Stereological Assessment of Nephroprotective Effects of *Trachyspermum ammi* Essential Oil against Carbon Tetrachloride-Induced Nephrotoxicity in Mice

Evaluación Estereológica de los Efectos Nefroprotectores del Aceite Esencial de *Trachyspermum ammi* Contra la Nefrotoxicidad Inducida por Tetracloruro de Carbono en Ratones

Mohammad Hossein Farzaei¹; Mohammad Mahdi Zangeneh¹²; Nader Goodarzi³ & Akram Zangeneh¹²


SUMMARY: *Trachyspermum ammi* (*T. ammi*) has been used in folk medicine as anti-inflammatory, antipyretic, antibacterial, anti-fungal agent. The present study was conducted to investigate the protective effect of *Trachyspermum ammi* (*T. ammi*) essential oil against CC1₄ induced nephrotoxicity in mice. Thirty-five mice were divided into five groups as follows; positive control received olive oil 1 mL/kg/ip, negative control received CC1₄ 1 mg/kg/ip + 0.5 mL distilled water orally and tree treatment groups which received CC1₄ similar to the negative control and 200, 800 and 1600 µg/kg of *T. ammi* essential oil, respectively. All treatments were done twice a week (Saturday and Wednesday) for 45 days. On the last day, blood was sampled for urea and creatinine assessment and the left kidney was removed for stereological estimations. Essential oil of *T. ammi* at high dose significantly (p ≤ 0.05) decreased serum levels of creatinine and urea in comparison with CC1₄-treated group. Total volume of the kidney, cortex, proximal convoluted tubules (PC), glomerulus, vessels and interstitial tissue as well as total length of PC and vessel were significantly (p ≤ 0.05) increased following CC1₄ administration and were restored toward normal levels at high dose of *T. ammi*. Also, high dose of *T. ammi* improved glomerular loss significantly (p ≤ 0.05) as compared with CC1₄-treated group. Due to the chemical composition of *T. ammi* essential oil such as tymol, *p*-cymene, *γ*-terpinene which are antioxidant, it can be concluded that the essential oil of *T. ammi* can ameliorated renal injury induced following CC1₄ toxicity via its antioxidant components.

KEY WORDS: *Trachyspermum ammi*; Carbon tetrachloride; Essential oil; Stereology; Kidney.

INTRODUCTION

The kidneys participate in adjustment of endocrine system, blood pressure, osmolality situation, reabsorption of vital micronutrients, extracellular fluid volume, whole-body homeostasis, acid-base balance, electrolyte concentrations, and excretion of wastes (Musabayane, 2012). Some of the renal disorders such as glomerulonephritis, interstitial nephritis are mainly caused by toxic chemicals. Carbon tetrachloride is a toxic chemical that can cause damage to many tissues including kidney through free radicals generation and lipid peroxidation (Rechnagel et al., 1989; Kumar et al., 2005; Khan & Ahmed, 2006; Masuda, 2006). It seems that free radicals- induced lipid peroxidation to be one of the major causes of cell membrane damage resulting in a series of pathological situations by causing acute and chronic renal injuries (Satyanarayana et al., 2001; Manna et al., 2006; Adewole et al., 2007). Furthermore, there are many reports of documented case studies which indicate that CC1₄ can produces renal diseases through alteration of antioxidant status in humans (Javier Perez et al., 1987; Manna et al.). Therefore, in the present study, the CC1₄ has been used to induce acute nephrotoxicity.

Recently, medicinal plants as a source of useful chemical compounds received much attention for treatment of many diseases and promotion of human health (Zangeneh et al., 2016). Due to recent developments in methodology, medicinal herbs prepared and examined in various types such as essential oils (Tahvilian et al., 2016). Essential oil is a condensed hydrophobic liquid including aromatic compounds from herbs. Essential oils could be extracted

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from several parts like leaves, stems, flowers, and roots (Faramarzi et al., 2017). Essential oils can act as antioxidant and anti-inflammatory agents through inhibiting lipid peroxidation, scavenging free radicals and chelating metal ions. However, part of plant used, climatic condition and time of harvesting may contribute to their chemical composition and consequently their chemical activities (Miguel, 2010).

*Trachyspermum ammi* (T. ammi) commonly known as Ajowan caraway, is a wild bush that is widely distributed in Iran and India (Wadikar & Premavalli, 2012). It contains many substances which can act together to prevent several diseases. *T. ammi* genus’s anti-oxidative and anti-inflammatory properties make it a logical adjuvant to improve wound healing (Thangam & Dhananjayan, 2003; Bera et al., 2004). Based on the literatures, *T. ammi* has been used in folk medicine as an anti-inflammatory, antipyretic, antibacterial, anti-fungal, antiviral, anti-diabetic, antinociceptive, and analgesic agent (Dashti-Rahmatabadi et al., 2007; Rasooli et al., 2008; Hejazian et al., 2008).

As far as we know, there is a very little data about chemical composition and nephroprotective properties of *T. ammi* essential oil grows in Kermanshah province, west of Iran. Hence, the aim of the present study was to specify the chemical composition of *T. ammi* essential oil obtained from Kermanshah city, west of Iran and also to investigate its nephroprotective effects against CC1₄-induced nephrotoxicity. Histological change such as volume and length of renal tubules and glomerular number were estimated stereologically. Renal function was assessed thorough analysis of serum biomarkers.

**MATERIAL AND METHOD**

**Plant sample collection.** In the empirical-experimental study, medicine plant collected from Kermanshah city, west of Iran.

Table I. The components of *T. ammi* Essential oil that analyzed by GC/MS.

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>Area (%)</th>
<th>RI (Retention Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Thujene</td>
<td>0.6</td>
<td>927</td>
</tr>
<tr>
<td>2</td>
<td>α-Pinene</td>
<td>0.3</td>
<td>934</td>
</tr>
<tr>
<td>3</td>
<td>β-Pinene</td>
<td>1.8</td>
<td>976</td>
</tr>
<tr>
<td>4</td>
<td>β-Myrccene</td>
<td>0.6</td>
<td>990</td>
</tr>
<tr>
<td>5</td>
<td>p-Cymene</td>
<td>25.7</td>
<td>1029</td>
</tr>
<tr>
<td>6</td>
<td>γ-Terpinene</td>
<td>25.1</td>
<td>1064</td>
</tr>
<tr>
<td>7</td>
<td>Thymol</td>
<td>44.2</td>
<td>1299</td>
</tr>
<tr>
<td>8</td>
<td>Carvacrol</td>
<td>0.3</td>
<td>1302</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>98.6</td>
<td></td>
</tr>
</tbody>
</table>

**Essential oil extraction.** Essential oil from aerial part of *T. ammi* extracted by Clevenger apparatus. Briefly, in first 100 to 150 g of plant was add to the distillation flask (1L) which was join to a steam producer via a glass tube and to a condenser to resume the essential oil in a funnel tube. Components of the essential oil was purified from the plant material and evaporated into hot steam without burning the plant material itself. Then, steam containing the essential oil was compressed through a cooling system for 3 h and essential oil was withdrawn. The essential oil was purged through anhydrous Na₂SO₄, dehumidified essential oil. Then, the essential oil was accumulated in small and dull vials and stored in a refrigerator.

**Gas chromatography / mass spectrometry (GC/MS).** GC/MS (Shimadzu capillary GC-quadrupole MS system QP 5000 (with two fused silica capillary column DB-5 (30 µm, 0.25 µm i.d, film thickness 0.25 mm) and a flame ionization detector (FID) run in EI mode at 70 eV were used. Here on, injector temperature was 220 °C and detector temperature was set at 250 °C. Also in the device, helium was employed as carrier gas (1 µl/min). 1 ml of the essential oil was injected and analyzed with the column held initially at 60 °C for 2 min. Then, the amount of components of the essential oil is obtained. NIST standard reference database (AMDLA version 2.70) was used to interpret the mass spectral data. The constituents of essential oil is present in Table I.

**Animals and treatments.** Thirty five healthy male Balb/c mice weighing between 30 and 40 g were provided by laboratory animal center of Kermanshah University of Medical Sciences. The animals were kept under constant humidity and temperature. All animals were allowed free access to food and water *ad libitum* during the experiment. The animals were treated according to the standard directive as recommended in the Guide for Care and Use of Laboratory Animals and approved by the research authorities of Kermanshah University of Medical Sciences. The mice were divided randomly into five groups (n=7):

Group I (control): received 1 mL/kg olive oil intraperitoneally and 0.5 mL distilled water through gavages.

Group II: received 1 mg/kg CC₁₄ mixed with olive oil in the ratio of 5:5, intraperitoneally + 0.5 mL distilled water through gavages.

Group III: received CC₁₄ mixed with olive oil in the ratio of 5:5, intraperitoneally + ethanolic extract of *T. ammi* 200 µg/kg through gavages.

Group IV: received CC₁₄ mixed with olive oil in the ratio of 5:5, intraperitoneally + ethanolic extract of *T. ammi* 800 µg/kg through gavages.
Group V: received CC14 mixed with olive oil in the ratio of 5:5, intraperitoneally + ethanolic extract of *T. ammi* 1600 µg/kg through gavages.

All treatments were done twice a week (Saturday and Wednesday) for 45 days. On the last day of the experiment, blood samples were obtained from animal’s heart and centrifuged for collecting serum. Then the subjects weighed and sacrificed using chloroform inhalation. The left kidney of each animal was removed, weighed and fixed in 10 % neutral buffered formaldehyde. Tubular length estimation needs isotropic uniform random sections. Therefore, after 72 h fixation, the kidneys were cut using orientator method. Totally, 7–10 slabs were collected from each kidney. The slabs were embedded in paraffin and sections (5 µm thicknesses) were prepared and stained by Periodic Acid Schiff (PAS) method.

Due to the tissue shrinkage following fixation and tissue processing, this should be considered for estimating reference volume. A circle was punched by a trocar from a kidney slab and its area was calculated before (AB) and after (AA) tissue processing. Finally, the tissue shrinkage was estimated using following formula (Nyengaard, 1999):

\[
\text{Volume shrinkage} = 1 - \left( \frac{AA}{AB} \right)^{1.5}
\]

Total volume of kidney (reference volume) was estimated using:

\[
V_{\text{final}} = V_{\text{primary}} \times (1 - \text{volume shrinkage})
\]

Stereological study

**Volume estimation.** Each sampled section was analyzed using a videomicroscopy system equipped with a microscope (Olympus CX2, Japan) linked to a video camera (Dinocapture ver.5, dino-lit.com 30.5 mm), a P4 PC computer, and a flat monitor to determine the parameters. The point probe (composed of 20 points) was superimposed upon the images of the tissue sections viewed on the monitor, and a volume density (V;) of renal cortex, medulla, glomeruli (GLOM), proximal convoluted tubule (PC), distal convoluted tube (DC), collecting ducts (CD), Henle’s loop (LH), vessels (V), and interstitial tissue (IT) were estimated using point-counting method and the following formula (Fig. 1) (Gundersen et al., 1988):

\[
V_v = \frac{P_{\text{structure}}}{P_{\text{reference}}}
\]

Where ‘’P structure and P reference ‘’ were the number of test points falling on the structure’s profile and on the reference space, respectively. 10-14 microscopic fields were examined in each kidney. The absolute volume of the parameters was estimated by multiplying the fractional volume by the final volume of the kidney to prevent the reference trap (Nyengaard; Mandarim-de-Lacerda, 2003).

**Length estimation.** The length density of the renal tubules and vessels, was estimated using an unbiased counting frame (740 x 740 µm) (Nyengaard; Mandarim-de-Lacerda). The counting frame was superimposed on the monitor live images and tubule profiles completely inside the counting frame or partly inside the counting frame but only touching the top and right lines were counted. The length density (Lv) of the each tubule was calculated as:

\[
L_v = 2 \times \frac{\Sigma Q}{a(\text{frame}) \times \Sigma \text{frame}}
\]

Where Σ Q denotes the total number of the tubule profiles counted per mouse kidney, a(frame) equals the area associated with a frame, 547600 µm², and Σ is the total number of frames counted. Finally, the total length of each tubule, L, was calculated by multiplying the length density (Lv) by the final volume of the kidney (Fig. 2).

**Number estimation.** Total number of glomeruli per kidney was estimated using physical dissector method. From each kidney, section pair 20 µm apart (the first and fifth sections) was obtained. Two separate projecting systems with similar equipment were used. Two dissector probe (740 x 740 µm) with exclusion lines (the left and lower margins) and inclusion lines (the right and upper margins) were superimposed on the images of the first section as reference section (Fig. 3a) and fifth section as look-up section (Fig. 3b) at the total magnification 135x. A glomerulus was counted if it was presented in the reference section but not in the look up section and didn't touch the exclusion lines. At least 100 glomeruli per kidney were counted. The numerical density of the glomeruli was estimated using:

\[
N_v = \frac{\Sigma Q^*}{a(\text{frame}) \times h \times \Sigma P}
\]

Where ΣQ* denotes the number of counted glomeruli, a (frame) is the area of the dissector frame, Σ P is the sum of studied field and h is the dissector height. Total glomerular number was estimated by multiplying the numerical density (Nv) by the reference volume (renal cortex).
were done through one-way ANOVA followed by Tukey's post-hoc test. \( P \leq 0.05 \) was considered as significant.

**Statistical analysis.** All data are expressed as mean and standard deviation. Statistical comparison between group means were done through one-way ANOVA followed by Tukey's post-hoc test. \( P \leq 0.05 \) was considered as significant.
RESULTS

Chemical composition of *T. ammi* via GC/MS. Chemical composition of *T. ammi* essential oil is presented in Table I. The main constituents of *T. ammi* essential oil are Tymol (44.2 %), p-Cymene (25.7 %) and γ-Terpinene (25.1 %) and β-Pinene (1.8 %). Other compounds including a-Thujene, a-Pinene, b-Myrcene and Carvacrol were found to be less than 1 % of the oil.

Effects of CCl4 and *T. ammi* on the weight and volume. The data of the kidney weight, mean absolute volume of kidney and its subcomponents in control and treated groups are shown in Tables II, III and IV. The results showed that the kidney weight and volume were increased 44 % and 55 % (p ≤ 0.001) respectively in - treated mice in comparison with the control group. Volume of the cortex increased 62 % (p ≤ 0.001) in this group but the medulla increased 40 % which was not significant (p > 0.05) compared to the control group. Treatment of - treated mice with high dose (1600 µg/kg) of *T. ammi* significantly (p ≤ 0.05) improved the kidney weight and consequently kidney volume in comparison with low and moderate dose (200 and 800 µg/kg). Further, the volume of the cortex was decreased significantly (p ≤ 0.05) in group which received high dose compared to the - treated group, whereas, medulla volume shows no significant decrease (p > 0.05).

Table II. The kidney weight (mg), absolute volume of the kidney (mm³), and absolute volume (mm³) of cortex and medulla of the control and experimental groups treated with *T. ammi* essential oil. Results are given as means ± standard deviation.

<table>
<thead>
<tr>
<th>Groups (n=7)</th>
<th>Kidney weight</th>
<th>Kidney volume</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.2 ± 1.4</td>
<td>110.5 ± 2.9</td>
<td>77.77 ± 4.3</td>
</tr>
<tr>
<td>CCl4</td>
<td>195 ± 15.6*</td>
<td>171 ± 21.5*</td>
<td>125 ± 11.8*</td>
</tr>
<tr>
<td>200 T + CCl4</td>
<td>191 ± 18*</td>
<td>160 ± 14.7*</td>
<td>125.2 ± 8.6*</td>
</tr>
<tr>
<td>800 T + CCl4</td>
<td>184 ± 22.5*</td>
<td>151 ± 11.8*</td>
<td>123 ± 12*</td>
</tr>
<tr>
<td>1600 T + CCl4</td>
<td>153 ± 35**</td>
<td>125.8 ± 17**</td>
<td>101.2 ± 14**</td>
</tr>
</tbody>
</table>

* p ≤ 0.05 v.s control group. ** p ≤ 0.05 v.s CCl4 treated group

Table III. Absolute volume (mm³) of the proximal and distal convoluted tubules (PCT, DCT), collecting ducts (CD), loop of Henle (LH), vessels (VES) and interstitial tissues (IT) in the control and experimental groups treated with *T. ammi* essential oil. Results are given as means ± standard deviation.

<table>
<thead>
<tr>
<th>Groups (n=7)</th>
<th>PC</th>
<th>DC</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68.5 ± 4</td>
<td>16.5 ± 3</td>
<td>20.9 ± 4</td>
</tr>
<tr>
<td>CCl4</td>
<td>122.2 ± 12*</td>
<td>22.5 ± 5.7</td>
<td>25.4 ± 5.1</td>
</tr>
<tr>
<td>200 T + CCl4</td>
<td>115 ± 18</td>
<td>20.1 ± 5.1</td>
<td>21.2 ± 6.3</td>
</tr>
<tr>
<td>800 T + CCl4</td>
<td>112.8 ± 8.9**</td>
<td>17.5 ± 2.8</td>
<td>23.7 ± 3.4</td>
</tr>
<tr>
<td>1600 T + CCl4</td>
<td>88 ± 13**</td>
<td>14.8 ± 3.1</td>
<td>21.7 ± 5.2</td>
</tr>
</tbody>
</table>

* p ≤ 0.05 v.s control group ** p ≤ 0.05 v.s CCl4 treated group

Table IV. Absolute volume (mm³) and number of the glomeruli (GLOM) in the control and experimental groups treated with *T. ammi* essential oil. Results are given as means ± standard deviation.

<table>
<thead>
<tr>
<th>Groups (n=7)</th>
<th>Parameters Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.002 ± 0.0001</td>
<td>28990.4 ± 1851.5</td>
</tr>
<tr>
<td>CCl4</td>
<td>0.005 ± 0.0003*</td>
<td>22474.8 ± 856.3*</td>
</tr>
<tr>
<td>200 T + CCl4</td>
<td>0.004 ± 0.0001*</td>
<td>22675 ± 1055</td>
</tr>
<tr>
<td>800 T + CCl4</td>
<td>0.004± ± 0.0002*</td>
<td>23556 ± 1168**</td>
</tr>
<tr>
<td>1600 T + CCl4</td>
<td>0.002± ± 0.0002**</td>
<td>26881 ± 1014**</td>
</tr>
</tbody>
</table>

* p ≤ 0.05 v.s control group. ** p ≤ 0.05 v.s CCl4 treated group.
The volume of PC, GLOM, vessels and interstitial tissue were increased 80 %, 150 %, 83 % and 64 % (p \( \leq 0.05 \)), respectively in - treated mice compared to the controls (Tables III and IV). The volume of CD and LH did not show significant differences (p > 0.05). Treatment with high dose (1600 \( \mu \text{g/kg} \)) of \textit{T. ammi} significantly (p \( \leq 0.05 \)) decreased the volume of PC and GLOM compared to the - treated group.

**Effects of and \textit{T. ammi} on glomerular number.** The obtained results showed that glomerular numbers per kidney in treated group were significantly (22.5 %) lower than that of the control animals. Co-administration of 1600 \( \mu \text{g/kg} \) of \textit{T. ammi} and prevented the decrease of glomerular numbers significantly (p \( \leq 0.05 \)) in comparison with the -treated group (Table IV).

**Effects of and \textit{T. ammi} on length of renal tubules and vessels.** The length of PC and vessel increased 51 % and 45 %, respectively (p \( \leq 0.05 \)) after administration. However, the increase in length of DC, CD and LH were not significant (p > 0.05) in -treated mice compared to the controls. High dose of \textit{T. ammi} could significantly improve the length of PC and vessels toward normal values (Table V).

**Effects of and \textit{T. ammi} on serum profile.** Serum concentration of urea and creatinine are presented in Table VI. Concentration of urea and creatinine was increased significantly (p \( \leq 0.05 \)) in serum with -induced nephrotoxicity as compared to the control group. Co-administration of and different doses of \textit{T. ammi} significantly improved the changed levels of urea and creatinine causing a subsequent recovery towards normalization. However, there was no significant difference between different doses of \textit{T. ammi}.

**DISCUSSION**

The present study showed the ameliorative potential of \textit{T. ammi} essential oil against - induced acute nephrotoxicity through stereological assessments and serum profile analysis. It has been documented that renal cortex contains higher amounts of cytochrom P450 than other organs and this increase the affinity of kidney for. Hence, has been commonly used for acute renal failure induction in experimental animals (Ronis et al., 1998).

Although, renal functional impairment following treatment can be recognized by high serum levels of urea and creatinine (Ozturk et al., 2003), it should be noted that creatinine concentration is a more potent indicator as it increase in the initial stages of renal disease. Furthermore, increase in serum level of creatinine can lead to the structural damage of nephrons (Gilbert et al., 1989). In the study, administration of (1 mg/kg body weight) result in significant increase of serum urea and creatinine as it was previously reported by other studies (Ogeturk et al., 2005; Khan & Ahmed, 2009; Khan & Zehra, 2013; Sherkatolabasieh et al., 2017). The increased level of serum urea and creatinine were significantly improved toward normal levels in the high dose (1600 \( \mu \text{g/kg} \) \textit{T. ammi} treated group when compared with the only treated animals. Similar studies have been
reported that various plant can improved -induced renal injury intoxication (Khan et al., 2010; Khan & Siddique, 2012; Sherkatolababasieh et al.). These results indicate protective effect of T. ammi against nephrotoxicity.

Our results showed that treated animals experienced noticeable renal hypertrophy which was mainly due to the cortical enlargement. Accordingly, proximal convoluted tubules and vessels were affected in respect of their volume and length. These alterations were alleviated significantly in the fifth group which received high dose of T. ammi.

In the present study, only treated mice showed glomerular hypertrophy. This event could be related to the vasconstriction and congestion of capillary tufts in the renal corpuscles. Glomerular hypertrophy was prevented in high dose group in comparison with the only treated group. There are some reports that another plant extract such as Oxalis corniculata (Khan & Zehra), Citharexylum spinosum (Khan & Siddique) and Sonchus asper (Khan et al., 2010) can inhibit glomerular hypertrophy induced through nephrotoxicity.

Another nephroprotective effect of T. ammi is its prevention of glomerular loss at high dose (1600 μg/kg). Embryologically, nephrogenesis stops after birth. Therefore, glomerular loss is a irreversible event. - induced nephrotoxicity has glomerular effect that can lead to loss of filtration rate, mesangial cell proliferation and apoptosis (Tavifi & Ahmadvand, 2011).

It is well established that induce Reactive Oxygen Species (ROS) which later can damage antioxidant barriers and start the process of lipid peroxidation (Halliwell & Gutteridge, 2007). Therefore, use of certain plant extract rich in antioxidant agents can ameliorate toxicity. The results of GC/MS analysis showed that Tymol (44.2 %), p-Cymene (25.7 %) and g-Terpinene (25.1 %) constitute the main components of T. ammi essential oil. Free radicals scavenging ability of these compounds were established previously. The antioxidant activity of Thymus marschallianus and Thymus proximus' essential oils which are mainly constituted by thymol, p-cymene andg-terpinene have been examined earlier by safranin method (Jia et al., 2010). The activity of both essential oils were reported to be dose dependent which is consistent with the present results. Furthermore, antioxidant activity for essential oils of some other plants containing thymol, p-cymene andg-terpinene such as Thymus vulgaris, Salvia officinalis, Origanum vulgare (Viuda-Martos et al., 2010), Satureja intricade (Jordan et al., 2010) and Lavandula angustifolia (Yang et al., 2010) have been approved. Therefore, it may said that the nephroprotective activity of essential oil of T. ammi is attributed to the presence of thymol, p-cymene and g-terpinene.

CONCLUSIONS.

Based on the obtained results, essential oil of T. ammi at high dose ameliorated renal structural changes and serum biomarkers disturbance induced followingintoxication. Thymol, p-cymene,g-terpinene and other antioxidant contents may justify its nephroprotective and therapeutic properties. Therefore, T. ammi can be considered as a new nephroprotective agent against toxicity.

ACKNOWLEDGEMENTS.

The authors are graffull to Mr. Moradi for technical assistance in essential oil preparation. The Kermanshah University of Medical Sciences is highly appreciated for financial support.


PALABRAS CLAVE: Trachyspermum ammi; Tetracloruro de carbono; Aceite esencial; Estereología; Riñón.
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Received: 27-09-2017
Accepted: 19-12-2017