IN-VITRO ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF MARCHANTIA POLYMORPHA IN KUMAUN REGION

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ABSTRACT

Ethanolic extract of Marchantia polymorpha (Family: Marchantiaceae) was assessed for its antioxidant and anti-inflammatory activity by in vitro methods. Anti-inflammatory activity was evaluated using Bovine serum albumin and antioxidant activity was evaluated using DPPH at different concentrations. Ascorbic acid and Diclofenac sodium were used as standard drugs for antioxidant and anti-inflammatory activity respectively. The results showed that Marchantia polymorpha ethanolic extract (MPEE) at a concentration range of 200-400 μg/ml significantly (p<0.01) and at the concentration range of 600-1000 μg/ml highly significantly (p<0.001) protects the protein denaturation. At the concentration of 1000 μg/ml, MPEE showed highly significant (p<0.001) (75.49%) and diclofenac sodium showed 85.09719% inhibition of protein denaturation action, but at the concentration of 100 and 50 μg/ml did not show significant (p>0.05) activity in protein denaturation action. A part from this in antioxidant activity EEMP showed 51.94% and ascorbic acid 66.267% inhibition from oxidation of DPPH at dose 50 μg/ml which is significant active (p<0.01). The results obtained in the present study indicate that ethanolic extracts of Marchantia polymorpha can be a potential source of anti-inflammatory as well as antioxidant agent.

KEYWORDS: Antioxidants, Bovine serum albumin, Egg albumin, Marchantia polymorpha, Antiinflammatory.
1. INTRODUCTION

Inflammation is the reaction of vascularized living tissue to local injury. The role of inflammation is to contain and isolate injury, to destroy invading microorganisms, inactivate toxins and to achieve healing and repair. However it may be potentially harmful, causing life threatening hypersensitivity reactions and progressive organ damage.\(^1\) In order to overcome mainly the NSAIDs (Non-Steroidal Anti-inflammatory Drugs) are prescribed. The activity of NSAIDs in rheumatoid arthritis and other inflammatory diseases does not seem to be only due to the inhibition of the production of endogenous prostaglandins (which could be affected at much lower doses than those required in these conditions), but also by preventing the denaturation of proteins (which act as antigens and leads to auto-immune diseases).\(^2\) These anti-inflammatory agents inspite of their potency in relieving pain and other consequences of inflammatory responses are also associated with some serious side effects, especially in elderly. NSAIDs on prolonged duration of usage may cause gastric bleeding, ulceration, bone marrow disturbance, kidney and liver dysfunction.\(^3\) However plant derived drugs is used to treat most of the inflammatory diseases which are difficult to treat with allopathic medicines. Even today 80% of the world population depends on plant derived medicines for the first line of primary health care because of least/no side effects.\(^4, 5\) When bovine serum albumin is heated it undergoes denaturation and expresses antigens associated to Type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus.\(^6\) There are emerging ethical issues with regards to the use of animals in the early stages of drug discovery for inflammatory diseases. Thus, the in vitro anti-denaturation (stabilization) effects of heat treated (immunogenic) bovine serum albumin (BSA) assay is being used for detecting a wide range of anti-inflammatory compounds.

Considering the potential role in food industry and human health, antioxidants are gaining popularity all across the globe. Antioxidants are defined as a substance that even in small amounts, is capable of preventing or delaying the oxidation of easily oxidizable materials. Antioxidant are also defined as a substance which are capable of inhibiting a specific oxidizing enzymes or a substance that reacts with oxidizing agents prior to causing damage to other molecules or a substance that sequesters metal ions or even a substance capable of repairing system such as iron transporting protein.\(^7\) As such, production of free radicals and other reactive oxygen species in the human body by numerous physiological and biochemical processes is reported\(^8\); however, overproduction of these could lead toward development of
diseases. In this context, an antioxidant can act at different levels by (i) decreasing localized oxygen concentration, (ii) preventing chain initiation by scavenging initiating radicals, (iii) decomposing lipid peroxides to peroxyl and alkoxyl radicals, (iv) decomposing peroxides by converting them to non radicals products, and (v) chain breaking to prevent continued hydrogen abstraction.\textsuperscript{[9]}

*Marchantia polymorpha* L. (Marchantiaceae) is a common thallus liverwort species known to produce a wide array of distinctive compounds and several bisbibenzyls, including marchantin A. Marchantin A has been reported to possess diverse biological activities, such as antifungal\textsuperscript{[10]}, antimicrobial\textsuperscript{[11]}, anti-inflammatory\textsuperscript{[12]}, antioxidative, and skeletal muscle relaxing\textsuperscript{[13]} activities. Askawa demonstrated that marchantin A was efficient against fungi of several species\textsuperscript{[14]} and inhibited LPS-induced iNOS in RAW264.7 cells.\textsuperscript{[12]}

2. MATERIALS AND METHODS

2.1 Plant material

*Marchantia polymorpha* was collected in month of September, 2016 from Dogaon Village, Dist. Nainital, North-East India. The collected sample was cleaned, then spread out in shade and dried and then reduced to coarse powder with an electric blender.

2.2 Extraction and sample preparation

Ethanolic extract was prepared by cold maceration method. By vacuum rotavapor (Perfit) the ethanolic extract of *Marchantia polymorpha* were concentrated (to remove ethanol) under reduced pressure and then dried in vacuum desiccator. Afterward dried extract was kept in refrigerator (4 ± 2°C) and this *Marchantia polymorpha* extract was used for CIPE in-vivo study.

2.3 Drugs and chemicals

Ethanol and methanol were purchased from Molychem. DPPH and ascorbic acid were procured from Central Drug House. Diclofenac sodium was procured from Yerrow chem. Bovine serum albumin and Tris base were procured from Himedia.

2.4 Phytochemical screening of ethanolic extracts

Freshly prepared crude extract of *Marchantia polymorpha* was subjected to qualitative phytochemical analysis for the presence of various classes of active chemical constituents.
such as tannins, saponins, glycosides, flavonoids, alkaloids, terpenes and steroids etc. using standard procedures.\textsuperscript{[15]}

2.5 Determination of Total Phenolic Content

The total phenolic content of the \textit{Marchantia polymorpha} extract was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth.\textsuperscript{[16]} Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of the plant extract (100 µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using double beam UV-VIS spectrophotometer (UV Analyst-CT 8200). The total phenolic content was determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

2.6 Determination of Antioxidant Activity by DPPH scavenging assay

The free radical scavenging activity extract of \textit{Marchantia polymorpha} extract and of standard solution (ascorbic acid) were investigated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method as reported in the literature.\textsuperscript{[17]} The assay mixture contained 2 mL of 1.0 mmol/L DPPH radical solution prepared in methanol and 1 mL of standard or extract solution of different concentrations (10-500 µg/mL).

The solution was rapidly mixed and incubated in dark at 37 °C for 20 min. The decrease in absorbance of each solution was measured at 517 nm using UV/Vis spectrophotometer. Ascorbic acid, a well known antioxidant was used as positive control while DPPH radical solution with 1 mL ethanol was taken as blank. The percentage of radical scavenging (%) was calculated by the following formula.

\[
\% \text{ Free radical scavenging activity} = \frac{Ac - As}{As} \times 100.
\]

Where Ac=Absorbance of control at 517 nm; As= Absorbance of sample.

The concentration of sample required to scavenge 50% of the DPPH free radical (IC\textsubscript{50}) was determined from the curve of percent inhibitions plotted against the respective concentration.
2.7 Evaluation of *in-vitro* anti-inflammatory activity

2.7.1 Inhibition of protein denaturation method

The anti-inflammatory activity of *Marchantia polymorpha* extract was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al\[18\] and Sakat et al\[19\] followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 º C for 20 min, after cooling the samples the turbidity was measured at 660nm.( UV Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows: **Percentage inhibition** = (Abs Control –Abs Sample) X 100/ Abs control.

2.8 Statistical analysis

The results are expressed as mean ±SEM. Student’s t-test was used to analyze level of statistical significance between groups. P<0.05 was considered statistically significant.

3. RESULTS

3.1 Phytochemical screening

Preliminary phytochemical analysis of the extract showed the presence of major classes of phytochemicals such as tannins, flavonoids, cardiac glycosides etc (Table 1). Saponins, alkaloids, protein and amino acids were not detected in the extract.

Table 1: Preliminary phytochemical analysis of *Marchantia polymorpha* extract.

<table>
<thead>
<tr>
<th>Phytochemical Test</th>
<th>Name of the test</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>Benedict's Test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Molisch's test</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>Million's Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Xanthoprotein test</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Wagner's Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mayer's Test</td>
<td>-</td>
</tr>
<tr>
<td>Tannins &amp; Phenolic Compound</td>
<td>Iodine</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Tyrosine</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>Salkowski Reaction</td>
<td>+</td>
</tr>
<tr>
<td>Test for hexose sugar</td>
<td>Tollen's Phloroglucinol test</td>
<td>+</td>
</tr>
<tr>
<td>Test for Organic Acids</td>
<td>Test for oxalic acid</td>
<td>+</td>
</tr>
</tbody>
</table>

+: present, -: absent
3.2 Total phenolic content
The total phenolic content expressed in terms of GAE and yield (%) of extract was found to be (19.31±1.79) mg of GA/g and 8.69% (w/w) respectively. The total phenolic contents were calculated using the following linear equation based on the calibration curve of gallic acid; A=0.008X+0.0727, R²= 0.9967
Where A is absorbance and X is amount of gallic acid in µg.

3.3 DPPH free radical scavenging activity
The scavenging effect of different concentration of Marchantia polymorpha extract on the DPPH free radical was compared with standard anti-oxidant, ascorbic acid. The results were expressed as inhibition (%) shown in Table 2. Extract showed a dose dependent scavenging activity. However, their scavenging ability was found to be non significant (P>0.05) in comparison to ascorbic acid.

Table 2: Absorbance of DPPH free radical of Marchantia polymorpha extract/ ascorbic acid at 517nm.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Ascorbic acid</th>
<th>EEMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.873933333 ± 0.01302</td>
<td>0.8943 ± 0.009252</td>
</tr>
<tr>
<td>10</td>
<td>0.763133333 ± 0.01713</td>
<td>0.7959 ± 0.02196</td>
</tr>
<tr>
<td>20</td>
<td>0.6644 ± 0.006502</td>
<td>0.7709 ± 0.01445</td>
</tr>
<tr>
<td>30</td>
<td>0.578366667 ± 0.03064</td>
<td>0.6499 ± 0.02066</td>
</tr>
<tr>
<td>40</td>
<td>0.431133333 ± 0.02115</td>
<td>0.5609 ± 0.01494</td>
</tr>
<tr>
<td>50</td>
<td>0.2948 ± 0.005335</td>
<td>0.4298 ± 0.0113</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=3.

Graph 1. Absorbance of DPPH free radical of Marchantia polymorpha extract/ ascorbic acid at 517nm.
Table 3: Percentage inhibition of DPPH free radical of *Marchantia polymorpha* extract/ascorbic acid at 517nm.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>Ascorbic acid</th>
<th>EEMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>12.67831261</td>
<td>11.00301912</td>
</tr>
<tr>
<td>20</td>
<td>23.97589442</td>
<td>13.79850162</td>
</tr>
<tr>
<td>30</td>
<td>33.82027615</td>
<td>27.32863692</td>
</tr>
<tr>
<td>40</td>
<td>50.66748036</td>
<td>37.28055462</td>
</tr>
<tr>
<td>50</td>
<td>66.26744984</td>
<td>51.94006486</td>
</tr>
</tbody>
</table>

Graph 2: Percentage inhibition of DPPH free radical of *Marchantia polymorpha* extract/ascorbic acid at 517nm.

3.4 *In-vitro* anti-inflammatory activity

3.4.1 Bovine serum albumin

The inhibitory effects of different concentrations of *Marchantia polymorpha* extract on protein denaturation are shown in Table 3. *Marchantia polymorpha* extract (100-500 µg/mL) showed significant inhibition of denaturation of egg albumin in a dose dependent manner. The *in-vitro* anti-inflammatory activity of the extract was comparable to the diclofenac sodium, a reference drug (100 and 200 µg/mL). A significant difference in the inhibition of thermally induced protein denaturation was observed in case of extract when compared with standard drug at concentration of 100 µg/mL. Though at concentration of 200 µg/mL, inhibition activity of extract and diclofenac sodium were comparable.
Table 4: Percent inhibition of protein denaturation by Diclofenac sodium and extract

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
<th>Diclofenac sodium</th>
<th>EEMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>24.21706 ± 2.17</td>
<td>18.08855 ± 1.388</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>44.06048 ± 0.7512</td>
<td>28.38823 ± 1.634</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>51.41739 ± 3.395</td>
<td>54.46814 ± 1.467</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>64.9703 ± 0.8504</td>
<td>65.69924 ± 1.174</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>76.2149 ± 1.526</td>
<td>71.38229 ± 0.9324</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>85.09719 ± 0.8651</td>
<td>75.49946 ± 1.337</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=3.

Graph 3. Percent inhibition of protein denaturation by Diclofenac sodium and extract.

4. DISCUSSION

Medicinal plants since ancient time are lauded for their diverse pharmacological actions which could be attributed to the presence of secondary plant metabolites such as alkaloids, flavonoids, glycosides, tannins, steroids etc. Some of these plants are important source of natural antioxidants that have been shown to reduce the risk and progression of certain acute and chronic diseases such as cancer, heart diseases and stroke by scavenging free radicals which are implicated in the pathogenesis of many diseases.\[20,21\]

The results of preliminary phytochemical screening confirmed the presence of various classes of secondary metabolites in the Marchantia polymorpha extract including poly phenols (tannins and flavonoids). Plant polyphenols, produced either from phenylalanine or from its precursor shikmic acid, are important dietary antioxidants because they possess an ideal structural chemistry for free radical scavenging activity. Numerous in-vitro studies have conclusively shown their antioxidant potential in protecting against many diseases.\[22\] The
present study indicated that of *Marchantia polymorpha* is rich in polyphenols (19.31 mg/g of GAE of dry extract), but their total phenolic content is found to be lesser than the previously reported result in extract.[23] DPPH free radical scavenging activity is an easy and widely used method for testing in-vitro antioxidant activity of natural compounds or plant extracts.[24] DPPH is a stable free radical at room temperature, purple in color. Its reduction capability to accept an electron or a hydrogen radical from antioxidants is determined by measuring decrease in its absorbance values at 517 nm. DPPH radical scavenging activity of *Marchantia polymorpha* extract was compared with standard ascorbic acid in this study. Although standard antioxidant had higher scavenging activity at all tested concentrations than the extract, the extract still showed good free radical scavenging activity. The free radical scavenging property of *Marchantia polymorpha* may be one of the mechanisms by which this plant is effective as a traditional medicine. The consumption of the *Marchantia polymorpha* can be beneficial in preventing oxidative stress related degenerative diseases. Inflammation is a very common symptom of many chronic diseases. It is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. Inflammation is a protective attempt by the body to remove injurious stimuli as well as initiate the healing process for the tissue.[25] Non steroidal anti-inflammatory drugs are commonly used for the management of inflammatory conditions, but these are associated with many unwanted side effects such as gastric irritation, ulcer etc.[26] Medicinal plants used in traditional medicine to treat anti inflammatory conditions seem a viable and logical alternative in search of safe and effective anti-inflammatory agents. *Marchantia polymorpha* is commonly used traditional medicine in South Asian countries to treat inflammatory conditions; hence, a simple and viable protein denaturation bioassay method was selected to evaluate its potential as anti-inflammatory drug. It is a well known fact that denaturation of tissue proteins lead to inflammatory and arthritic diseases.[27] Natural products that can prevent protein denaturation therefore, would be worthwhile for development of anti-inflammatory drug therapy. *Marchantia polymorpha* extract and reference drug diclofenac sodium exhibited dose dependent percentage inhibition of heat induced protein denaturation in fresh egg albumin. Percent inhibition of protein denaturation with respect to control is a measure of protein stabilization.[28] Although *Marchantia polymorpha* extract showed a moderate free radical scavenging activity, its effect on inhibition of protein denaturation was found to be comparable with the standard drug diclofenac sodium. Thus it can be concluded that antiinflammatory activity of *Marchantia polymorpha* could be due to their high phenolic content. The results of our study suggest that *Marchantia polymorpha* are rich in phenolic
compounds and has a good antioxidant activity. It can be used as a natural source of antioxidants to prevent the progression of many diseases. *Marchantia polymorpha* extract also produced marked in-vitro anti-inflammatory activity that justifies its use in traditional system of medicine in Oman and other Asian countries. However, further detailed investigations are needed to ascertain the mechanisms and constituents behind its anti-inflammatory actions.

5. **Conflict of interest statement**

We declare that we have no conflict of interest. Acknowledgements Authors are thankful to Department of Pharmaceutical sciences, Kumaun University, Bhimtal Campus, Nainital, for providing the infrastructure and necessary research facilities to carry out the research work and UGC for providing fund.

6. **REFERENCES**


