Anti-Platelet, Anti-Hypercholesterolemic and Anti-Oxidant Effects of Ethanolic Extracts of Brassica oleracea in High Fat Diet Provided Rats

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Abstract: Cabbage is commonly used as a vegetable in several cuisines and as a herbal remedy in many ailments. In this study, some in vitro and in vitro tests were carried out. Biochemical parameters like total serum cholesterol, triglycerides, LDL and HDL were estimated enzymatically. Anti-platelet assay was performed by using ADP as an agonist in the aggregometer. Lipid peroxidation was calculated in serum using TBARS assay, while the anti-oxidant effect of cabbage was evaluated through the use of DPPH free radical scavenging assay. As compared to the control, the ethanolic extract of cabbage decreased total lipid, total cholesterol, triglycerides and LDL, while increased serum HDL. It also significantly increased the anti-atherogenic index ($p<0.05$). Furthermore, it significantly inhibited platelet aggregation as compared to the control group ($p<0.05$). TBARS assay decreased lipid peroxides to a considerable extent than the control. DPPH free radical scavenging assay showed strong inhibition of free radical at a concentration of 5mg/mL of water extract, as well as ethanolic extract at the same concentration. These results suggest that cabbage possesses certain medicinal effects that can potentially contribute to the prevention and treatment of various life-threatening conditions, like myocardial infarction, atherosclerosis and other associated modalities.

Key words: Atherosclerosis • LDL • HDL • Cabbage • Platelet aggregation

INTRODUCTION

Diseases are generally considered to be the consequences of severe disorders and abnormalities in body homeostasis [1]. In other words, severe disorders are mostly accompanied by many modalities which in turn worsen the condition. The major examples of such debilitating conditions are diabetes mellitus, cardiovascular diseases and kidney stone diseases. Among them, cardiovascular diseases, especially atherosclerosis, stand as one of the leading causes of deaths in both the developed and developing countries [2].

Numerous research studies have concluded that the initiation and progression of atherosclerosis is primarily due to severe impairments in different physiological body functions, such as elevated levels of cholesterol in the blood (hypercholesterolemia), oxidation of LDL cholesterol and its deposition in the arteries (oxidative stress) [3] plaque formation [4] vascular dysfunction, [5] and platelet aggregation [6]. Hypercholesterolemia has been accepted as the major issue in the initiation and progression of atherosclerosis, with \textit{“lower the cholesterol level, the better”} now becoming a widely accepted notion [7]. Among many research studies on this topic, the \textit{“Framingham Study”} clearly demonstrated the causal relation between elevated levels of cholesterol in the blood and atherosclerosis [8]. Furthermore, several recent studies have clearly demonstrated that hypercholesterolemia leads to the development of oxidative stress which, along with other factors, eventually results in the pathogenesis of atherosclerosis [9].

Scientists have concluded that the overproduction of reactive oxygen species (i.e. oxidative stress) plays a pivotal role in the oxidation of LDL molecules, which get accumulated in the layers of blood vessels and are considered as foreign bodies by macrophages. After the macrophages engulf these molecules, they are converted into the foam cells [10]. As a result of the proliferation of smooth muscle cells and calcification, these foam cells are converted into a hard substance known as plaque, which
increases in size at a snail's pace and produces resistance in the normal blood flow [11]. The plaque then ruptures and platelets start aggregating around it, which permanently blocks the blood flow [12]. Platelets release adenosine diphosphate (ADP), which facilitates platelet aggregation due to its direct effect on the plasma membrane. Platelets also release arachidonic acid when exposed to ADP, which is sequentially converted into thromboxane A2 via oxidation of arachidonic acid by cyclooxygenase and thromboxane A2 synthase. During this pathway, the released thromboxane A2 acts as a positive feedback mediator for the activation and aggregation of platelets which cause blood clotting [13]. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin and indomethacin generally block this pathway by inhibiting either one or both of the enzymes and their therapeutic effects can be explained by their direct inhibition of thromboxane and Prostaglandin (PG) biosynthesis [14]. Due to the similarity between the action of NSAIDs and herbal extracts, it has been hypothesized that some plants may also bring about this therapeutic effect, in part at least, by inhibiting PG biosynthesis.

Aspirin is globally accepted as a cardio-protective medicine, owing to its effective anti-platelet activity. Although it stands as the first drug of choice for cardiologists around the world, the side effects and drug resistance of aspirin limits its application to a great extent [15]. As a result, scientists have been dedicating their time and efforts to explore other treatment options for cardiovascular diseases.

For centuries, herbs have been fulfilling both nutritional and medicinal necessities of human beings [16]. Herbs are considered not only as preventive agents against different health associated problems, but also as curative agents against many life-threatening diseases [17]. Likewise, compounds isolated from natural sources can protect our body from different pathological conditions [18]. Among them, cabbage (Brassica oleracea) has been used as a vegetable as well as a herbal remedy for the treatment and prevention of various diseases in different parts of the world [19]. Many scientific studies have been carried out to assess the potential medicinal effects of cabbage and the results have clearly shown its beneficial effects in different conditions, particularly cholesterol metabolism [20]. These promising findings encouraged us to design the experiment under discussion, in order to ascertain the possible beneficial role of cabbage in treating the various modalities that lead to the progression and the severity of atherosclerosis.

**MATERIALS AND METHODS**

**Preparation of Ethanolic Extract of Cabbage:** Fresh cabbage was purchased from Empress Market Saddar (Karachi, Pakistan), were dried under shade and extracted with 80% ethanol. The extract was then filtered and dried by lyophilization (yield: 13.6% of dry weight).

**Animals and Diets:** Eighteen wistar rats were taken from the animal house of International Centre of chemical and Biological Sciences (ICCBS) (University of Karachi, Pakistan) and handled under the ethical guidelines of the institution. After two weeks of acclimatization, these rats were randomly divided into three groups: each group comprised of six rats and was kept in well ventilated cages in a controlled environment with regular diurnal cycles at an ambient temperature of 25 °C. Group A was treated as the control and the rats were provided normal lab diet. Group B served as the positive control group and was provided a high fat diet consisting of 20 percent animal fat (butter). The third group was the test and was also provided a high fat diet in the same ratio; however, ethanolic extracts of cabbage (500mg/kg of body weight) were also given by gavage to this group. After a period of 12 weeks, the rats were fasted overnight, anesthetized and then dissected. The blood was collected through cardiac puncture. For platelet aggregation assay, blood was collected in sterile tubes containing sodium citrate (3.8% w/v), whereas for the biochemical studies and measurement of thiobarbituric acid reactive species, the blood was collected in separate sterile tubes with no preservative. Serum was separated by centrifuging it at 1,000 rpm for 10 minutes.

**Biochemical Assay:** Total serum cholesterol, triglyceride, LDL and HDL were estimated enzymatically by using kits (Globe diagnostics, Italy). The color intensity of test samples was monitored in a spectrophotometer (Lambda 25 Perkin Elmer).

**Platelet Aggregation Assay:** Nine parts by volume of the blood samples were collected in sterilized tubes and clotting was prevented by adding one part by volume of sodium citrate (3.8% w/v). This mixture was then centrifuged at 1,000g. A supernatant was separated which contained platelets (i.e. Platelet Rich Plasma (PRP)), while the remaining pallet was re-centrifuged at 1,800g and then another supernatant was collected which did not contain platelets (i.e. Platelet Poor Plasma (PPP) [21].
Aggregation was monitored through the use of dual channel aggregometer (Model 400 Chronolog Corporation USA.). 450\mu L volume of PRP and PPP was used throughout the course of this experiment. Prior to the experiment, 0\% and 100\% light transmittance was adjusted with PRP and PPP respectively. Aggregation was induced by using 20\mu L ADP (5\mu M) as an agonist of aggregation. The percent inhibition of aggregation was calculated by using the following formula [22].

\[ \text{Percent Inhibition of Aggregation} = \frac{A-B}{A} \times 100 \]

Where A is the maximum aggregation recorded by control sample and B is the maximum aggregation after the addition of cabbage.

**Serum TBARS Assay:** Serum TBARS assay is an indirect measurement of oxidative stress by calculating lipid peroxidation species in the body. This was conducted similar to the methodology described above, but with slight modifications [23]. Briefly, serum was separated in sterile tubes by the above-mentioned method and 100\mu L serum was mixed with 500 \mu L trichloroacetic acid (TCA), which was then vortexed vigorously. Next, 200 \mu L of thiobarbituric acid (TBA) was added to it and the mixture was placed on heating blocks for 30 minutes at 95\(^\circ\)C. 800\mu L of n-butanol was then added and vortexed vigorously, after which the n-butanol layer was separated by placing the mixture in a centrifuge (Eppendorf 5810 R) at 6,000 g for 10 minutes. A supernatant was carefully separated from each tube and the optical density (100\mu L) of each supernatant was measured in 96 well plate (Sarstedt Trecan) ELISA reader. The values for malondialdehyde (MDA) products were calculated by comparing them to the MDA standard curve. The standards of 2.5\mu L, 5 \mu L, 10 \mu L and 20 \mu L were prepared and the results were expressed in terms of nMol/mL of serum [23].

**DPPH Assay:** DPPH assay was also carried out to investigate the antioxidant potential of ethanolic and water extracts of cabbage. 5mg/ml of ethanolic extract and 5 mg/ml of water extract were used in the preparation of this assay. Ethanolic and water extracts were prepared directly by adding 5mg crude extract in ethanol and water respectively, whereas the anti-oxidant assay was carried out through the previously described method [24].

**Anti-atherogenic Index:** The Anti-atherogenic index (AAI) is an important measure to elucidate the anti-atherogenic potential of a herb. This was calculated as follows [25].

AAI (%) = 100 x [HDL/Total cholesterol-HDL]

**Statistical Analysis:** The results were presented as mean \pm SEM and Mann-Whitney U-test was applied to the data and a difference of p<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

Cardiovascular diseases, particularly atherosclerosis, are considered to be the major causes of medical complications among humans that can lead to death and it has been estimated that atherosclerosis would be the number one cause of deaths worldwide by the year 2020 [26]. This condition is a result of a series of impairments in the normal body functions, most important of which are overproduction of cholesterol, redox imbalance and vascular dysfunction [27].

This study aimed at evaluating the preventive role of ethanolic extracts of cabbage in the modalities which eventually lead to atherosclerosis. Certain biochemical parameters like serum total cholesterol, triglyceride, LDL and HDL were measured to assess the preventive or curative role of this herb in hypercholesterolemia. Table 1 shows that ethanolic extracts of cabbage significantly reduce serum cholesterol to a considerable extent (p<0.05). The results obtained on the other parameters of total lipid, serum triglyceride, LDL and HDL also support this finding, though they exhibited a comparatively weaker relationship as compared to the serum cholesterol level. It is believed that more significant results could be obtained at higher doses than those used in this study.

In one of our earlier research studies, we had evaluated anti-platelet effects of many indigenous plants in vitro, including cabbage and found that cabbage has a high potential of inhibiting platelet aggregation in vitro [22]. Fig. 1 illustrates that in the present study, ethanolic extracts of cabbage showed promising anti-platelet effects in vivo using Adenosine Diphosphate (ADP) as an agonist of aggregation in the rat model, as it inhibited platelet aggregation induced by ADP at a very low concentration. Since ADP is considered a molecular agonist involved in platelet aggregation and the
Table 1: Effects of ethanolic extract (EE) of cabbage on lipid profile in diet induced hyperlipidemic rats. (mg/dL)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total lipid</th>
<th>TAG</th>
<th>Cholesterol</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>AAI(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>404.1±8.4</td>
<td>155.5±3.6</td>
<td>47.3±2.5</td>
<td>30.7±2.1</td>
<td>34.8±2.9</td>
<td>251.08±27.1</td>
</tr>
<tr>
<td>HFD Control</td>
<td>457.1±26.6</td>
<td>178.5±12.8</td>
<td>52.5±3.1</td>
<td>29.1±1.5</td>
<td>44.6±2.6</td>
<td>130.06±14.3</td>
</tr>
<tr>
<td>EE with HFD</td>
<td>422.6±7.6</td>
<td>170.4±4.7</td>
<td>40.3±1.7</td>
<td>28.1±0.5</td>
<td>42.9±3.3</td>
<td>259.05±41.1</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SEM of 6 male wistar rats. Significance of differences with respect to the control group was evaluated by Mann-Whitney U-test (*p<0.01)

Table 2: Effects of ethanolic extract (EE) of cabbage on MDA level in diet induced hyperlipidemic rats using TBARS assay

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA level (nmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>HFD Control</td>
<td>0.41±0.1</td>
</tr>
<tr>
<td>Test Sample</td>
<td>0.28±0.01</td>
</tr>
</tbody>
</table>

Results expressed as ±SEM of 6 male wistar rats

Table 3: Effects of ethanolic extract (EE) (5mg/mL) and water extract of cabbage (5mg/mL) on DPPH free radical scavenging assay

<table>
<thead>
<tr>
<th>Extract %</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Extract (WE)</td>
<td>92.1±1.21</td>
</tr>
<tr>
<td>Ethanolic Extract (EE)</td>
<td>92.4±1.07</td>
</tr>
</tbody>
</table>

Results expressed as SD ± of test run in triplicate

Fig. 1: Platelet anti-aggregation effects of cabbage.
Figure shows percentage of platelet aggregation. Bar no.1 represents percent aggregation in control group while bar no.2 represents percent aggregation in the group which was provided the ethanolic extract of cabbage. Significance of differences with respect to the control group was evaluated by Mann-Whitney U-test (*p<0.01)

The in vitro anti-oxidant activity of water and ethanolic extracts of cabbage was also evaluated in this study by using DPPH free radical scavenging activity. Table 3 reveals that the ethanolic and water extracts possess significant, as well as comparable anti-oxidant effects at the concentration of 5mg/mL. Therefore, these results not only confirm previous findings, but also highlight that ethanolic and water extracts of cabbage exhibit similar activity levels in this assay system.

The life-threatening condition atherosclerosis is believed to be a result of high cholesterol levels in blood, oxidative stress, platelet aggregation and vascular dysfunction. The aim of this study was to evaluate the possible beneficial effects of cabbage in the prevention of the early events that lead to the initiation and progression of atherosclerosis. The results of this study show that cabbage possesses a promising potential in the prevention and treatment of atherosclerosis, as well as other related modalities like hypercholesterolemia, platelet aggregation and oxidative stress. On the basis of these findings, further studies will be carried out on molecular basis to investigate broader approaches and applications in the treatment and prevention of these debilitating medical conditions.

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