Indeed, a recent study suggests that increased of body temperature used as a therapeutic target in the treatment of thyroid hormone disorder (Grimaldi et al., 2014). Many secondary metabolites supply from exogenous sources (extract) is confirmed with the resultant feedback response leading to increased thyroid stimulating hormone (TSH) production by the pituitary gland and elevated serum TSH and is reflected on the increased stimulation of production of T3 and T4 hormones (Boelen et al., 2011). Some studies reported that bioactive compounds in C. limon seeds extract increase of thyroid gland responsible for T4 production, and increase of enzymes activity in target tissues metabolize T4 to T3, or T3 to reverse T3 (rT3) occurs intracellular (Economidou et al., 2011). While, liver in body also metabolizes the thyroid hormones and helps regulate their thyroid functions and disrupt in these hormone productions may influence the liver function, and liver disease can affect thyroid hormone metabolism (Malik and Hodgson, 2002).

Recent study demonstrated that the bioactive compounds present in C. limon extract, increase of an intracellular enzyme activates that stimulate thyroid hormone production or regulated three-fold by these compounds (Da-Silva et al., 2007). Da-Silva et al., (2007) suggested that flavonoids compound (Kaempferol) which increases the rate of T3 production by 2.6-fold. Seasonal variation also effects the thyroid hormone production like exposure to cold, cases of small elevation in TSH, T3 in human healthy compare to exposed to long periods of cold. (Do et al., 2004). Also, the important of natural compounds in Citrus sp. extracts on thyroid hormones secretion may be due to its antioxidant effect (Monforte et al., 1995). Antioxidant ability of C. limon plant are belong to the phenolic compounds content (De Pascale, 2008). Work by (Haman et al., 2006) suggested that isoflavonoids compounds present in C. limon plant structurally similar T3 and T4 and could act as these hormones influencing the hypothalamus–pituitary feedback system.

**Table 1:** Effect of L-thyroxine and types of extract of C. limon on thyroids hormone

<table>
<thead>
<tr>
<th>Extracts</th>
<th>T3 nmol/1</th>
<th>T4 nmol/1</th>
<th>TSH nmol/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G1) Healthy</td>
<td>1.8±0.333</td>
<td>46.9±1.285</td>
<td>0.05±0.005</td>
</tr>
<tr>
<td>(G2) Control (L-Thyroxine only)</td>
<td>1.92±0.015</td>
<td>47.56±0.688</td>
<td>0.06±0.005</td>
</tr>
<tr>
<td>(G3) C. limon (Water)+ L-Thyroxine</td>
<td>1.80±0.008</td>
<td>44.36±0.608</td>
<td>0.07±0.003</td>
</tr>
<tr>
<td>(G4) C. limon (Ethanol)+ L-Thyroxine</td>
<td>1.84±0.008</td>
<td>44.36±0.384</td>
<td>0.05±0.005</td>
</tr>
</tbody>
</table>

**LSD** 0.063 2.652 0.017

Effect of L-thyroxine drug and extract of C. limon (seeds) on Calcium and cholesterol levels: Among calcium levels, results in the figure 2 showed that there was no significant difference (P < 0.05) when comparing the group G2 (Hyperthyroid) 12.2±0.371 with the control group G1 (Healthy) 13.3±0.497. The results also showed there was no significant difference (P < 0.05) in Ca^{2+} when comparing both groups G3 14.1±0.145 (the group that was orally received L-thyroxine and water extract of C.limon plant), and G4 14.5±0.317 (the group that was orally received L-thyroxine and alcohol extract of C.limon plant) with the control group G2 for calcium level (Ca^{2+}). The results in the figure (2) showed that there was a significant difference (P < 0.05) of cholesterol levels when
comparing the group $G_2$ $96.3 \pm 0.348$ (the group that was orally received L-thyroxine) with the healthy group $G_1$ $93.1 \pm 0.440$ that has a significant increase ($P < 0.05$).

![Figure 2](image)

**Figure 2:** Effect of L-thyroxine drug and *C. limon* (seeds) extract on Calcium levels: $G_1$: Healthy, $G_2$: Hyperthyroid, $G_3$: Hyperthyroid + *C. limon* water extract, $G_4$: Hyperthyroid + *C. limon* ethanol extract.

The results in figure 3 also showed there was a significant difference ($P< 0.05$) when comparing both groups $G_3$ $99.3 \pm 0.404$ (the group that was orally received L-thyroxine and water extract of *C. limon* plant) $a$ that has a significant increase ($P < 0.05$) and $G_4$, $95.3 \pm 0.513$ (the group that was orally received L-thyroxine and alcohol extract of limon plant) with the control group $G_2$, which was no significant increase ($P< 0.05$) that for cholesterol level.

The present study agreements with study by Ding *et al.*, (2007) and Khan, (2012). Found that treatment with avocado and AVOE plant extracts, Our, result where, increased in cholesterol, T3, T4 as compared to CCl4 drug group. These results suggest that AVOE and avocado plants can protect liver and thyroid tissue against oxidative damage, may be due to antioxidant effects of its natural compounds. Present studies demonstrate that *C. limon* plant extracts possesses hypocholesterolemic and hypolipidemic, causes a decrease in lipid peroxidation and an increase in plasma antioxidant capacity (Abdullah and Al-A, 2012). Based on this result, observed that the effect of *C. limon* extracts on thyroid hormone secretion, this may be due to the natural compounds present in *C. limon* especially vitamin C, phenolic and Steroids compounds interferes with iodine uptake and the synthesis of thyroid hormones T3 and T4. (Malik and Hodgson, 2002).

![Figure 3](image)

**Figure 3:** Effect of L-thyroxine drug and extract of *C. limon* (seeds) on cholesterol levels: $G_1$: Healthy, $G_2$: Hyperthyroid, $G_3$: Hyperthyroid + *C. limon* water extract, $G_4$: Hyperthyroid + *C. limon* ethanol extract. Cellular toxicity [Lethal dose 50 (LD50)]

All experimental rats treated with both type of water and alcoholic ethanolic and methanolic extract of *C. Limon* seeds for the concentration (200, 250 and 300mg/kg b.w) did not show any abnormal behavior or sings of toxicity and rats activity, there was no significant change in the body weights and
did not record any death-rate after 30 minutes and during the two days post treatment. Present studies showed that no signs of toxicity and no expressed changes in their physio-pathological activity.

Conclusion
The results of hormones showed that the extract of *C. limon* has a significant effect (p < 0.05) in the levels of thyroid hormones compared to healthy rats. As well as for the water extract at 200mg / kg of animal weight where a significant difference more (p<0.05) on all the parameters studied compared to the alcoholic extract.

REFERENCES


