EVALUATION OF METHANOLIC EXTRACT OF *PHYSALIS MINIMA* FRUITS FOR IMMUNOMODULATORY ACTIVITY

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ABSTRACT

Traditional plants are used to treat several ailments. They are rich source of variety of phytoconstituents. The present phytochemical studies have addressed extracting, isolating and identifying bioactive compounds of plants. In the present study, *Physalis minima* unripe fruits were extracted with methanol and screened for active group of chemical constituents by different analytical methods. The literature survey revealed that there is no evaluation of immunomodulatory activity of the fruits. So, the present work was aimed to evaluate the immunomodulatory activity of methanolic extract of unripe fruits of *Physalis minima*. From research studies in the past and present HPLC and LC-MS data, it can be assumed that the extract possess steroidal alkaloids, which are responsible for immunostimulant properties.

KEY WORDS

Phytochemical, methanolic extract, *Physalis minima*, HPLC, LC-MS, Immunomodulatory activity

INTRODUCTION

Immunomodulatory activity means the biological or pharmacological effects of compounds on humoral or cellular aspects of the immune response. For maintaining a disease-free state, modulation of immune response either through stimulation or suppression is required. There are certain agents, which are apart from being specifically stimulatory or suppressive, have been shown to possess activity to modulate pathophysiological processes and are hence addressed as immunomodulatory agents. They are also known to be biological response modifiers like haemopoietic drugs. Drugs may modulate immune mechanism by either suppressing or by stimulating in any of the following ways: by antigen recognition and phagocytosis, by lymphocyte proliferation/differentiation, by synthesis of antibodies, by antigen-antibody interaction, by release of mediators due to immune response, modification of target tissue response.

*Physalis minima* is a perennial herb belonging to the family Solanaceae, commonly known as pygmy ground cherry, wild cape gooseberry, native gooseberry. It is pantropical annual herb possess cream to yellowish flower followed by edible yellowish fruit encapsulated in papery cover which turns straw brown on maturity. The results of the preliminary phytochemical analyses in the chloroform, diethyl ether, ethanol, ethyl acetate and methanol extracts of stem, leaves and unripe fruits showed presence of Alkaloids, flavonoids, cardiac glycosides, phenols, saponins, steroids, tannins and terpenoids. Reducing sugars were unable to be separated in all the solvent extracts of *P.*minima. Amount of phenols eluted by the organic solvents was very low in all the plant parts.
The past studies reported that the plant possess diuretic activity, anti-inflammatory, analgesic, antipyretic, antibacterial, antidiabetic activities. The literature survey revealed that there is no detailed study of chemical constituents using analytical methods such as HPLC, I.R., and LC-MS. So, the present work was aimed to study the detailed chemistry of active principles present in the methanolic extract of unripe fruits of Physalis minima.

MATERIALS AND METHODS

Collection of Plant Material
The unripe fruits of Physalis minima were collected from the fields of Jammikunta, Karimnagar, Telangana, India. The plant parts were authenticated and deposited at the herbarium of University College of Pharmaceutical Sciences, Satavahana University, Karimnagar, Telangana, India.

Preparation of the extract
The unripe fruits of Physalis minima (2.0kg) were kept for maceration with methanol for seven days. The extracts were concentrated in desiccators.

Chemicals
All the chemicals used for the investigation were of analytical grade.

Drugs
In the present study Levamisole was used as an immunostimulating agent.

Antigenic material
All the above groups mice were antigenically challenged with SRBC (0.5x109cells/ml/100 g) on the 5th day intraperitoneally.

Detection of phytoconstituents
The extract was tested for phytoconstituents by preliminary tests, separated the constituents by HPLC and identified molecular weight by LC-MS.

Screening of immunomodulatory activity
Methods
- Carbon clearance test
- Humoral antibody titre
- Delayed type hypersensitivity

METHOD OF EVALUATION
Detection of Phytoconstituents
The extract was tested for the presence of Carbohydrates, Tannins, Flavonoids, Alkaloids, Anthocyanin and Betacyanin, Glycosides, Proteins, Steroids and Phytosterols, Phenols.

Chromatography

a. Thin-layer chromatography (TLC)
TLC was used for detecting the class of compounds present in the sample.

b. Preparative high-performance liquid chromatography
This technique was used to identify the specific constituent present in the sample, which was isolated by column chromatography.

HPLC conditions
Column: Hypersil BDS-C18 (150X4.6mm, 5μ)
Mobilephase: A: 0.1% TFA in Water (50%)
B: 0.1% TFA in ACN (50%)
Flowrate: 1.0 ml/min
Column temp: 35°C
Run time: 40min
Programme (Isocratic)
Diluent:MeOH
Sample Preparation: 1.0 mg/mL in diluent
Vail: 96
Injection Volume: 10 μL

b. Liquid Chromatography-Mass Spectroscopy (LC-MS)
Method: D:\Methods\General-5.lcm
Method Parameters: Column: Hypersil BD S C-18 150 X 4.6 mm, 5 μm
Mobile Phase:A:Acetonitrile
Mobile Phase: B: 5mM Ammonium acetate in water
Gradient Time: -0.01 10.0 30.0
B%: - 95 10 10
Flow Rate: 1.0 mL/min
Sample Preparation: in MeOH: ACN
Note: Filtered sample was taken

Screening of immunomodulatory activity

Carbon clearance test
Swiss albino mice were divided into five groups which were administered drug for 5 days orally. On the last day, mice were injected with 0.1ml Indian ink via the tail vein. Blood samples were withdrawn at 0min and 15min. A 50μL blood sample was mixed with 4ml, 0.1% Sodium carbonate solution and the absorbance of this solution was determined at 660nm. The phagocytic index K was calculated using the following equation:
K= (Log OD1-Log OD2/15
where OD1 and OD2 are the optical densities at 0 and 15min respectively.

Delayed type hypersensitivity
Cell-mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their...
products (lymphokines). The cell mediated immune response was assessed by DTH reaction, i.e. Footpad reaction.\textsuperscript{18-20}

**Humoral antibody titre**

The animals were immunized by injecting 0.1ml of SRBCs suspension, containing $1 \times 10^8$ cells, intraperitoneal on day 0. Blood samples were collected in micro centrifuge tubes from individual animal by retro orbital puncture on day 7. Briefly, equal volumes of individual serum samples of each group were pooled. To serial two-fold dilutions of pooled serum samples made in 25μl volume of normal saline, in U-bottomed micro titration plates were added 25μl of freshly prepared 1% suspension of SRBCs in saline. After mixing, the plates were incubated at 37ºC for 2h and examined visually for agglutination. The reciprocal of the highest dilution of the test serum causing visible haemagglutination was taken as the antibody titre.

### RESULTS AND DISCUSSIONS

The unripe fruits of *Physalis minima* were extracted with methanol and screened for active group of chemical constituents using primary phytochemical tests. The extract showed positive results for alkaloids (Table 1-2).

#### Table 1: Percentage yield of various extract of unripe fruits of Physalis minima

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Extract</th>
<th>Yield (%w/w)</th>
<th>Extract colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methanol soluble</td>
<td>0.20</td>
<td>brown</td>
</tr>
</tbody>
</table>

#### Table 2: Detection of Phytoconstituents

<table>
<thead>
<tr>
<th>Dried leaves extracted with Organic solvents</th>
<th>Alkaloids (Mayer’s test)</th>
<th>Glycosides (borntrager’s test)</th>
<th>Carbohydrates (Molisch test)</th>
<th>Steroids (Liebermann–Buchard test)</th>
<th>Phytosterols</th>
<th>Tannins (FeCl₃ test)</th>
<th>Phenols (FeCl₃ test)</th>
<th>Saponins (Foam test)</th>
<th>Flavonoids (H₂SO₄ test)</th>
<th>Proteins (Ninhydrin test)</th>
<th>Anthocyanins</th>
<th>Betacyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates present; - indicates absent

**Thin-layer chromatography (TLC)**

**TLC analysis**

- Chloroform: Ethanol was used in the ratio of 9:1, orange coloured spot was observed on TLC\textsuperscript{21,22} (Fig.1)
### Table

<table>
<thead>
<tr>
<th>Peak#</th>
<th>Ret. Time</th>
<th>Area</th>
<th>Area %</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>3.361</td>
<td>33.286</td>
<td>0.278</td>
<td>2.690</td>
</tr>
<tr>
<td>15</td>
<td>4.000</td>
<td>10112</td>
<td>0.090</td>
<td>2.793</td>
</tr>
<tr>
<td>16</td>
<td>4.167</td>
<td>103.293</td>
<td>0.917</td>
<td>2.903</td>
</tr>
<tr>
<td>17</td>
<td>4.257</td>
<td>155.434</td>
<td>1.202</td>
<td>2.966</td>
</tr>
<tr>
<td>18</td>
<td>4.360</td>
<td>466464</td>
<td>4.125</td>
<td>3.038</td>
</tr>
<tr>
<td>19</td>
<td>4.552</td>
<td>20632</td>
<td>0.183</td>
<td>3.157</td>
</tr>
<tr>
<td>20</td>
<td>4.676</td>
<td>21085</td>
<td>0.214</td>
<td>3.254</td>
</tr>
<tr>
<td>21</td>
<td>4.788</td>
<td>69159</td>
<td>0.614</td>
<td>3.336</td>
</tr>
<tr>
<td>22</td>
<td>4.937</td>
<td>121544</td>
<td>1.079</td>
<td>3.440</td>
</tr>
<tr>
<td>23</td>
<td>5.090</td>
<td>11385</td>
<td>0.990</td>
<td>3.525</td>
</tr>
<tr>
<td>24</td>
<td>5.149</td>
<td>50164</td>
<td>0.445</td>
<td>3.588</td>
</tr>
<tr>
<td>25</td>
<td>5.205</td>
<td>16152</td>
<td>0.144</td>
<td>3.627</td>
</tr>
<tr>
<td>26</td>
<td>5.900</td>
<td>88369</td>
<td>0.764</td>
<td>4.111</td>
</tr>
<tr>
<td>27</td>
<td>6.557</td>
<td>90897</td>
<td>0.807</td>
<td>4.568</td>
</tr>
<tr>
<td>28</td>
<td>7.428</td>
<td>23147</td>
<td>0.205</td>
<td>5.109</td>
</tr>
<tr>
<td>29</td>
<td>7.940</td>
<td>19912</td>
<td>0.177</td>
<td>5.462</td>
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<tr>
<td>30</td>
<td>8.001</td>
<td>7385</td>
<td>0.066</td>
<td>5.575</td>
</tr>
<tr>
<td>31</td>
<td>8.267</td>
<td>56281</td>
<td>0.500</td>
<td>5.760</td>
</tr>
<tr>
<td>32</td>
<td>8.483</td>
<td>27741</td>
<td>0.246</td>
<td>5.910</td>
</tr>
<tr>
<td>33</td>
<td>8.583</td>
<td>24184</td>
<td>0.215</td>
<td>5.980</td>
</tr>
<tr>
<td>34</td>
<td>8.708</td>
<td>47690</td>
<td>0.423</td>
<td>6.067</td>
</tr>
<tr>
<td>35</td>
<td>8.800</td>
<td>14950</td>
<td>0.133</td>
<td>6.131</td>
</tr>
<tr>
<td>36</td>
<td>8.964</td>
<td>20301</td>
<td>0.180</td>
<td>6.246</td>
</tr>
<tr>
<td>37</td>
<td>9.169</td>
<td>23346</td>
<td>0.207</td>
<td>6.388</td>
</tr>
<tr>
<td>38</td>
<td>9.248</td>
<td>14508</td>
<td>0.129</td>
<td>6.443</td>
</tr>
<tr>
<td>39</td>
<td>9.661</td>
<td>43738</td>
<td>0.388</td>
<td>6.731</td>
</tr>
<tr>
<td>40</td>
<td>10.395</td>
<td>107518</td>
<td>0.954</td>
<td>7.247</td>
</tr>
<tr>
<td>41</td>
<td>10.576</td>
<td>10662</td>
<td>0.094</td>
<td>7.369</td>
</tr>
<tr>
<td>42</td>
<td>10.747</td>
<td>60955</td>
<td>0.538</td>
<td>7.488</td>
</tr>
<tr>
<td>43</td>
<td>11.641</td>
<td>75464</td>
<td>0.670</td>
<td>8.111</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>11265869</td>
<td>100.00%</td>
<td></td>
</tr>
</tbody>
</table>
HPLC analysis
Results of HPLC analysis of methanolic crude extract of unripe fruits of Physalis minima, at 330 nm, shows presence of active constituents as evidenced by the chromatogram obtained at retention time 1.548, 1.797, 2.117, 6.217 with corresponding retention area 1916775, 196535, 24826, 86070, 2224206.

LCMS analysis
HPLC coupled with different detection methods e.g. UV, MS provided a preliminary information about the content and nature of constituents found in the active extracts i.e., steroidal alkaloids. By selective ion monitoring in LC/MS or even LC/MSMS, it is possible to achieve the detection of specific target molecules - those, for example, which have already been found to exhibit a particular activity. The recent introduction of other hyphenated techniques such as LC/NMR will render the on-line structure determination of metabolites even more accurate and rapid.

Screening of immunomodulatory activity
The animals were screened using the haemagglutinating antibody titre to assess humoral immune response and Carbon clearance test to assess scavenging activity. The animals were also evaluated for delayed type hypersensitivity by the difference between the pre and post challenge footpad thickness. They have shown significant immunostimulant properties i.e., immunodulatory activity for all the methods used. The data were analyzed using statistical methods and compared to that of the standard drug, obtained values at a dose of 200mg/kg body weight (Table 3).

Table 3: Effect of methanolic extract of unripe fruits of Physalis minima by Carbon Clearance test, Humoral Antibody (HA) Titre and Delayed Type Hypersensitivity (DTH) response

<table>
<thead>
<tr>
<th>Treatment dose</th>
<th>Carbon Clearance test</th>
<th>DTH response</th>
<th>HA Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.065±0.2</td>
<td>09.00±0.011</td>
<td>3.20±0.51</td>
</tr>
<tr>
<td>Std (Levamisole)</td>
<td>0.069±0.1</td>
<td>09.84±0.013</td>
<td>3.36±0.54</td>
</tr>
<tr>
<td>TME (200mg)</td>
<td>0.073±0.1</td>
<td>12.09±0.020</td>
<td>5.05±0.22</td>
</tr>
<tr>
<td>TME (400mg)</td>
<td>0.075±0.2</td>
<td>12.88±0.032</td>
<td>7.38±0.45</td>
</tr>
<tr>
<td>TME (600mg)</td>
<td>0.079±0.2</td>
<td>14.86±0.036</td>
<td>10.90±0.51</td>
</tr>
</tbody>
</table>

CONCLUSION
The HPLC and LC-MS of the methanolic crude extract of unripe fruits of Physalis minima showed the presence of active constituents i.e., steroidal alkaloids. Based on the past literature survey and present study results, it can be assumed that the extract possesses steroidal...
alkaloids, which are responsible for stimulant properties. So, it has been concluded that the extract of unripe fruits of Physalis minima could be used as a drug to strengthen immunity to fight against various infections.

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