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Research Article
Membrane Stabilization Activity of Amino Acids Rich Chromatography Fractions from *Pleurotus pulmonarius* (Fr.) Quel. (Pleurotaceae)

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Abstract

Background and Objective: Amino acids are not just important for protein synthesis but are important for whole body homeostasis. Mushrooms aside being used for food and for medicine are also good sources of amino acids that can be used as a nutraceuticals. This study evaluated the Aqueous Ethanol Extract (AEE) of the defatted *Pleurotus pulmonarius* and its amino acids containing fractions for their Membrane Stabilization (MS) properties. **Materials and Methods:** Air dried fruiting bodies (150 g) of *P. pulmonarius* were defatted with n-hexane and dichloromethane successively prior to extraction with 80% aqueous ethanol. Fractionation of AEE was done by using column chromatography and the amino acids derivative rich fractions identified using TLC with ninhydrin as spray reagent. *In vitro* MS assay was done using the heat induced human erythrocytes haemolysis model. **Results:** The AEE afforded 2 amino acids derivative containing fractions T2 and T3 in addition to T1 devoid of amino acids constituents. Trend in MS activity (IC_{50} μg mL^{-1}), AEE 524.8)>T2 (403.4)>T3 (40.5)>Aspirin (6.8)>Indomethacin (0.37) was obtained. The TLC analysis for T2 has amino acids at R_f 0.23 similar to that obtained for glycine in addition to two other amino acids at R_f 0.28 and 0.38. T3 have two amino acids spot at R_f 0.08 and 0.13. **Conclusion:** The MS activity justified *Pleurotus pulmonarius* as a potential source of agents for the development of nutraceutical and drug lead compounds for the management of diseases associated with inflammation and oxidative stress.

Key words: *Pleurotus pulmonarius*, amino acids derivatives, anti-inflammation agents, nutraceutical, antioxidants, inflammatory diseases, protein synthesis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The desire and search for natural anti-inflammatory agents have in recent times increased due to their obvious advantages considering the drawbacks of the non-steroidal anti-inflammatory agents[1-3]. Complications from inflammation are highly associated with the pathophysiology and mortality in chronic diseases like cancer, rheumatoid arthritis, septic shock, heart attack and diabetes among others[4]. When inflammation response is triggered, the activation and release of inflammatory mediators sets in the cells. This will cause vasodilation and increase permeability of blood vessels leading to the leakage of plasma proteins and fluids into the injured tissues[5] which could be prevented through membrane stabilization. The stabilization of lysosomal membrane which is structurally similar to the human red blood cell membrane is important to prevent the inflammatory processes[6]. It has been shown that amino acids are not just important for protein synthesis, but also take up regulatory functions both in the cell and tissue levels[7,8]. Edible and medicinal mushroom species are known to be a good source of food nutrients and biologically active compounds that aids the proper functioning of the body physiology thereby improving the health[9]. The anti-cancer, anti-inflammatory and other health promoting benefits of several polysaccharide protein complexes and related amino acids derivatives from several species of the mycoflora have been documented[10-15]. *Pleurotus* species (Pleurotaceae) have been reported to be a good source of amino acids[16] and protein[15]. Aside being edible, the medicinal properties of several *Pleurotus* species such as: the anti-inflammatory potential of *Pleurotus eryngii*[15], antioxidant properties of oyster mushrooms *Pleurotus ostreatus* and *Pleurotus sajor-caju* and their amino acids[16], analgesics activity of *Pleurotus ostreatus* extracts and fractions[17], inhibition of colitis-associated colon carcinogenesis in mice by glucans from *Pleurotus pulmonarius*[18] and the immunomodulatory activity of pleuran a polysaccharide from *Pleurotus ostreatus*[19] and anti-inflammatory properties of exopolysaccharides from *Pleurotus sajor-caju*[20] are documented. Thus, they can serve as functional foods as well as drug lead source. As a follow up to the previous report on the bioactivities of *Pleurotus pulmonarius* extracts and isolated compounds[21]. Also, considering the reported role of mushrooms amino acids and polysaccharides derivatives in the management of diseases of inflammation[15-20], this present study was aimed at evaluating the Aqueous Ethanol Extract (AEE) of the defatted *Pleurotus pulmonarius* and its amino acids containing fractions for their Membrane Stabilization (MS) properties.

MATERIALS AND METHODS

Sample collection, identification and processing: The investigations were carried out in the laboratories of Pharmacognosy and Phytotherapy Department during the period of March, 2015-December, 2017. Fresh fruiting bodies of *Pleurotus pulmonarius* were purchased in the month of March, 2015 from Dilomats farm, Rivers State University, Port Harcourt. Nigeria and authenticated by the taxonomist of the Department of Plant Science and Biotechnology, University Port Harcourt with a voucher specimen UPH/P/1287 deposited in the same Herbarium. The fruiting bodies were thoroughly cleaned removing all soil and other foreign debris, cut into small pieces and spread on clean platform to air dry under a current of air at ambient temperature and light for 4 days. The dried fruiting bodies were then pulverized using an electric blender and stored in air tight container until further use.

Reagents, solvents, equipment and biological specimen: Microscope, Autoclave, Desiccator chamber, VLC column, UV lamp, pH meter, Silica gel TLC plate, Volumetric flasks, Beakers, Silica gel 200-400 mesh, Filter paper (Whatman No. 1), Fume chamber, Refrigerator, Sterile test tubes, Centrifuge, Varian 680-IR spectrophotometer, N-hexane, Chloroform, Methanol, Ethanol, Dichloromethane, N-butanol, Acetic acid, Acetone, Ammonia 10%, Sodium chloride, Potassium chloride, Distilled water, Sterile phosphate buffer saline and standard reference drugs: Indomethacin, Aspirin, Glycine, Tryptophan and biological substrates: Fresh human blood intravenously collected from healthy human volunteers into heparinized tubes to prevent coagulation.

Ethical approval: Ethical approval for the collection and use of fresh human blood was sought for and obtained from the University of Port Harcourt Teaching Hospital research ethics committee (Ref No: UPTH/ADM/90/S.II/VOL.XI/581).

Extraction: To achieve exhaustive removal of lipoidal metabolites[22] without denaturing of polar thermo labile metabolites, the cold extraction[23] protocol was adopted with modification. Briefly, the sample (150 g) was defatted by successive cold maceration in n-hexane and dichloromethane as solvents for three consecutive days each with agitation at interval of 6 h and fresh replacement of solvent every 24 h. The defatted marc obtained from the successive maceration was air dried in a fume cupboard and the marc was similarly extracted with 80% aqueous ethanol for 3 days with fresh replacement of solvent[24] every 24 h. The aqueous ethanol

extract was pooled together, concentrated using a rotary evaporator and further dried in a vacuum desiccator to obtain the 80% Aqueous Ethanol Extract (AEE) of the defatted *P. pulmonarius* used for the study.

Fractionation of the defatted Aqueous Ethanol Extract (AEE) of *P. pulmonarius*: The 80% Aqueous Ethanol Extract (AEE) of the defatted *P. pulmonarius* was fractionated using Vacuum Liquid Chromatography (VLC). Briefly, the AEE (2.0 g) was loaded into the column dry packed with normal phase silica gel (200-400 mesh size, KCM light, India) as stationary phase. The mobile phase gradient (500 mL) used comprised chloroform: methanol (10:0, 9:1, 8:2, 7:3, 6:4 v/v). The eluents were collected at intervals of 50 mL and pooled based on the similarities in the Retardation factor (R_f) of the resolved spots, characteristic colours under the UV light (254-365 nm) and chromogenic reagents[25] (ninhydrin for amino acids detection and iodine) on evaluation using Thin Layer Chromatography (TLC). Mobile phases used on silica gel HF_{254} pre-coated TLC plates include: n-butanol: Acetic acid: H_2O (4:1:1 v/v/v), $CHCl_3$: MeOH (9:1 V/V) and n-butanol: Acetone: Acetic acid: H_2O (7:4:4:4 v/v/v/v). Based on the similarities in the R_f of the resolved amino acids spots from TLC, two pooled amino acids derivative containing fractions T2 and T3 were obtained in addition to a non-amino acids containing fraction T1 not used in this study.

***In vitro* membrane stabilization assay:** This was done by following the report of heat induced Human Red Blood Cells (HRBCs) haemolysis model[26] with modification as briefly described. A 5 mL fresh human whole blood was collected in an Ethylene Diamine Tetraacetic Acid (EDTA) centrifuge bottle and centrifuged at 2000 rpm for 5 min. The packed cells were washed three times with equal volume of normal saline and a 40% v/v suspension was made with isotonic buffer solution of pH 7.4 (composition of isotonic buffer (g L^{-1}): NaCl 4.4 g, NaH_2PO_4 (1.6 g), Na_2HPO_4 (7.6 g). To separate 5 µL aliquot of the erythrocytes suspension, 5 mL of each concentrations of the test samples, AEE (100-100,000 µg mL^{-1}, T2: (100-1000 µg mL^{-1}), T3: (10-100 µg mL^{-1}), Aspirin (1-100 µg mL^{-1}) and Indomethacin (0.1-10 µg mL^{-1}) were added and mixed gently. Two replicates from each concentration were incubated on a water bath regulated at 54°C for 20 min, while others were incubated for the same duration at a temperature between 0-4°C and all centrifuged for 3 min. The haemoglobin content in supernatant solution was estimated based on their optical density by using spectrophotometer at 540 nm. The percentage of haemolysis was calculated by using the formula[26]:

$$\text{Inhibition of hemolysis (\%)} = \left[1 - \frac{OD_2 - OD_1}{OD_3 - OD_1}\right] \times 100$$

Where:

OD_1 = Absorbance of test sample unheated
OD_2 = Absorbance of test sample heated
OD_3 = Absorbance of control sample heated

The median inhibitory concentration (IC_{50}) was extrapolated by regression analysis from a plot of inhibition (%) of heat induced hemolysis against concentration.

Statistical analysis: One-way Analysis of Variance (ANOVA) and student t-test was carried out using Statistical Package for the Social Sciences (SPSS version 10.0). The difference between means was considered significant at $p<0.05$. Analysis was carried out in duplicate and the result was expressed as Mean±SD.

RESULTS

Extraction and fractionation: Table 1 shows the results of the extraction and fractionation of the defatted fruiting bodies of *P. pulmonarius.* The AEE (yield 2.634 g) afforded two amino acids derivative containing fractions T2 and T3 with the yield: T2 (0.719 g) and T3 (0.492 g). T1 eluted with chloroform: methanol 9:1 v/v mobile phase gradient with yield of 0.016 g was also obtained but devoid of amino acids constituents as confirmed from the qualitative thin layer chromatogram shown in Fig. 1 and 2.

Identification of amino acids-rich fraction: From Fig. 1 and 2, fraction T2 showed the presence of 3 constituents under UV light 254 nm (R_f: 0.67, 0.61, 0.55 and 0.45) in addition to 3 amino acids when sprayed with ninhydrin (R_f: 0.38, 0.28 and 0.23). Fraction T3 showed the presence of 2 spots after reacting with ninhydrin spray (R_f: 0.08 and 0.13). This confirmed the presence of

Table 1: Yield of the extract and fractions from the defatted fruiting bodies of *P. pulmonarius*

Extract and fraction	Yield (g)	Yield (%)	Eluting mobile phase
AEE	2.634	1.756	Not applicable
T1	0.016	0.011	Chloroform: Methanol 9:1 v/v
T2	0.719	0.479	Chloroform: Methanol 8:2 v/v
T3	0.492	0.328	Chloroform: Methanol 7:3-6:4 v/v

AEE: 80% aqueous ethanol extract , T1, T2 and T3 are chromatography fractions

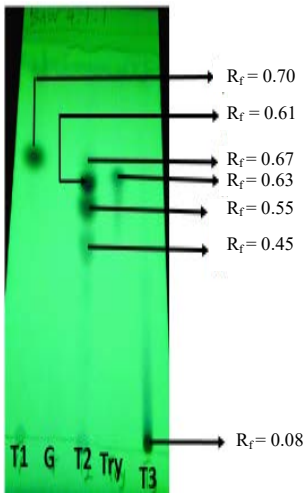

Fig. 1: TLC profile of the chromatography fractions T1, T2 and T3 under UV 254 nm
G: Glycine, Try: Tryptophan, R_f: Retardation factor

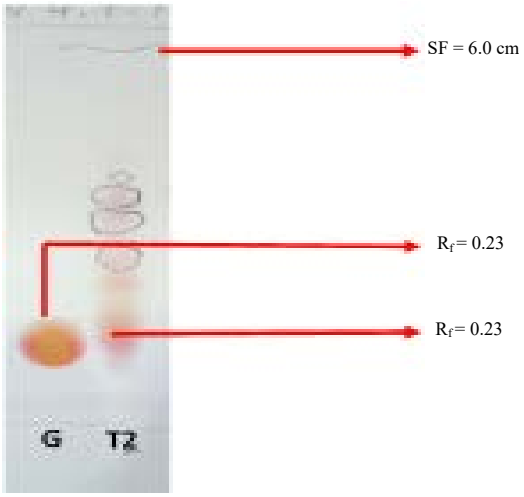

Fig. 3: TLC chromatogram of fraction T2
R_f: Retardation factor

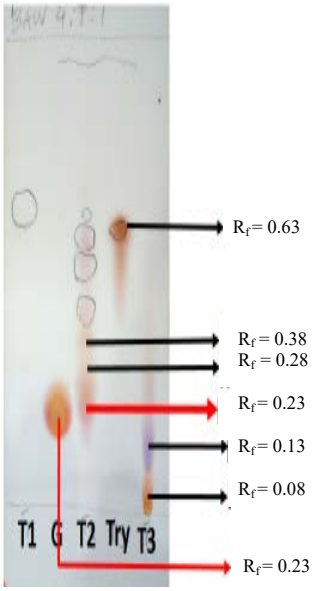

Fig. 2: TLC profile of the chromatography fractions T1, T2 and T3 with Ninhydrin reagent spray
G: Glycine, Try: Tryptophan, R_f: Retardation factor

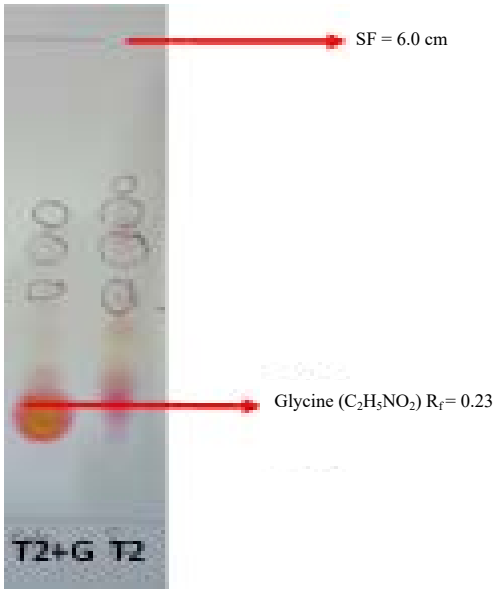

Fig. 4: TLC chromatogram of fraction and glycine spiked T2 sample (T2+glycine mixture)
R_f: Retardation factor

amino acids in T2 and T3 while fraction T1 has a resolved component (R_f = 0.7) when viewed under UV light 254 nm which is devoid of amino acid components since it did not give a colour reaction after spraying with ninhydrin reagent. The amino acid constituent of T2 (R_f = 0.23) corresponded to that of pure amino acid glycine (R_f = 0.23) used as reference standard, by reacting similarly with ninhydrin spray as well as the non-detection of this spot in both chromatogram under 254 nm UV light. Furthermore, the spiked mixture of T2 and glycine (T2+G) spotted alongside fraction T2 gave the same R_f value of 0.23 and indicated no separation in band of glycine or T2 from the T2+G (Fig. 3, 4).

Membrane stabilization activity profile: The results of the MS of the AEE T2, T3 and the reference drugs aspirin and indomethacin as shown in Fig. 5-9 which indicated that the AEE (Fig. 5) and its amino acids containing chromatography fractions T2 (Fig. 6) and T3 (Fig. 7) significantly ($p<0.05$)

inhibited the heat induced hemolysis of HRBCs in a concentration dependent manner. Based on the model used in this study, the membrane stabilization activity profile observed for AEE at the concentration range of 100-10,000 μg mL^{-1} was 28.3-87.3%. For T2 at the concentration range of 100-1000 μg mL^{-1} was 35- 81.7%, for T3 at the concentration range of 10-100 μg mL^{-1} was 20-90% while for the reference drugs: aspirin at the concentration range of 1-100 μg mL^{-1} was 14.3- 86.03% and indomethacin at the concentration range of 0.1-10 μg mL^{-1} was 23.33- 81.67% (Fig. 5-9). The observed trend of MS IC_{50} (Fig. 10) is AEE (524.814 μg mL^{-1})>T2 (403.392 μg mL^{-1})>T3 (40.451 μg mL^{-1})>Aspirin (6.765 μg mL^{-1})>Indomethacin (0.372 μg mL^{-1}).

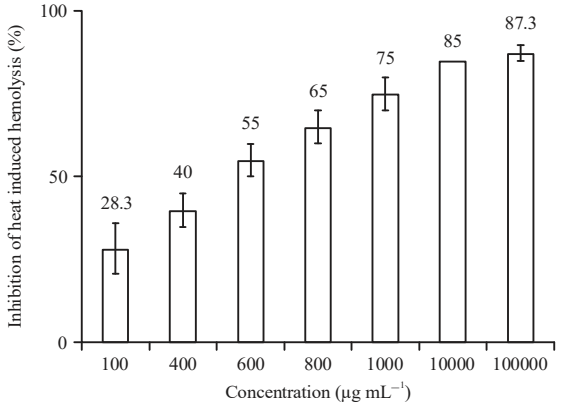

Fig. 5: Concentration-dependent membrane stabilization activity of the 80% Aqueous Ethanol Extract (AEE) of defatted fruiting bodies of *P. pulmonarius*

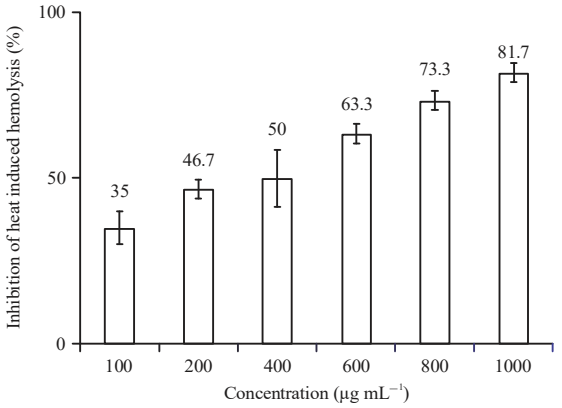

Fig. 6: Concentration response membrane stabilization activity profile of the chromatography fraction T2 from the 80% Aqueous Ethanol Extract (AEE) of the defatted fruiting bodies of *P. pulmonarius*

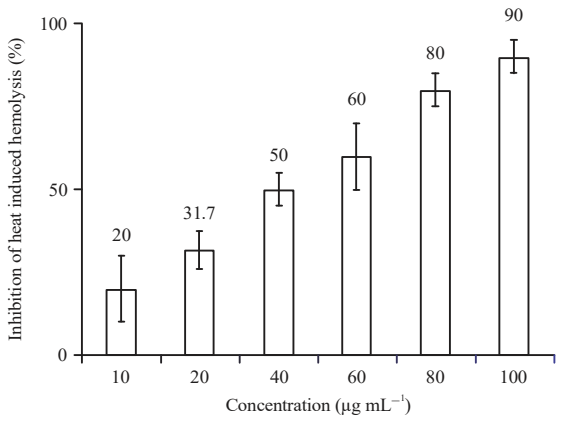

Fig. 7: Concentration response membrane stabilization of the chromatography fraction T3 from the 80% Aqueous Ethanol Extract (AEE) of the defatted fruiting bodies of *P. pulmonarius*

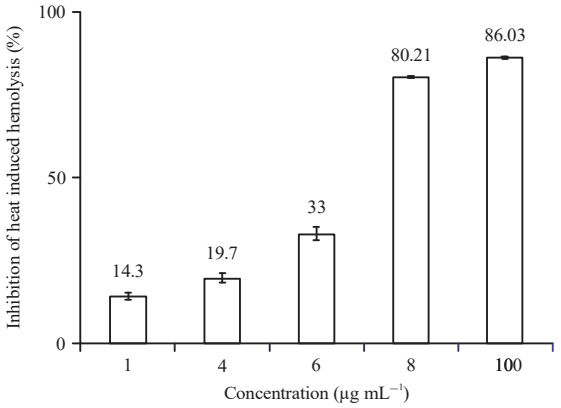

Fig. 8: Concentration response membrane stabilisation activity of the reference drug aspirin

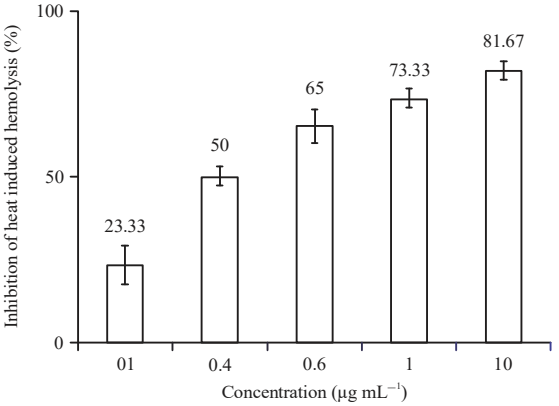

Fig. 9: Concentration response membrane stabilization activity of the reference drug indomethacin

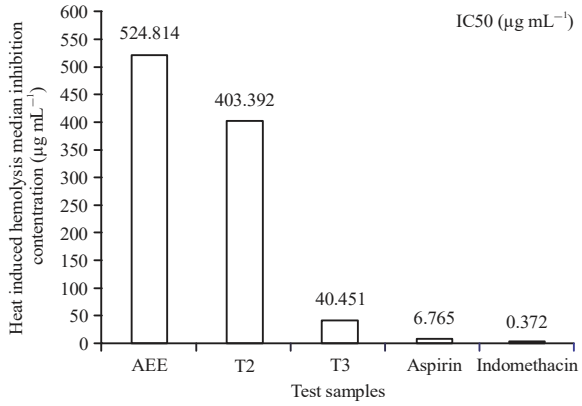

Fig. 10: Heat induced haemolysis median inhibition concentration for AEE, T2, T3, Aspirin and Indomethacin

AEE: Aqueous ethanol extracts of defatted *P. pulmonarius* (80%), T2 and T3: Amino acids containing fractions from AEE

DISCUSSION

The stabilization of lysosomal membrane is important in the prevention of complication due to inflammatory response in tissue damage[6], because the lysosomal membrane is structurally similar to the human red blood cell membrane[6], the *in vitro* human erythrocyte stabilization assay model[26] is used to screen for anti-inflammatory agents that act by preventing the rupture of the lysosomal membrane thereby preventing tissue damage associated inflammation process[6]. In this study the Aqueous Ethanol Extract (AEE) of the defatted *Pleurotus pulmonarius* and its amino acids containing fractions were evaluated for their Membrane Stabilization (MS) properties using the *in vitro* human erythrocyte stabilization assay model[26]. The two amino acids containing fractions T2 and T3 were found to be more active that the parent Aqueous Ethanol Extract (AEE) with the fraction T3 being the most active. Although they were not as active as the reference drugs aspirin and indomethacin as seen from their IC_{50} values in Fig. 10, at the high concentration of 100 µg mL^{-1}, a significantly better ($p<0.05$) membrane stabilization activity for T3 (90.0%, Fig. 7) was observed when compared with that of the reference non-steroidal anti-inflammation drug aspirin (86.02%, Fig. 8). The membrane stabilization result corroborated with reports that most anti-inflammatory properties of mushrooms, especially *Pleurotus* spp. are due to the presence of polysaccharide protein complexes, glucan polysaccharides and amino acids[17-19]. As noted from a previous report, the aqueous ethanol extract of *P. pulmonarius* exhibited good antioxidant compared to n-hexane and dichloromethane extracts[21] and these results are highly correlated with the MS activities of AEE. These activities are important in investigation and screening of molecules that can serve as a good lead for drugs for the treatment of diseases associated with inflammation such as; cancer and arthritis among others. Most reported anti-inflammatory property from mushrooms extract have been linked to some polysaccharide protein complexes[26,27], amino acids[28] and water soluble polysaccharide[20,29] (especially β-glucan). The detection of the amino acid glycine among other yet to be identified amino acids from the TLC examination could also offer a plausible rationale for the trend in membrane stabilization properties[30]. Glycine is functional amino acid that have been documented to have cytoprotective and inflammatory effects[31] among other health promoting benefits. Its ability to function as: stimulatory or inhibitory neurotransmitter[32], reduce the infiltration of inflammatory cells, synovial hyperplasia, edema and ankle swelling in PG-PS induced arthritis rats[33], protect against inflammatory effects (intestinal injury) following trinitrobenzene sulfonic acid or dextran sulfate sodium induced colitis in rats[34].

CONCLUSION

This report is suggestive that the AEE and its amino acids containing fractions T2 and T3 from *P. pulmoarius* are promising source for drug lead substance for the development of good anti-inflammatory medicines and nutraceuticals that could serve as a useful alternative for treating many chronic diseases linked to inflammation. The identification of the amino acid glycine from TLC analysis is being reported for the first time and could serve as a biomarker for the standardization of this mushroom. Also this study is a further validation of the traditional uses of mushroom in the treatment of diseases of inflammation and ageing as well as its use as a nutraceuticals containing functional amino acids.

SIGNIFICANCE STATEMENT

This study discovered that the amino acids rich fraction from the fruiting bodies of *P. pulmonarius* can be beneficial for the management of diseases of inflammation. This study will help the researcher to uncover the critical areas of the role of membrane stabilization by mushroom-derived amino acids in the prevention and management of tissue damage due to inflammation that many researchers were not able to explore. Thus a new theory on the role of glycine and other *P. pulmonarius* derived amino acids in the management of diseases of inflammation may be arrived at.

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