In vitro anticoagulant and antiinflammatory activities of Geastrum fimbriatum Fr., namely as Earthstar fungus

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Abstract: Mushrooms have great potential to be used as food and pharmaceutical sources. Most of the non-edible mushrooms contain biologically active metabolites that are functional for modern medicinal applications. Within the present study, anticoagulant and antiinflammatory activities of Geastrum fimbriatum Fr. (Syn. Geastrum sessile (Sowerby) Pouzar), a mushroom naturally grown in Turkey, were investigated. The in vitro anticoagulant activity of the ethanolic extract obtained with a soxhlet apparatus determined by activated partial thromboplastin time (APTT) and prothrombin time (PT) assays using commercial reagents. The antiinflammatory activity of the extract was determined by lipoxygenase inhibition assay. When compared with the negative control DMSO, G. fimbriatum extract exhibited significant anticoagulant effects in the APTT test that evaluates the intrinsic coagulation pathway. The ethanolic extract found to prolong the coagulation time. However, no inhibition was observed in the PT test which evaluates the extrinsic coagulation pathway. The extract showed 12.92% inhibition on the lipoxygenase enzyme activity. Overall, G. fimbriatum ethanolic extract exhibited potent antiinflammatory activity besides being a potential source of anticoagulant. Further analysis is required to evaluate the medical use of Geastrum mushrooms from a pharmaceutical point of view.

1. INTRODUCTION

Health problems in heart and blood vessels and also thrombosis are the major causes of death in the World [1]. It is reported by World Health Organization [2] that an estimated 17.5 million people died from cardiovascular diseases (CVDs) in 2012, representing 31% of all global deaths. It is supposed that 7.4 million of those people died due to coronary heart disease and 6.7 million died because of stroke [2]. CVDs are a group of disorders of the heart and blood...
vessels such as coronary, cerebrovascular and rheumatic heart diseases, deep ven thrombosis and pulmonary embolism [2]. Most require anticoagulant therapy is generally required for thromboembolic processes. Recently researchers are focusing on developing specific and potent anticoagulant and antithrombotic agents [1]. Anti-coagulants that mentioned above present some restrictions to their use, and some are used under a critical control due to hemorrhagic risk and limitation in the administration, however, they have effective use [3]. That’s why investigating new, safer, and more effective anti-coagulant drugs, with less hemorrhagic risk and fewer side effects or interaction with drugs and food has been gaining much importance [4].

Inflammation is a complex biological process, induced by microbial infection or tissue damage [5]. There are different types of rheumatic disorders such as rheumatic fever, rheumatoid arthritis, ankylosing spondylitis, polyarthritis nodosa, systemic lupus erythematosus and osteoarthritis, which are called as inflammatory diseases [6]. Inflammation is a body defense reaction which tries to eliminate or limit the spread of injurious agent. Inflammatory reaction involves various components that can contribute to the associated symptoms and tissue injury. These components may be listed as oedema, leukocyte infiltration, and granuloma formation [7].

Arachidonic acid is released from the cell membranes due to the cell damage associated with inflammation [8]. There are two metabolic pathways that arachidonic acid undergoes: the cyclooxygenase (COX) pathway involving cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) to produce the prostaglandins and thromboxanes [9]; and the lipoxygenase (LOX) pathway, involving 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX), to produce the leukotrienes and hydroperoxy fatty acids [10,11]. Lipoxygenases are involved in the biosynthesis of leukotrienes which have a key role in several inflammatory diseases such as cancer, arthritis, asthma and allergic diseases. For this reason, lipoxygenase inhibitors may be potentially used with their medicinal benefits in prevention of these inflammatory cases [12].

Lipoxygenases (LOXs) (LOX; EC 1.13.11.12) are the group of non-heme iron-containing dioxygenases which catalyze the biosynthesis of leukotrienes. Leukotrienes take part as initiators of inflammation so their inhibition is directly related to anti-inflammatory activity [13].

Mushrooms have been used since long times for the treatments of various diseases, but still it has not been approved in main stream science as drugs or medical treatments [14-16]. Both fruiting body and mycelia of different mushroom known to contain different compounds such as flavonoids, alkaloids, polysaccharides, polyglucans, polyphenol, steroids, terpenoids, polyketides and dietary fibers which exert several pharmacological activities [17-18]. Geastrum species are mushrooms of the class Gasteromycetes in which the hymenium is enclosed until spores are matured. This genus has cosmopolitan distribution especially in the sandy soil forests of Asia, Africa, Australia, Europe, Mexico, North America and South America [19-21]. Medicinal uses of Geastrum species have not been well documented but some members of this class have been reported to have bioactive potentials [22-23]. In the study reported the rare appearance of Geastrum species and its ethnomedicinal uses as wound healing by ethnic tribes of Northern Odisha, India [24]. Further, some species of Geastrum have also been reported to have antimicrobial activity from southern India [25].
In this study the anticoagulant and antiinflammatory activities of the ethanolic extract of *G. fimbriatum* were investigated. To our knowledge, this is the first study related to the anticoagulant and antiinflammatory activities of this mushroom.

2. MATERIALS AND METHODS

2.1. Mushroom Collection and Extraction Procedure

The mushroom samples were collected from Golcuk village (Gediz-Kutahya), Turkey and identified by Dr. Alli (Figure 1). The samples were brought to the laboratory, dried in an incubator at 40°C. The dried and powdered mushroom samples (20 g) were extracted with ethanol (Merck) (400 ml) using the Soxhlet apparatus at 4 h. The extract was evaporated and then kept in small sterile opac bottles under refrigerated conditions until used [26,27].

2.2. Anticoagulant Activity

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were performed using commercial human plasma (purchased from Sigma Aldrich). 10 mg/ml concentration of the *G. fimbriatum* extract was prepared with pure dimethyl sulphoxide (DMSO). The APTT and PT were measured via Pacific Hemostasis™ APTT and PT reagents (Thermo Fisher Scientific) according to the manufactures guidelines.

In the APTT test, the calcium chloride (0.02 M) prewarmed at 37°C. 100 µl of commercial plasma (Sigma, P9523) and 10 µl of the *G. fimbriatum* extract (10 mg/ml) transferred to the tube and also prewarmed at 37°C. After then, 100 µl of APTT-XL added to the tube and mixed. The plasma-reagent mixture was incubated at 37°C for 3-5 minutes (activation time). Forcibly added 100 µl prewarmed calcium chloride and coagulation times in the tubes were recorded [28,29].

In the PT test, 100 µl of commercial plasma (Sigma, P9523) and 10 µl of the *G. fimbriatum* extract (10 mg/ml) were transferred to the test tube. The mixture was incubated at 37°C for 1 min and then 200 µl of heated Thromboplastin-DS (at 37°C) added to the tube. Immediately the stopwatch was operated and coagulation times in the tubes were recorded [28,29].

Acetylsalicylic acid (0.5 mM final concentration) and pure DMSO were used as positive control and negative control, respectively.
2.3. Lipoxygenase Inhibition Assay

15-Lipoxygenase (15-LOX) inhibition activity was assayed using the Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s guidelines. 15-LOX from soybean was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Linolenic acid was used as a substrate [30].

Residual 15-LOX activity was determined after pre-incubation for 5 min at room temperature with 100 µg/ml G. fimbriatum extract. Firstly, 10 µl of G. fimbriatum extract (100 mg/ml), 90 µl of 15-LOX, 10 µl of 0.1 M Tris-HCl buffer (pH 7.4) and 10 µl of 1 mM linolenic acid were added to a plate and incubated for 5 min. Then, the reaction was stopped by adding 100 µl chromogen and the plate was incubated in a shaker for 5 minutes and the absorbance was measured at 550 nm. The 100% initial activity was obtained from the well which incubated with DMSO. Nordihydroguaiaretic acid (NDGA) at 100 µM final concentration was used as a positive control. Data analysis was performed according to the instructions supplied with the assay kit.

3. RESULTS

In this study, the anticoagulant and antiinflammatory activities of the ethanolic extract of G. fimbriatum were investigated. To carry out coagulation tests, two standard in vitro tests, including PT and APTT tests were applied. These two tests cover both intrinsic and extrinsic blood coagulation pathways. This assay was made with commercial human plasma, using acetylsalicylic acid as a positive control. The anticoagulant activity of the G. fimbriatum extract was shown in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PT (sec)</th>
<th>APTT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. fimbriatum extract</td>
<td>22</td>
<td>67</td>
</tr>
<tr>
<td>Negative control (DMSO)</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>23</td>
<td>71</td>
</tr>
</tbody>
</table>

The G. fimbriatum extract exhibited notable anticoagulant effects in the APTT test that was applied to evaluate the intrinsic coagulation pathway, compared with the negative control DMSO. The extract prolonged the time of coagulation. On the other hand, in the PT test that was applied to evaluate the extrinsic coagulation pathway, no inhibition was observed with the extract.

The antiinflammatory activity of the G. fimbriatum extract was determined with the lipoxygenase inhibition capacity. The extract showed 12.92% inhibition on the LOX enzyme activity (Table 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. fimbriatum extract</td>
<td>100 µg/ml</td>
<td>12.92</td>
</tr>
<tr>
<td>NDGA</td>
<td>100 µM</td>
<td>40.00</td>
</tr>
</tbody>
</table>
4. DISCUSSION

Enzyme promoting processes are involved in the blood coagulation [31]. At the end of these processes; fibrin is formed from fibrinogen and blood is transformed to gel state from colloidal state. An enzyme, the thrombase, effects the hemostasis and blood coagulation [32]. In order to explain the possible hemostatic mechanism, the compounds were tested for PT and APTT assays. PT is mainly applied to evaluate the extrinsic coagulation pathway while APTT is related to the intrinsic coagulation pathway [33]. When APTT prolongs, it suggests the inhibition of the intrinsic and/or the common pathway. On the other hand, PT prolongation gives information about the inhibition of the extrinsic and/or the common pathway [34].

Anticoagulants play a role in the prevention and treatment of thromboembolic disorders [35-36]. Anticoagulant drugs consisting of heparin and its derivatives, and vitamin K antagonists, have been the main anticoagulants in clinical practice. Despite their efficacy, major and life-threatening side effects of these agents have also been reported [37,38].

Anticoagulant drugs become crucial when there is a failure throughout the hemostasis; which leads to blood coagulation that could cause a vessel occlusion; unfractionated heparin (UFH), coumarin, low molecular weight heparin (LMWH), and the most recent drug introduced in the market - the synthetic pentasaccharide fondaparinux are the current medical options [3].

The use of anticoagulants is under a critical control as there are some restrictions for their use and also it needs to pay attention due to hemorrhagic risk and limitation in the administration [3]. Nowadays researchers focus on developing new, safer, and more effective anti-coagulant drugs, with less hemorrhagic risk and fewer side effects or interaction with drugs and food [4].

Most pharmaceutical drugs have side effects that may be experienced alongside their therapeutic actions [39]; for example, warfarin, a commonly used anticoagulant drug, demonstrates side effects that include bruising, bleeding gums, red or dark brown urine, red or black bowel motions, nosebleeds, haemoptysis, dyspnoea and dysphagia, heavier than usual menstrual periods, excessive wound bleeding, dark or blood stained vomit, severe headache and dizziness, unexplained pain, swelling and discomfort [40].

However, traditional remedies have long been known to have fewer adverse effects [41]. Therefore, the authors claim that the use of herbs with therapeutic effects and minimal side effects, could be the best option for the prevention and treatment of a wide range of diseases including cardiovascular disorders [42].

LOXs are the group of enzymes involve in the biosynthesis of leukotrienes that play an important role in the pathophysiology of various inflammatory diseases. The LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation (5-LOX, 9-LOX, 12-LOX, 15-LOX) [43].

LOX enzymes are directly related to inflammatory and allergic reactions due to the formation of the leukotrienes (LTs). LTs values may increase in the case of allergic rhinitis, rheumatoid arthritis, psoriasis, asthma and colitis ulcerosa. Inhibition of the lipoxygenase pathway prevents the production of LTs. As a useful tool for cancer treatment, therapeutic strategies for inhibition of L isoforms and/or their biologically active metabolites may be biologically and pharmacologically targeted by using lipoxygenase inhibitors [44].

Medicinal mushrooms are recognized with their extracts that profound health benefits and functionally used for traditional therapies. There are extensive studies that demonstrating the in vitro or in vivo biological activities of extracted and/or purified mushroom species such as antitumor and immunomodulating activity [45,46], anticancer [47,48], antioxidant [49], antiviral [50], antihypercholesterolemia [51], antidiabetic [52] effects.
There are drugs in the market that are used to treat antiinflammatory disorders but most of them are not toxically free. The use of antiinflammatory drugs may lead to gastrointestinal problems which is a problem for medical industry [53]. Thus, investigation of a natural antiinflammatory drug with functional properties is a necessity for pharmaceutical technology. Similar to our study, Guerradore et al. [54] reported that Geastrum saccatum extract inhibited the enzyme cyclooxygenase and had a promising antiinflammatory activity. Geastrum species have also antioxidant [54,55], antibacterial [56], and cytotoxic [54] activities. Although there are not any studies about G. fimbriatum, a nature mushroom growing in Mugla. The present study presents an important view about the anticoagulant and antiinflammatory activities of G. fimbriatum that potentially active.

5. CONCLUSION

Studies with mushrooms have been developing recently and it is figured out that potent properties of secondary metabolites from different mushroom species show great biological activities. Besides being an important dietary source, mushrooms are strong pharmaceuticals that reveals multiple therapies to diseases. There is a growing interest in active metabolites that are obtained from natural sources as an alternative to synthetic drugs. Anticoagulant and antiinflammatory characteristics of an agent enhance its functionality. In the present research, the potential use of mushroom extract as an anticoagulant and/or antiinflammatory agent has been evaluated. From the results of the study, it can be concluded that G. fimbriatum extract is endowed with effective anticoagulant and antiinflammatory activities. Comprehensive studies may be required to investigate the health-related activity of this mushroom.

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6. REFERENCES


