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EVALUATION OF PHYTOCHEMICALS AND *IN* VITRO ANTI-INFLAMMATORY, ANTI-DIABETIC ACTIVITY OF THE WHITE OYSTER MUSHROOM, *PLEUROTUS FLORIDA*

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ABSTRACT

Phytochemicals which are present in the human diets possess a number of beneficial effects on human health. Mushrooms are invaluable sources of useful therapeutic agents. Phytochemical screening of edible white oyster mushroom, *Pleurotusflorida* reveals the presence of phenols (61.85mg catechol equivalent), flavonoids 2.78mg , alkaloids 1.92mg, terpenoids 0.08 mg , tannins 0.52mg, saponins 0.05mg and glycosides 0.12mg in 100g dried powder. Methanol extract of the mushroom was assessed for its anti-inflammatory and anti-diabetic by *In* vitro methods. Anti-inflammatory activity of mushroom was investigated by Human Red Blood Cell Membrane (HRBC) stabilization method. Results showed that the extract exhibited significant anti-inflammatory activity of 43.50% at the concentration of 1.0mg/ml which is comparable to that of the standard diclofenac sodium. The mushroom extract also showed significant anti- diabetic (26.5%) activity by inhibition of α-amylase (E C 3.2.1.1) at the concentration of 1.0 mg/ml. From the result, it s concluded that white oyster mushroom,*Pleurotusflorida*can be administered for its anti-inflammatory and anti-diabetic activities.

Keywords: Phytochemical, anti-inflammatory, anti-diabetic, *Pleurotus florida*, Diclofenac.

INTRODUCTION

Mushrooms are abundantly available throughout the world, and are mainly focused than ever before because they have the capability of producing many benefits indeed to mankind especially in the line of medicine. White oyster mushroom, *Pleurotusflorida* has been regarded as an edible mushroom for many years [1], and are being cultivated widely. This mushroom has unique flavor, rich nutrients and phytochemicals. Phytochemicals are naturally occurring compounds that works with nutrients to act against diseases or more specifically provides protection against diseases [2]. It has been reported that the anti-oxidant activity of phytochemicals in plants are responsible for their therapeutic effect against cancer, cardiovasclar diseases, inflammation lowering cholesterol and glucose [3]. Some of the most recently isolated and identified phytochemicals from mushrooms have been demonstrated to possess significant anti-tumor, anti-inflammatory, hepatoprotective and anti-diabetic properties [4]. Oyster mushrooms are also reported to be a rich source of phenolics, alkaloids and flavonoids and are responsible for anti-heliminthic activity [5]. Inflammation is a multi-faceted response mediated by the activation of cells of immune system in response to the invasion by a foreign body. Inflammation can also accelerate cancer and chronic inflammation which is regarded as an essential factor for the progression of the neoplastic process [7]. For chronic diseases such as osteoarthiritis and rheumatoid arthiritis, lifelong dependency on anti-inflammatory drugs is necessary. The most widely used non-steroidal anti-inflammatory drugs (NSAID's) cause several side effects. Also it is well known that the incidence of diabetes mellitus is high all over the world, especially in Asia [8].

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Different types of synthetic oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, but have side effects, including hematological coma and disturbances of liver and kidney [9]. Hence, the search for effective anti-inflammtory and anti-diabetic agents of natural origin that could be used on a long term basis without any side effects is a priority.

Keeping in view the wide distribution and easy cultivation of White Oyster mushroom, namely *Pleurotus florida*, the present study was conducted to evaluate its phytochemicals and to investigate the anti-inflammatory and anti-diabetic activity *invitro*.

MATERIALS AND METHODS

Standards and reagents

Folin-ciocalteu phenol reagent**,** 3, 5-dinitro salicylic acid, potassium tartarate, Catechol, Tannic Acid Standard, Saponin Standard, Folin-Denis Reagent, Buljet's Reagent, Sodium Carbonate, Ammonium Hydroxide, sodium Hydroxide, Magnesium Carbonate were obtained from Sigma (St Louis, Mo, USA). All other chemicals and solvents used were of analytical grade.

Sample Collection and preparation

Fruit bodies of *Pleurotusflorida*, were collected from the mushroom house, Department of Biology, Gandhigram Rural Institute – Deemed University, Gandhigram - Dindigul, Tamilnadu. Freshly harvested mushrooms were solar dried and finely powdered. Mushroom powder was stored in an air tight container in a refrigerator for phytochemical analysis. Twenty five grams of the dried powder were extracted successively with methanol over night using soxhlet apparatus. Extract was filtered through vacuum filter and the filtrate was concentrated in vacuum evaporator. The methanol extract was used for further pharmaceutical studies.

Determination of Total Phenols

The total phenolic content was determined according to the method described by Siddhuraju and Manian[10]. 0.1ml of mushroom extracts were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as the catechol equivalents.

Determination of Alkaloids

Alkaline precipitation gravimetric method described by Harborne [11] for determination of alkaloid was followed. 10gm of the sample was dispersed in 10% acetic acid solution in ethanol to form a ratio of 1:10 (10%). The mixture was allowed to stand for 4h at 28ºC. It was later filtered via Whatman No. 42 filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation and treated with drop wise addition of Conc. aqueous NH_4OH until the alkaloid was precipitated. The alkaloid precipitated was received in a weighed filter paper, washed with 1% ammonia solution dried in the oven at 80ºC. The resulting weight difference gave the weight of alkaloid in the sample.

Determination of Flavonoids

Flavonoids were determined according to the method of Harbone. 5gram of the sample was boiled in 50ml of 2M HCl solution for 30min under reflux. It was allowed to cool and then filtered through Whatman No 42 filter paper. A measured volume of the extract was treated with equal volume of ethyl acetate starting with a drop. The flavonoid precipitated was recovered by filtration using weighed filter paper. The resulting weight difference gave the weight of flavonoid in the sample.

Determination of Tannins

The method by Harbone was used for the determination of tannin. 0.2 g of finely ground sample was taken in a 50 ml beaker. 20 ml of 50% methanol was added and covered with paraffin film and placed in a water bath at 77-80^{o}C for 1 h and stirred with a glass rod to prevent lumping. The extract was filtered using a double layered Whatman No.1 filter paper into a 100 ml volumetric flask using 50% methanol to rinse. This was made up to mark with distilled water and thoroughly mixed. 1 ml of sample extract was pipetted into 50 ml volumetric flask, 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na2CO3 were added and mixed properly. The mixture was made up to mark with distilled water, mixed well and allowed to stand for 20 min. Standard tannic acid solutions of range 0-10 ppm were treated similarly as 1 ml of sample above. The absorbances of the tannic acid standard solutions as well as samples were read after colour development on a spectrophotometer at a wavelength of 760 nm.

Determination of Saponin

The Spectrophotometric method described by Brunner [12] (1984) was used for saponin analysis. 1 g offinely ground sample was weighed into a 250 ml beaker and 100 ml Iso butyl alcohol was added. The mixture was shaken on a UDY shaker for 5 h to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No. 1 filter paper into a 100 ml beaker and 20 ml of 40% saturated solution of magnesium carbonate was added. The mixture obtained with saturated MgCO3 was again filtered through a Whatman No 1 filter paper to obtain a clear colourless solution. 1 ml of the colourless solution was pipetted into 50 ml volumetric flask and 2 ml of 5%FeCl3 solution was added and made up to mark

with distilled water. It was allowed to stand for 30 min for blood red colour to develop. 0-10 ppm standard saponin solutions were prepared fromsaponin stock solution. The standard solutions were treated similarly like the sample above. The absorbance of the samples as well as standard saponin solutions was read after colour development on a spectrophotometer at a wavelength of 380 nm.

Determination of Gylcosides

Glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy [13]. 1g of the fine powder of *P. florida* was soaked in 10ml of 70% alcohol for 2hrs and then filtered. The extract obtained was then purified using lead acetate and Na2HPO4solution before the addition of freshly prepared Buljet's reagent (containing 95ml aqueous picricacid + 5ml 10% aqueous NaOH). The difference between the intensity of colours of theexperimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

***In Vitro* Anti-inflammatory Assay**

Human red blood cell membrane stabilization method [14] was used for this study. The blood (10 ml) was collected from the healthy human volunteer who was not taken any NSAID's for 2 weeks prior to the experiment. Aseptically blood was transferred to the heparinized centrifuged tube. The tubes were centrifuged at 3000rpm for 10min and were washed three times with equal volume of isosaline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline. Various concentrations of extracts were prepared (0.5mg/ml,1.0mg/ml) using distilled water and to each concentration 1ml of phosphate buffer, 2ml isosaline and 0.5 ml of packed cell suspension were added and incubated at 37ºC for 30 min and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant solution was read at 560nm. The percentage haemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization was calculated using the following formula.

% Membrane Stabilization =

$$\frac{\text{100 - OD of Extract treated sample}}{\text{OD of Control}} \times 100$$

Assay for α-amylase inhibition

The bio assay method was adopted and modified from Sigma-Aldrich (www.sigmaaldrich.com). A starch solution (0.1%w/v) was obtained by stirring 0.1 g of potato starch in 100ml of 16mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5mg of α-amylase in 100ml of distilled water. The colorimetric reagent was prepared by mixing sodium potassium tartarate solution and 96mM 3, 5-dinitro salicylic acid solution. Both control and mushroom extracts (0.5mg/ml & 1.0mg/ml) were added with starch solution and left to react with α-amylase solution under alkaline condition at 25ºC. The reaction was measured over3 minutes. The generation of maltose was quantified by the reduction of 3, 5-dinitro salicylic acid to 3- amino-5-nitro salicylic acid. The reaction is detectable at 540nm. The anti-diabetic activity was investigated through the inhibition of α-amylase, an enzyme that made the digestion of starch and so reduced the glucose absorption. The α-amylase inhibition was expressed as a percentage of inhibition and calculated by the following equation.

% Reaction =

$$\frac{\text{(Maltose) Test}}{\text{(Maltose) Control}} \times 100$$

% Inhibition = 100 - % reaction ±SD

Statistical analysis

All experiments were conducted in triplicates and the parameters were given as means ± standard error. Both mean and standard deviation were performed where appropriate, using the statistical package within Microsoft® Excel Version 2007.Ink.

RESULTS

Phytochemical Analysis

The mushroom sample tested for the evaluation of phytochemicals showed (Table-1) phenol in terms of catechol equivalent, flavonoids and alkaloids as major constituents. Tannins, glycosides in small amounts and saponins, terpenoids in vestigial amounts (≤ 0.10g).

***In vitro* anti-inflammatory activity**

The methanol extract of *P.florida* tested for HRBC membrane stabilization was found to be concentration dependent (Table-2). Red blood cell membranes are similar to the lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity. The maximum activity at 1.0mg/ml for the methanol fraction was 43.50%. This is comparable to that of the standard diclofenac sodium.

***In vitro* anti-diabetic activity**

The anti-diabetic activity was investigated through the inhibition of α- amylase, an enzyme that digests starch. The results (Table-3) revealed 20.7% and 26.5% of inhibition for the methanol extract of *P.florida*at the concentration of 0.5mg/ml and 1.0mg/ml respectively.

Table 1. Phytochemical Analysis of *P. florida*

S.No	Parameters	Result mg/100g of sample
1	Phenols	61.85mg catechol Equivalent
2	Alkaloids	1.92
3	Flavonoids	2.78
4	Terpenoids	0.08
5	Saponins	0.05
6	Tannins	0.52
7	Glycosides	0.12

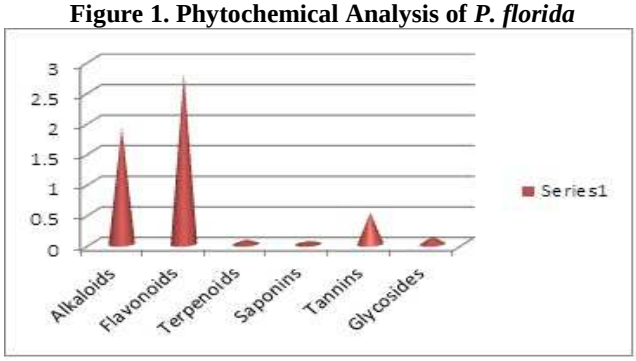
Table 2. *In vitro* anti-inflammatory activity of methanol extract of *P. florida*

S.No	Concentration mg/ml	% Protection	
		Mushroom Extarct	Standard
1	0.25	09.14	-
2	0.50	18.66	72.31
3	1	43.50	-

Table 3. *In vitro* anti-diabetic activity of methanol extract of *P. florida*

S.No	Concentration mg/ml	% Inhibition
1	0.25	13.78
2	0.50	20.7
3	1	26.5

Figure 1. Phytochemical Analysis of *P. florida*



DISCUSSION

For millennia, mushrooms have been valued by mankindas an edible and medicinal source. Attempts have been madein many parts of the world to explore the mushrooms andtheir metabolites for the treatment of a variety of humanailments. But still a vast wealth of mushrooms has not beenexplored which may contain active medicinal properties ofcuring a number of diseases. Therefore, the pharmaceutical screening studies should be concentrated on the mushrooms for their potential values. The presentinvestigation was carried out by *in vitro* anti-inflammatory and anti-diabetic analysis to characterize the White oyster mushroom *Pleurotus florida* as important raw drug material.

The total phenolic content was estimated to contain 61.85mg catechol equivalent, alkaloids, flavonoids, tannins, terpenoids, saponins and glycosides which varies quantitatively from low to highly present. These results on *P.florida* are in correlation with the previous work on mushrooms [15]. Alkaloids and flavonoids have been isolated from hundreds of species of mushroom that are effective against many chronic diseases [16] and the phytochemical constituents of *Pleurotus florida* confirmed it to be one among these. The medicinal value of the mushroom may be due to the presence of secondary metabolites [17].

These metabolites present in the human diet possess a number of beneficial effects on human health such as anti-oxidant, anti-allergic, anti-viral, anti-diabetic and anti-carcinogenic [18]. The myoconstituent triterpenoid in *Ganoderma lucidum* might be responsible for its anti-oxidant and anti-inflammatory activity [19]. Saponins cause cytotoxic effects and are beneficial in

cholesterol and blood sugar lower [20]. The relationship between the oxidative stress and inflammation has been investigated and reported in many edible mushrooms [21]. With this background knowledge as supporting evidence *in vitro* pharmaceutical study was carried out.

Thus from the HRBC assay, stabilization of the RBC's membrane was evaluated for the anti-inflammatory activity of *P. florida*. The extract was effective in inhibiting the heat induced haemolysis at different concentrations. These provide evidence for membrane stabilization as a mechanism of their anti-inflammatory effect. This extract may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and proteases, which upon extracellular release causes further tissue inflammation and damage [22]. The results obtained were in concurrence with the anti-inflammatory activity of the *S.colais* [23] and *S.cochinchnensis* [24] they exhibited membrane stabilization effect which is concentration dependent. Inhibition of α-amylase assay was used to evaluate the *in vitro* anti-diabetic activity of the *P.florida*. The activity was investigated through the inhibition of α-amylase, an enzyme that made the digestion of starch and so reduced the glucose absorption. Similar results were obtained on the anti-diabetic activity *in vitro* studies on *Amaranthus caudatus* seeds [25].

The medicinal values of mushrooms therefore may be attributed to the presence of these phytochemicals. Thus in nutshell, myoconstituents of *Pleurotus florida* might be responsible to show anti-diabetic and anti-inflammatory effects *in vitro*. Furtherer studies with animal models, on isolation and identification of the active compounds may provide a better source for developing new therapeutic agents against harmful allopathic drugs.

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