IN VITRO CYTOTOXICITY EVALUATION OF NOVEL COMBINED PLANT CRUDE AQUEOUS EXTRACT OF LICORICE AND MACA

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ABSTRACT

Recently, researchers have shown an increased interest in the potential positive effects of Glycyrrhiza glabra (licorice) and Maca extracts. However, there has been no detailed investigation of the effects of mixing these two plants extracts. Therefore, the present research explores for the first time, the effects of the mixture of these two extracts. The aim of the current study is to figure out the cytotoxicity of this mixture on the sensitive system of the mammal’s body.

This study, therefore, set out to assess the cytotoxicity effect of the aqueous crude extract of Licorice, Maca, and the effect of their extracts mixture on mice normal spleenocytes by MTT assay. The results show that the cells growth inhibition of both extracts and their mixture was 0% compared to control. The mixture medium (12.5/2.5mg) has the best viability with 245.0% compared to Licorice medium alone (12.5mg) with viability 223.1% and Maca medium alone (2.5mg) with viability 133.6 %. The most obvious finding to emerge from this study is that the mixtures of licorice and Maca extracts have no harmful effects at a dose of (12.5/2.5mg). Furthermore, the mixture data can be applied for orally used in the treatment of infertility status.

Keywords: cytotoxicity, MTT assay, licorice, maca

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INTRODUCTION
**Glycyrrhiza glabra** (Licorice) is a native plant in Eurasia, western Asia, northern Africa \(^{(1)}\), belonging to the family Leguminosae. There is wide consumption of this popular herb and has many pharmaceutical functions including anti-inflammation, anticarcinogenic, antiviral, antiulcer, detoxification and many other activities \(^{(2)}\). Licorice extract and its derivatives are classified as safe (GRAS) according to the U.S. Food and Drug Administration (FDA) for human and animal feeds \(^{(3)}\). It contains more than 20 tri-terpenoids and 300 flavonoids \(^{(4)}\), the two most important chemical constituents are glycyrrhizin and flavonoids which have an anti-inflammatory effect.\(^{(5)}\)

It has been reported that glycyrrhizic acid is recommended to treat female’s sterility for its pharmacological characteristics, moreover, its flavonoids decrease hyperglycemia and fat accumulation in polycystic ovaries \(^{(6)}\). Consuming a low dose of licorice root extract by pregnant female mice increases offspring numbers and weights with no mutation effect on the young male gonads \(^{(5)}\). Gg extract support fertilization rate and normal early cleavage of mice embryo in vitro \(^{(7)}\). *Lepidium meyenii* Walpers (Maca), a Peruvian plant belong to the *Brassicaceae* family (Cruciferae). It used as food supplements and for medical beneficial for centuries in Peru. Previously, whether in vivo or in vitro studies, Maca aqueous extract had been indicated as safe to use and do not cause hepatotoxicity \(^{(8)}\).

The primary metabolites include protein, carbohydrate, lipids, fibers, Free fatty acid (linoleic, palmitic, oleic acids), amino acids (leucine, arginine). While, the secondary metabolites includes macaridine, macaene, alkaloids, sterols, glucosinolates, macamides which is a novel compounds of Maca that have not been found in another plant so far \(^{(9)}\). Maca boiled aqueous extract has been reported to increase sperm count in testis, epididymis, vas deference \(^{(10)}\), and improves sexual behavior in male rodents \(^{(7)}\), it was used as plant fertilizer and in fungal medium \(^{(11, 12)}\). There are no previous studies on the impacts of both Licorice and Maca extracts on cell viability. Thus, the aim of the current study is to detect the cytotoxicity of this mixture on mice spleenocytes.

**MATERIALS AND METHODS**

**Plants extraction**

Licorice roots were obtained from local markets in Baghdad, air dried and grinded into powder. The hot aqueous extract of licorice was prepared according to Harborne \(^{(13)}\). About 250 g of dried powdered were extracted by the soxhlet (Sigma, USA) for 10 hours using 300 ml distilled water. Then, filtered and drying by lyophilize apparatus. The residue was kept in dark container at 4°C until usage.

Maca powder (Healthworks®, USA) 100% raw and certified organic was obtained from Peru and supplied commercially from the USA. According to the traditional method, 100g dried maca hypocotyls
mixed with 2 liters of water, boiled at 100°C for 2 hours, and cooled and filtered. Then, the extract was kept in vials in 4°C until use (10).

**A preliminary analysis of secondary metabolites in crude aqueous extracts**

The secondary metabolites in the crude aqueous extracts of both licorice and Maca were detected by using 1ml from both plants extract and the tests were done according to the methodology reported by Harborne (13) as shown in Table (1).

**Preparation of mice Splenocytes**

The mice were sacrificed by cervical dislocation and their spleen harvested into petridish and washed twice with RPMI washing media. The cells gently macerated with 5 ml of washing media, then pipetted into a centrifuge tube at 2000 rpm for 10 min. The supernatant containing cells was aspirated to another centrifuge tube; this step was repeated twice with 10ml washing media and centrifuged. The supernatant was mixed with 3 ml D.W. for 4 min. to eliminate R.B.C. then centrifuged. This step was repeated twice with washing media. The supernatant cells were added to 20 ml media then the cells were counted by using an inverted microscope. The hematocytometer was used to account at least 106 splenocytes. Finally, the cells were aspirated into a 96-well cell culture plate with 100 µl per well and incubated at 37°C in a fully humidified atmosphere of 5% CO2 for 24hrs.

**MTT ASSAY**

The cytotoxicity influence of licorice and Maca and their mixture, on splenocytes, was demonstrated by colorimetric cell viability MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay according to (14, 15) in Biotechnology Research Center/Nahrain University, Baghdad-Iraq

For this assay, cells were treated with 100µl of different concentrations of plant extracts (50mg, 25mg,12.5mg licorice), (10mg,5mg, 2.5mg maca), (25/5mg, 12.5/2.5mg, 6.25/1.25mg licorice/maca mixture) and incubate at 37c in 5% co2 for 24h then 10µl of filtered sterilized MTT solution (5mg/ml) was added to every well and incubated for 4hrs. in 370C, then the medium was removed and 50µl DMSO (1% dimethyl sulfoxide) was added to each well to solubilize formazan crystals, incubate 10 min. Absorbance was read at 620nm using inverted microscope reader ELISA (Avusturya® Microplate Reader), the percentage of cell viability and growth inhibition were calculated using the below formula:

\[
\text{Cell viability} \% = \frac{\text{absorbance sample}}{\text{absorbance control}} \times 100\%
\]

\[
\text{Growth inhibition} \% = \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}} \times 100\%
\]
Statistical analysis

The results reported and mean values performed in triplets. Data analysis of percentage viability from O.D. values using Microsoft Excel 2010 \(^{(15)}\).

RESULTS AND DISCUSSION

Medicinal plants have indefinite ability to synthesis bioactive compounds that have effectiveness and fewer side effects compare to synthetic drugs \(^{(16)}\). In previous study, *Paeonia lactifora* was used together with *Glycyrrhiza glabra* to augment the therapeutic effect, for example, the prescription 'Shaoyaoganaotang' \(^{(17)}\). The analysis of secondary metabolites in crude aqueous extracts of both licorice and maca shows a positive reaction for each of these active compounds: alkaloids, saponins, tannins, flavonoids, terpenes, steroids, Sugars (table 1).

**Table 1: Secondary metabolites in the crude aqueous extracts of both licorice and Maca.**

<table>
<thead>
<tr>
<th>Active compound</th>
<th>Reagents</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkaloids</td>
<td>Mayers reagent</td>
<td>White precipitant +</td>
</tr>
<tr>
<td></td>
<td>Wagners reagent</td>
<td>Brown precipitant +</td>
</tr>
<tr>
<td>saponins</td>
<td>Foam</td>
<td>Foam appearance +</td>
</tr>
<tr>
<td></td>
<td>HgCL(_2)</td>
<td>White precipitate +</td>
</tr>
<tr>
<td>tannins</td>
<td>FeCL(_2)</td>
<td>Orange precipitant +</td>
</tr>
<tr>
<td></td>
<td>Lead acetate solution</td>
<td>Yellow precipitant +</td>
</tr>
<tr>
<td>flavonoids</td>
<td>H(_2)SO(_4)</td>
<td>Red precipitate +</td>
</tr>
<tr>
<td></td>
<td>HCL+Mg</td>
<td>Red precipitate +</td>
</tr>
<tr>
<td>terpenes</td>
<td>Chlorophorm+H(_2)SO(_4)</td>
<td>Reddish-brown precipitant +</td>
</tr>
<tr>
<td></td>
<td>Anas aldehyde detector</td>
<td>Brown precipitant +</td>
</tr>
<tr>
<td>steroids</td>
<td>Acetate-anhydride +H(_2)SO(_4)</td>
<td>Purple then blue then green +</td>
</tr>
</tbody>
</table>
This study was performed to assess the cytotoxic activity of the aqueous crude extract of licorice and maca in a 1:1 combination at different concentrations on the spleenocytes using the colometric cell viability MTT assay. Values of cell viability percentage of spleenocytes at various extracts concentrations after 24hrs. Incubation is shown in Table-2 and Figure 1.

From the MTT assay results, it can be determined the percentage of cell viability for the three different concentrations of each and both aqueous crud extract of licorice and Maca. The effect of both licorice and maca on the proliferation of spleenocytes expressed as % cell viability (Table 2, Figure 1).

Table 2: *In vitro* Cytotoxicity analysis of crude aqueous extract of Licorice and MACA and the combination of them.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration mg/ml</th>
<th>Absorbance 620nm</th>
<th>Cell Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>50</td>
<td>0.674</td>
<td>612.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.388</td>
<td>352.7</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>0.245</td>
<td>223.1</td>
</tr>
<tr>
<td>M</td>
<td>10</td>
<td>0.147</td>
<td>134.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.139</td>
<td>126.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.147</td>
<td>133.6</td>
</tr>
<tr>
<td>G+M</td>
<td>25/5</td>
<td>0.375</td>
<td>341.2</td>
</tr>
<tr>
<td></td>
<td>12.5/2.5</td>
<td>0.269</td>
<td>245.0</td>
</tr>
<tr>
<td></td>
<td>6.25/1.25</td>
<td>0.181</td>
<td>164.8</td>
</tr>
<tr>
<td>CON</td>
<td>0</td>
<td>0.110</td>
<td>100</td>
</tr>
</tbody>
</table>

G: licorice, M: maca, CON: control.
Nine different concentrations were (50mg, 25mg, and 12.5mg licorice), (10mg, 5mg, and 2.5mg Maca), (25/5mg, 12.5/2.5mg, 6.25/1.25mg licorice/Maca mixture). The combined extract of licorice and Maca at (25/5 mg) produces 341.2 % cell viability, (12.5/2.5 mg) produces 245.0 % cell viability, (6.25/1.25 mg) produces 164.8% cell viability. The concentration of licorice and Maca (12.5/2.5 mg) crude extract mixture yields the value of 245.0% which is the best cell viability compared to licorice alone 12.5 mg with 223.1 %, and to Maca alone 2.5 mg with 133.6% as they were separately (Figure 1). Viability percentage values of spleenocytes treated with licorice were found to increase with increase in concentrations, at 12.5mg concentration viability values were 223.1 %, at 25mg the viability value increase to 352.7 %, at 50mg the viability increases to 612.7%, the viability percentage value of all concentrations were higher than control.

Different plant extracts yield different activity on spleenocytes, this may be due to spleenocytes sensitivity to the active compounds in the two extracts. Licorice root extract with MACA treated group shows a high increase in growth when compared with other groups. Its possible synergism effect between licorice and Maca perhaps due to their mostly similar active compounds that together activated and enhanced the beneficial effect of the two extracts. Licorice extract provided a wide range of active ingredients that provide essential nutrients to the cells, it contains Ca++, glucose, potassium, vitamin C, vitamin E, fructose, Zn++, amino acid, sucrose, and many other substances all these substances stimulate normal cell growth (7). The licorice contains antioxidant compounds like flavonoids which have been reported that it has antioxidant activity 100 times stronger than vitamin E (4), also it contains Glycyrrhizin which has a similar mechanism to steroids as it is a phytohormone that increases protein production and growth (18).
Adding a mixture of licorice and pentoxifylline to the medium of cryopreserved semen sample improves some sperm function parameters with no sperm DNA damage after in vitro activation (19). Maca, on the other hand, contains protein, carbohydrate, lipids, fibers, Free fatty acid( linoleic, palmitic, oleic acids), amino acids (leucine, arginine) which all essential for cell growth. Also contains macaridine, macaene, alkaloids, sterols, glucosinolates, macamides (9). Maca extract increases some sperm function parameters in vasectomized mice (20).

CONCLUSION

The results of the present study showed that the two extracts of licorice and MACA are not toxic at a dose of (12.5/2.5mg) but biologically active and should be studied further for their potential to develop as a pharmaceutical drug.

ACKNOWLEDGMENT

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ETHICALCLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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