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## Journal Pre-proofs

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**Anti-angiogenic and anti-inflammatory activity of the truffle '*Tuber aestivum*' extracts and a correlation with the chemical constituents identified therein**

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**Abstract**

Fungi are a huge source of unexplored bioactive compounds. Owing to their biological activities, several fungi have shown commercial application in the health industry. *Tuber aestivum* Vittad. is one such edible fungi with an immense scope for practical biological applications. In the present study, the anti-angiogenic activity of petroleum ether and ethanol extracts of *T. aestivum* was investigated using the chick chorioallantoic membrane assay and compared to the positive controls silibinin and lenalidomide. Both the extracts showed a dose-dependent anti-angiogenic response. The extracts were also assessed for their anti-inflammatory potential by lipoxygenase-inhibition assay. The IC<sub>50</sub> values for LOX inhibition assay, computed by the Boltzmann plot, were 368.5, 147.3 and 40.2 µg/mL, for the petroleum ether extract, ethanol extract, and the positive control ascorbic acid, respectively. The ethanol extract of *T. aestivum* showed superior anti-angiogenic and anti-inflammatory activity than the petroleum ether extract. Compositional investigation of the extracts by GC-MS revealed the presence of various bioactive compounds. The compounds were correlated to their anti-angiogenic and anti-inflammatory activity based on a meticulous literature search.

**Keywords**

Truffles; *Tuber aestivum*; chick chorioallanotic-membrane (CAM) assay; anti-angiogenic; LOX-inhibitors; anti-inflammatory

## 1. Introduction

Fungi are presumed to have vast medicinal potential and yet remain under investigated with regard to such properties. Numerous fungi have displayed properties such as anticancer and immune stimulation. Several edible fungi have proven to be a great source of bioactives such as polysaccharides and proteins that display antitumor, antioxidant and immunological properties (Sun, Zhang, & Fang, 2019). Truffles are a category of fungi that have been primarily used in gourmet cuisine but have lately also been researched for their various properties and applications. The truffles belonging to the genera '*Tuber*' are ascomycetes that show a symbiotic association as ectomycorrhizae with trees and shrubs (Angelini, Tirillini, Properzi, Rol, & Venanzoni, 2015). Few truffle species found in European regions such as the *Tuber maculatum* and *Tuber aestivum* Vittad. have a peculiar aroma profile and are highly priced (Nadim et al., 2015). Truffles have been used in pickles, sauces, butters and creams. Additionally, they are also known for their antioxidant potential (Patel, Rauf, Khan, Khalid, & Mubarak, 2017). Researchers have also focused on controlling the post-harvest deterioration of truffles for prolonged storage as well as to maintain their nutritional and aroma profile (Wang & Marcone, 2011).

In traditional medicine, higher fungi have proved to be a valuable repository of novel therapeutic compounds and hence in recent decades, their study has gained importance in the scientific community (Aly, Debbab, & Proksch, 2011). Truffles have been used not just as food but also as a medicinal source in diverse cultures. Native Saharan Desert people of Algeria have used desert truffles for increasing fertility, and for the treatment of eye ailments and fatigue (Bradai, Neffar, Amrani, Bissati, & Chenchouni, 2015).

Scientists working with truffles have not only been exploring their applications in food, but have also shown growing interest in their biological properties. In recent years, the research concerned with truffles has become more diverse. The nutritional profile of truffles has been observed to be diverse for each truffle species. The traditionally studied biological properties of truffles include antiviral and

antimicrobial activities, along with antioxidative and anti-inflammatory properties being a recent focus (Wang & Marcone, 2011). Truffles are also credited with immunosuppressant (Patel, 2012), anticarcinogenic, anti-mutagenic, hepatoprotective, and anti-inflammatory properties (Wang & Marcone, 2011). Exploring truffles for their various biological properties could lead to possible biotechnological applications.

Medicinal use of truffles has been observed for a long time. Truffles are a rich source of bioactive compounds. For instance, polysaccharides from truffle *T. indicum* have been shown to prevent the PC12 cells (pheochromocytoma of the rat adrenal medulla) on exposing to H<sub>2</sub>O<sub>2</sub> stressor (Patel et al., 2017). Moreover, several truffle species have shown antimicrobial properties on agar-well diffusion assays. Truffle bioactives have also demonstrated anticancer, antidepressant and immune modulatory properties (Patel et al., 2017). Polysaccharides from truffles such as *T. aestivum* have also displayed anti-hyperglycemic activity (Mudliyar et al., 2019). Unlike bacterial diseases, viral diseases cannot be cured by antibiotics, thus instigating a need to discover new sources of antiviral compounds. Apart from the extracts, compounds found in edible fungi have also proven useful as antiviral (Sun et al., 2019). A major part of the approved drugs involves compounds of natural origin. Since there are several unexplored natural sources of such drugs, there is still a huge scope to discover new or less explored therapeutic compounds (Aly et al., 2011).

The chick chorioallantoic membrane (CAM) assay has been widely used for assessing the anti-angiogenic activity of bioactives. The use of CAM assay as an alternative to the mouse model offers several advantages such as short observation periods (days), inexpensive, easy reproducibility and high throughput, requirement of smaller quantities of the samples for the study, and direct visualization of the effects (Lokman, Elder, Ricciardelli, & Oehler, 2012). However, in case of the mouse model, the observation periods to study the impact are comparatively longer (weeks to months). Although the

biology and physiology of mouse model is well known, it is comparatively complex (Lokman et al., 2012).

The present study aimed at assessing the medicinal applications of *T. aestivum* by assaying the anti-inflammatory and anti-angiogenic activity. The anti-inflammatory assessment was performed by studying the soybean lipoxygenase-inhibition activity of the petroleum ether and ethanol extracts, whereas the anti-angiogenic activity was studied using the chick chorioallantoic membrane assay. Moreover, the inhibition of LOX was correlated to the suppression of angiogenesis. The extracts were further analyzed using GC-MS in order to determine the active compounds present therein.

## **2. Materials and Methods**

### **2.1 Enzymes and Chemicals**

Lipoxygenase enzyme from soybean (type **V**), linoleic acid, silibinin standard, and lenalidomide standard were purchased from Sigma-Aldrich. All the other chemicals and reagents used were of analytical reagent grade.

### **2.2 Truffle strains**

*Tuber aestivum* were purchased from an Italian market and shipped to Juva, Finland in vacuum packaged and frozen conditions.

### **2.3 Methods**

#### **2.3.1. Preparation of the extracts of *Tuber aestivum***

The truffle *T. aestivum* was collected and cleaned using tap water followed by distilled water. This was followed by chopping the truffles into thin slices and drying in a ventilated oven at 40 °C, for 24 h. After drying, the sample was powdered in a mechanical electric blender, sieved (A.S.T.M 20) and stored in airtight plastic bags at 4 °C until further use. The extracts were prepared by swirling 10 g dried truffle powder with 100 mL extraction solvent (petroleum ether or ethanol) at 150 rpm using a shaking incubator (Incu-shaker 10L-H2010, Benchmark scientific) at room temperature (26±2 °C), for 24 h. This

was followed by filtering the extract using Whatman filter paper (grade 1) and centrifuging at 1792 g for 20 min at 4 °C. The supernatant was dried using a rotatory evaporator at 30 °C to obtain the extracts. These extracts were stored in a refrigerator (4 °C) until further use.

### 2.3.2. Compositional analysis of the extracts by GC-MS

The analysis of the petroleum ether and ethanol extracts of *T. aestivum* was carried out using a GC-MS method reported by Seppänen-Laakso, Nygren, & Rischer (2017). The derivatization of bound fatty acids (such as glycerolipids, glycerophospholipids, and sterol esters) was carried out by transesterification with sodium methoxide. Briefly, 250 µL of 0.5 M sodium methoxide solution prepared in methanol was added to 5 mg of sample spiked with internal standards (C17:0 TAG and C17:0 FFA). The mixture was vortexed and heated at 45 °C for 5 min in a block heater. This was followed by addition of 500 µL of 15% sodium hydrogen sulfate and the mixture was vortexed. To this mixture, 250 µL petroleum ether was added and vortexed followed by centrifugation for 1 min at 7800 × g. The supernatant containing petroleum ether was separated and the sample was dried under nitrogen flow. The residue so obtained was further used for the derivatization of sterols, free fatty acids and other polar compounds, which was carried out by trimethylsilylation. The residue was dissolved in 50 µL of dichloromethane followed by addition of 25 µL mixture of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (1%). The mixture was heated at 80 °C for 20 min. The sample was cooled and added to a vial insert for GC-MS analysis.

The GC-MS analysis was carried out using an Agilent 6890 GC (gas-chromatograph) with Agilent 5973N MSD (mass selective detector). The volume of the sample injected was 1 µL. The column used for the analysis was an Agilent DB-5MS (30 m, ID 0.25 mm, 0.25 µm). The oven temperature was programmed from 70 to 250 °C at a rate of 7 °C/min, for a run time of 45 min. The injector temperature was set to 290 °C and that of the MS source and quadrupole was 230 and 150 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.2 mL/min.



### **2.3.3. Chick chorioallantoic membrane assay to test the anti-angiogenic activity of *Tuber aestivum* extracts**

The anti-angiogenic activity of the petroleum ether and ethanol extracts of *T. aestivum* was tested using the chick chorioallantoic membrane assay, by the method given by Seow et al. (2011) with slight modifications. Briefly, fertilized chicken eggs were incubated at 37 °C for 3 days and were rotated frequently during this period. On the third day, the eggs were candled using an electric bulb to locate and confirm the development of the embryo. The non-viable eggs were discarded at this stage. The viable eggs were further incubated until the fifth day, with frequent rotation. On the fifth day the eggs were swabbed with a disinfectant in a laminar air flow unit, and the albumin was aseptically removed from the pointed-end of the egg, by making a hole. The hole was sealed aseptically using a parafilm. The egg was placed horizontally and a window was cut using a hand-held electric drill/cutter machine. This window provided an access to the CAM for placing the sample. A photograph was taken of the region of the blood vessels where the sample would be placed. Filter paper discs containing pre-loaded and dried, extracts or positive controls of different concentrations (50, 100, and 200 µg/disc) were placed carefully and aseptically on the target blood vessel region. A photograph was taken again after placing the disc for the ease of location on the seventh day. The eggs were sealed aseptically by a surface-sterilized parafilm and a cloth-tape. The eggs were re-incubated at 37 °C until day 7. On day 7, the window was opened and the filter paper disc was removed using sterile forceps. A photograph of the same region of blood vessels was taken. As depicted in Table 1 (Seow et al., 2011), based on the change in the blood vessels, a score was assigned to each sample for its anti-angiogenic effect. The results were compared with that of a control egg, which did not receive any treatment with extract or standard compound. Eggs containing a blank filter paper disc were also studied. For each sample tested, 18 eggs were used at the start, assuming around 40% mortality that is inevitable to the process. This would yield around 8-10 viable eggs per test

sample. For every test sample, the average score was considered to interpret the anti-angiogenic effect as depicted in Table 2 (Seow et al., 2011).

#### **2.3.4. *In vitro* anti-inflammatory study of *Tuber aestivum* extracts by LOX inhibition assay**

The anti-inflammatory potential of the petroleum ether and ethanol extracts of *T. aestivum* was assessed using 15-LOX inhibitory assay, as per a reported protocol (Lyckander & Malterud, 1992). Ascorbic acid was used as a positive control. Linoleic acid was used as a substrate for 15-LOX. The samples (extracts and ascorbic acid) were dissolved in DMSO. For a test reaction, 2.5  $\mu\text{L}$  of test compound was added to 97.5  $\mu\text{L}$  enzyme solution (400 U/mL) prepared in 0.2 M borate buffer of pH 9. The mixture was incubated for 5 min. DMSO (2.5  $\mu\text{L}$ ) was used in the control reaction. Linoleic acid solution (100  $\mu\text{L}$ , 250  $\mu\text{M}$ ) was added to all the reaction mixtures. To prepare the blank sample, 2.5  $\mu\text{L}$  DMSO was added to 97.5  $\mu\text{L}$  borate buffer followed by 100  $\mu\text{L}$  substrate solution. Absorbance was recorded at 234 nm using a spectrophotometer. The decrease in the absorbance was a direct measure of the inhibitory activity of the samples. Percentage inhibition of 15-LOX was calculated by using the following formula:

$$\% \text{ Inhibition} = [(A_c - A_t)/A_c] \times 100$$

Where,  $A_c$ : Absorbance of control

$A_t$ : Absorbance of test

IC50 values of the extracts and positive control (ascorbic acid) for the inhibition of 15-LOX were computed by Boltzmann sigmoidal function plotted using Origin Pro 8.5 (OriginLab).

#### **2.3.5. Statistical analysis**

The experimental data was subjected to statistical analysis using the IBM<sup>®</sup> SPSS<sup>®</sup> version 20. The data was subjected to Tukey's HSD test. Data with P-value less than 0.05 was considered as statistically significant. Data is represented as mean along with its standard deviation.

### **3. Results and Discussion**

### 3.1. Composition of the extracts of *T. aestivum* and its correlation with the anti-angiogenic and anti-inflammatory activity

The chemical composition of the petroleum ether and ethanol extracts of *T. aestivum* was determined using GC-MS analysis. Since these extracts would consist of a plethora of compounds, an untargeted approach was selected, where we aimed at carrying out their chromatographic separation first (by GC) followed by their detection by MS. Since the approach used to determine the bioactives was an ‘untargeted’ one, MS was preferred over other analytical techniques such as NMR. Furthermore, GC-MS provides good reproducibility and a highly reproducible fragmentation, thus offering a potent tool for the identification of a range of compounds at the same time (Emwas, 2015). In contrast to NMR, MS is a highly sensitive technique and is able to detect compounds that are present at remarkably low concentrations (picomole to femtomole) (Emwas, 2015). The presence of numerous compounds in the extracts, found at very low concentration, and their possible synergistic contribution towards the biological activities also favored MS as an appropriate analytical technique. The GC-MS chromatograms are shown in Figure S1 of supplementary material. Both the extracts consisted primarily of fatty acids with chain length of 16-20 carbons. The obtained GC-MS results were subjected to a NIST08 GC-MS spectral library search to get suggestions for the identification of the minor compounds. Such studies with untargeted approach to determine the chemical composition of truffles have been reported (Longo et al., 2017). For the abundant fatty acids shown in Table 5, the similarity indices (quality) varied from 95 to 99 (max 100), but for the minor constituents the indices were substantially lower as expected. In addition, a thorough literature search was carried out in an attempt to understand the correlation between the compounds detected using GC-MS and the observed anti-angiogenic and anti-inflammatory activities. The proposed compounds in the petroleum ether and ethanol extracts of *T. aestivum* using the NIST08 GC-MS library search are presented in Tables 3 and 4, respectively. The GC-MS analysis revealed the presence of various compounds in both petroleum ether and ethanol extracts that have been reported for

anti-angiogenic and/or anti-inflammatory activity. The compounds detected included phenolics and non-phenolics. For instance, the petroleum ether extract of *T. aestivum* showed presence of compounds such as (2E)-2-(methoxyimino)butanoate,  $\delta$ -aminolevulinic acid (ALA), 9,10-anthracenedione, 1-amino-4-hydroxy, and 4-(2,4,5-trimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one. The ethanol extract showed presence of compounds such as porphine derivative, lanostanes (triterpenoid), and naringenin-7-O-neohesperidoside. The presence of the major C16-20 fatty acids in the truffle species such as *T. melanosporum*, *T. sinense*, *T. aestivum*, and *T. indicum* has been confirmed and compared (Tang, Li, Li, Wan, & Tang, 2011). The petroleum ether extracts also showed the presence of docosanoic and octadecatrienoic acid, the presence of which in truffle species has been reported earlier (Tang et al., 2011). The structures of the detected biomolecules were considered and correlated to their anti-angiogenic and anti-inflammatory activity based on a thorough literature search, and is discussed in the subsequent sections.

The relative amounts of fatty acids in the extracts were also determined and are presented in Table 5. Oleic acid dominated the fatty acid profile in petroleum ether extracts, whereas linoleic acid was detected in highest relative amount in the ethanol extracts. It is known that the fungi growing in colder environments show presence of high proportions of unsaturated fatty acids which aids in modulating the fluidity of their cell membranes in harsh climate (Shah et al., 2020). Additionally, the dominance of linoleic and oleic acid in the fatty acid profiles of truffles has been reported (Shah et al., 2020; Yan, Wang, Sang, & Fan, 2017). The proportion of free fatty acids was higher in ethanol extract (30.4%) than in petroleum ether extract (18.4%) while that of hydroxyoctadecanoic acids (HODE) was higher in the latter extract.

### **3.2. Anti-angiogenic effect of *Tuber aestivum* extracts**

The anti-angiogenic activity of petroleum ether and ethanol extracts of *T. aestivum* was studied using the CAM assay. Silibinin and lenalidomide were used as positive controls. The motive behind

having two positive controls was to have a comparison of relatively polar and non-polar drugs. As depicted in Table 1 and Table 2, a semi-quantitative system was used to score the anti-angiogenic activity of the extracts and controls. Both, silibinin and lenalidomide showed minor changes in blood vessels at the concentrations of 50 and 100  $\mu\text{g}/\text{disc}$ . Increasing the concentration of silibinin to 200  $\mu\text{g}/\text{disc}$  lead to a ‘good’ anti-angiogenic effect (average score  $1.13\pm 0.33$ ), where we observed absence of capillaries under the disc-area along with reduction in the growth of blood vessels (Figure 1). Silibinin has been reported to suppress the formation of blood capillaries of venous endothelial cells of human umbilical cord on matrigel. Silibinin has been reported to act by decreasing the secretion of vascular endothelial growth factor (VEGF); the key mediator of angiogenesis (Yang et al., 2005). Anti-angiogenic treatment to tackle tumors leads to tumor hypoxia, which in turn causes alterations in the cellular metabolism, de-differentiation of metastatic cells, formation of reactive oxygen species (ROS) and elevated tendency of metastasis (Wang et al., 2015). Considering their antioxidant property, phytochemicals such as silibinin could not only act as anti-angiogenic factors, but could also reduce tumor hypoxia, thus reducing the deleterious side effects of anti-angiogenic treatment.

In case of lenalidomide, the observed effect was ‘strong’ (average score  $1.63\pm 0.48$ ) at a concentration of 200  $\mu\text{g}/\text{disc}$ , where there was complete absence of capillaries, no visibility of microvessels and convergence of large blood vessels (Figure 1). Lenalidomide acts on angiogenesis by multiple inhibitory modes on endothelial cells during normoxic and hypoxic conditions (Lu et al., 2009). Similar to silibinin, lenalidomide acts by impeding the expression of VEGF (Qu, Jiang, Wu, & Ding, 2016) and has been reported for its anti-angiogenic activity in CAM (De Luisi et al., 2011).

The petroleum ether and ethanol extracts of *T. aestivum* showed anti-angiogenic effect in a dose-dependent manner. As can be seen from Table 6, the anti-angiogenic effect observed for both extracts was ‘good’ with average scores of  $1.19\pm 0.50$  and  $1.31\pm 0.56$  at the concentration of 200  $\mu\text{g}/\text{disc}$ . It is noticeable that these scores were comparable to that of the positive controls silibinin and lenalidomide at

the same concentration (200 µg/disc). In strong contrast, we did not observe any anti-angiogenic effect when a blank disc was used (Figure 1).

The NIST08 GC-MS library search of the spectra and molecular weights of the minor compounds showed presence of several possible biologically active compounds. There are many routes to target cancer progression, one of which is blocking the process of angiogenesis. Matrix metalloproteinases (MMPs) are extracellular matrix enzymes that play a role in degradation of collagen and other proteins and also aid in the process of angiogenesis. For this reason, MMPs and their inhibitors are an important target in anticancer therapy (Jabłońska-Trypuć, Matejczyk, & Rosochacki, 2016). Photodynamic therapy (PDT) has been used to target MMPs and regulate their activity. Amongst the various compounds found in petroleum ether extract, we observed the presence of  $\delta$ -aminolevulinic acid (ALA) (compound 6, Table 3) which has been studied in combination with PDT (Filip et al., 2008; Wei, Ma, Liu, & Zhang, 2013) and hyperthermotherapy (HTT) (Takahashi et al., 2013) for anticancer therapies. The findings of Wei et al. (2013) and Filip et al. (2008) are suggestive of PDT to be effective anticancer therapy when used in combination with ALA. Takahashi et al. (2013) reported an improvement in the efficacy of HTT when evaluated in combination with ALA.

In the petroleum ether extract, an anthraquinone compound, 9,10-anthracenedione, 1-amino-4-hydroxy (compound 8, Table 3), was detected. Anthraquinones such as damnacanthal (García-Vilas, Pino-Ángeles, Martínez-Poveda, Quesada, & Medina, 2017) and emodin (Ma et al., 2015) have already been reported for their potent anti-angiogenic activity. García-Vilas et al. (2017) reported on the inhibition of three kinases involved in angiogenesis, *viz.* vascular endothelial growth factor receptor-2 (VEGFR-2), hepatocyte growth factor receptor (HGFR) and focal adhesion kinase (FAK), by the anthraquinone damnacanthal. The activity of damnacanthal has been studied using *in vitro* assays and showed specific effects such as the inhibition of tubulogenesis, production of extracellular matrix remodeling enzyme, and proliferation, survival, and migration of endothelial cells (García-Vilas et al.,

2017). Ma et al., (2015) studied the *in vitro* and *in vivo* anti-angiogenic activities of the anthraquinone derivative, emodin, isolated from the root and rhizome of *Rheum palmatum* L. Their findings were suggestive of potent anti-angiogenic activity of emodin caused by the inhibition of MMPs and VEGFR-2. Further, the fatty acid profile of the petroleum ether and ethanol extracts of *T. aestivum* revealed the presence of oleic and linoleic acid acids in the highest amounts. Extracts of the fungus *Alternaria alternata* have shown presence of these fatty acids in high amounts and have been reported for their anti-angiogenic potential assessed using CAM assay (Bendre & Gonjari, 2019).

The ethanol extract showed presence of a compound with porphine group (compound 1, Table 4). Porphyrin derivatives have been reported for their use in anti-cancer therapies such as PDT (Moylan, Scanlan, & Senge, 2015; Singh et al., 2015). We also detected the presence of a lanostane triterpene (compound 6, Table 4) in the ethanol extracts. Nguyen et al. (2015) reported the presence of lanostane triterpenes in the fungi *Ganoderma lucidum*. The authors also reported the potent anti-angiogenic activity of these compounds in the formation of capillaries in human umbilical vein endothelial cells.

Compound 12 (Table 4) composed of a benzothiophene group could also have contributed to the anti-angiogenic activity displayed by ethanol extract. Benzothiophene-based drugs have displayed various biological activities such as anti-inflammatory, anticancer, antimicrobial, and antidiabetic. Benzothiophene derivatives have also shown anti-angiogenic activity. For this reason it has been a compound that has gained major focus in drug discovery (Keri et al., 2017). The ethanol extract also showed presence of benzophenanthridine and naringenin derivatives (compounds 14 and 15, respectively, Table 4), both of which have been reported to show anti-angiogenic activity by inhibition of VEGF-induced angiogenesis (Pafumi et al., 2017; Xu et al., 2013).

### **3.3. Anti-inflammatory effect of *Tuber aestivum* extracts and its correlation with the inhibition of angiogenesis**

The enzyme arachidonate 5-LOX converts arachidonic acid to leukotrienes which in turn mediate inflammatory responses (Geronikaki et al., 2008). Furthermore, the products of LOX have been shown to upregulate the expression of VEGF which further promotes angiogenesis. For instance, products of 5-LOX such as 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene A4 (LTA4) have been shown to enhance the transcription of VEGF in human malignant mesothelioma model and human umbilical vein endothelial cells (Hamsin, Hamid, Yazan, Taib, & Yeong, 2014). The enhanced expression of LOX has also been associated with the upregulation of matrix metalloproteinases-2 (MMP-2) which is another key angiogenic factor. The inhibition of 5-LOX has been shown to reduce angiogenesis by suppressing the activity of MMP-2 and the expression of VEGF (Ye, Liu, Shin, Wu, & Cho, 2004). A suppression in VEGF-induced angiogenesis has also been achieved by the genetic and pharmacological inhibition of LOX (Kim, Kim, Choo, & Kwon, 2016). In recent years, researchers from across the globe have started focusing on developing inhibitors of lipoxygenase in the hope to tackle inflammatory response. Furthermore, considering the influence of LOX on the expression of pro-angiogenic factors, researchers are taking interest in LOX inhibitors as a means to tackle angiogenesis (Hamsin et al., 2014; Kim et al., 2016; Ye et al., 2004). Although the plant and animal LOXs differ in the number of amino acids, they share a great similarity in the topology and the active site (Chedea & Jisaka, 2011). For this reason, inhibition of soybean lipoxygenase has been widely used as a measure of the potential anti-inflammatory properties of drugs and extracts.

The anti-inflammatory activity of petroleum ether and ethanol extracts of *T. aestivum* was assessed by studying the inhibition of the enzyme lipoxygenase (from soybean). Ascorbic acid was used as a positive control to compare the results. As evident from Figure 2, the extracts showed a dose-dependent inhibition of LOX with increase in the concentration. The IC<sub>50</sub> values of petroleum ether and ethanol extract were 368.5 and 147.3 µg/mL, thus proving ethanol extract to be comparatively more potent. The positive control used in this study i.e. ascorbic acid demonstrated an IC<sub>50</sub> of 40.2 µg/mL.



Beara et al., (2014) studied the anti-inflammatory activity of methanol and aqueous extracts of *T. aestivum* Vittad. and *T. magnatum* Pico, using the *ex vivo* COX-1 and 12-LOX inhibition assays. Although they observed inhibition of these enzymes by *T. magnatum* extracts, no inhibition was observed by the extracts of *T. aestivum*. These results are in contradiction to what we have observed in our study. In the present study the petroleum ether and ethanol extracts of *T. aestivum* assessed for their anti-inflammatory activity showed significant inhibition of 15-LOX. This could be due to the difference in the composition of the extracts which is greatly influenced by the origin and maturity of the truffle (Shah et al., 2020) as well as the form and source of the lipoxygenase.

Ananthraquinone derivative in the form of 9,10-anthracenedione, 1-amino-4-hydroxy (compound 8, Table 3), was found in petroleum ether extract of *T. aestivum*. Anthraquinone derivatives have been reported to show LOX-inhibitory activity (Prinz & Müller, 1996). Chaaban et al., (2018) reported LOX inhibitory activities of novel quinoline derivatives that were even higher than that of zileuton which is an active inhibitor of lipoxygenase, and has shown anti-angiogenic effect by significantly reducing *in vitro* and *in vivo* VEGF-induced proliferation of human umbilical vein endothelial cells (HUVEC) has been reported (Lim, Park, Um, Lee, & Kwak, 2019). Compound 19 (Table 3) found in petroleum ether extract displayed a quinoline moiety, which could have contributed to a significant LOX-inhibitory activity. Compounds 6, 12 and 15 (Table 4) which could have added to the anti-angiogenic activity of the ethanol extract could have also contributed to the LOX-inhibitory activity. Yoshikawa et al., (2005) reported the inhibition of the inflammatory enzymes COX-1 and COX-2 by lanostanetriterpenoids isolated from the fungus *Fomitopsis pinicola*. The terpenoid compound ursolic acid has shown inhibition of lipoxygenase as well as downregulation of expression of MMP which contributes to angiogenesis (Kuttan, Pratheeshkumar, Manu, & Kuttan, 2011). Furthermore, the terpenoid boswellic acid found in the plants of the genus *Boswellia* have shown anti-inflammatory activity by inhibiting 5-LOX and consequential downregulation of MMP-9 and VEGF thus contributing to its anti-angiogenic activity (Kunnumakkara

et al., 2018). Similarly, benzothiophene derivatives have been reported for their COX-1/2 as well as 5-LOX inhibitory activity (El-Miligy, Hazzaa, El-Messmary, Nassra, & El-Hawash, 2017). Naringenin has also been reported for its 5-LOX-inhibitory potential (Wijaya, Jin, Nee, & Wiart, 2012). Compounds structurally similar to naringenin such as the flavonoids quercetin and baicalein have been reported for their anti-angiogenic activity by inhibiting LOX and ergo the angiogenic factors (Sagar, Yance, & Wong, 2006).

Further, hexadecanoic acid has been reported to show inhibition of phospholipase A<sub>2</sub> (Aparna et al., 2012) and lipoxygenase (Godara, Dulara, Barwer, & Chaudhary, 2019), which results in its anti-inflammatory activity and subsequently the possible inhibition of angiogenesis. Amongst the other compounds, the presence of hexadecanoic acid in petroleum ether and ethanol extracts must have contributed to the inhibition of 15-LOX.

Thus given the role of LOX and its products in inflammation and angiogenesis, exploring LOX inhibitors could pave a way towards developing an improved anti-inflammatory and anti-angiogenic therapy as well as promoting *T. aestivum* as a functional food. Further studies could be carried out on separation of the compounds present in the extracts of *T. aestivum* using chromatographic techniques and confirming their anti-angiogenic and anti-inflammatory activities.

#### **4. Conclusion**

The current study focused on testing the anti-angiogenic and anti-inflammatory potential of the petroleum ether and ethanol extracts of the truffle *Tuber aestivum* using the CAM and lipoxygenase inhibitory assays, respectively. The mass-spectra of the extracts demonstrated the presence of various compounds which could be responsible for the observed anti-angiogenic and anti-inflammatory activities. The biological activities of the petroleum ether and ethanol extracts could be attributed to the synergistic activity of the compounds therein. This study could be considered as a basis for promoting research area on the health effects of truffles and their use as a functional food.

## Notes

The authors have no conflict of interest

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## References

- Aly, A. H., Debbab, A., & Proksch, P. (2011). Fifty years of drug discovery from fungi. *Fungal Diversity*, 50, 3. <https://doi.org/10.1007/s13225-011-0116-y>
- Angelini, P., Tirillini, B., Properzi, A., Rol, C., & Venanzoni, R. (2015). Identification and bioactivity of the growth inhibitors in *Tuber* spp. methanolic extracts. *Plant Biosystems*, 149, 1000-1009. <https://doi.org/10.1080/11263504.2014.983575>
- Aparna, V., Dileep, K. V., Mandal, P. K., Karthe, P., Sadasivan, C., & Haridas, M. (2012). Anti-inflammatory property of *n*-hexadecanoic acid: Structural evidence and kinetic assessment. *Chemical Biology and Drug Design*, 80, 434-439. <https://doi.org/10.1111/j.1747-0285.2012.01418.x>
- Beara, I. N., Lesjak, M. M., Četojević-Simin, D. D., Marjanović, Ž. S., Ristić, J. D., Mrkonjić, Z. O., & Mimica-Dukić, N. M. (2014). Phenolic profile, antioxidant, anti-inflammatory and cytotoxic activities of black (*Tuber aestivum* Vittad.) and white (*Tuber magnatum* Pico) truffles. *Food Chemistry*, 165, 460-466. <https://doi.org/10.1016/j.foodchem.2014.05.116>
- Bendre, N. N., & Gonjari, G. R. (2019). Antiangiogenic potential of endophytic fungi *Alternaria alternata* isolated from *Lawsonia inermis* Linn. *Bioscience Biotechnology Research Communications*, 12, 820-828. <https://doi.org/10.21786/bbrc/12.3/40>
- Bradai, L., Neffar, S., Amrani, K., Bissati, S., & Chenchouni, H. (2015). Ethnomycological survey of

traditional usage and indigenous knowledge on desert truffles among the native Sahara Desert people of Algeria. *Journal of Ethnopharmacology*, 162, 31-38.  
<https://doi.org/10.1016/j.jep.2014.12.031>

Chaaban, I., Rizk, O.H., Ibrahim, T.M., Henen, S.S., El-Khawass, E.S.M., Bayad, A.E., El-Ashmawy, I.M., Nematalla, H.A. (2018). Synthesis, anti-inflammatory screening, molecular docking, and COX-1,2/-5-LOX inhibition profile of some novel quinoline derivatives. *Bioorganic Chemistry*, 78, 220-235. <https://doi.org/10.1016/j.bioorg.2018.03.023>

Chedea V.S. & Jisaka, M. (2011). Inhibition of soybean lipoxygenases – structural and activity models for the lipoxygenase isoenzymes family, in: Krezhova D. (Ed.) *Recent Trends for Enhancing the Diversity and Quality of Soybean Products*. (pp. 109-130) IntechOpen.  
<https://doi.org/10.5772/17976>

De Luisi, A., Ferrucci, A., Coluccia, A.M.L., Ria, R., Moschetta, M., De Luca, E., Pieroni, L., Maffia, M., Urbani, A., Di Pietro, G., Guarini, A., Ranieri, G., Ditunno, P., Berardi, S., Caivano, A., Basile, A., Cascavilla, N., Capalbo, S., Quarta, G., Dammacco, F., Ribatti, D., Vacca, A. (2011). Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma. *Clinical Cancer Research*, 17, 1935-1946.  
<https://doi.org/10.1158/1078-0432.CCR-10-2381>

El-Miligy, M. M. M., Hazzaa, A. A., El-Messmary, H., Nassra, R. A., & El-Hawash, S. A. M. (2017). New benzothiophene derivatives as dual COX-1/2 and 5-LOX inhibitors: Synthesis, biological evaluation and docking study. *Future Medicinal Chemistry*, 9, 443-468.  
<https://doi.org/10.4155/fmc-2016-0230>

Emwas, A. H. M. (2015). The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. In J. Bjerrum (Ed.), *Metabonomics. Methods in*

*Molecular Biology*, (pp. 161-193). Humana Press, New York, NY [https://doi.org/10.1007/978-1-4939-2377-9\\_13](https://doi.org/10.1007/978-1-4939-2377-9_13)

Filip, A., Clichici, S., Muresan, A., Daicoviciu, D., Tatomir, C., Login, C., Dreve, S., Gherman, C. (2008). Effects of PDT with 5-aminolevulinic acid and chitosan on Walker carcinosarcoma. *Experimental Oncology*, 30, 212-219.

García-Vilas, J. A., Pino-Ángeles, A., Martínez-Poveda, B., Quesada, A. R., & Medina, M. Á. (2017). The noni anthraquinone damnacanthal is a multi-kinase inhibitor with potent anti-angiogenic effects. *Cancer Letters*, 385, 1-11. <https://doi.org/10.1016/j.canlet.2016.10.037>

Geronikaki, A.A., Lagunin, A.A., Hadjipavlou-Litina, D.I., Eleftheriou, P.T., Filimonov, D.A., Poroikov, V. V., Alam, I., Saxena, A.K. (2008). Computer-aided discovery of anti-inflammatory thiazolidinones with dual cyclooxygenase/lipoxygenase inhibition. *Journal of Medicinal Chemistry*, 51, 1601-1609. <https://doi.org/10.1021/jm701496h>

Godara, P., Dulara, B. K., Barwer, N., & Chaudhary, N. S. (2019). Comparative GC-MS analysis of bioactive phytochemicals from different plant parts and callus of *Leptadenia reticulata* wight and arn. *Pharmacognosy Journal*, 11, 129-140. <https://doi.org/10.5530/pj.2019.1.22>

Hamsin, D. E. Z. A., Hamid, R. A., Yazan, L. S., Taib, C. N. M., & Yeong, L. T. (2014). *Ardisia crispa* roots inhibit cyclooxygenase and suppress angiogenesis. *BMC Complementary and Alternative Medicine*, 14, 1-9. <https://doi.org/10.1186/1472-6882-14-102>

Jabłońska-Trypuć, A., Matejczyk, M., & Rosochacki, S. (2016). Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 31, 177-183. <https://doi.org/10.3109/14756366.2016.1161620>

- Keri, R. S., Chand, K., Budagumpi, S., Balappa Somappa, S., Patil, S. A., & Nagaraja, B. M. (2017). An overview of benzo[b]thiophene-based medicinal chemistry. *European Journal of Medicinal Chemistry*, 138, 1002-1033. <https://doi.org/10.1016/j.ejmech.2017.07.038>
- Kim, T. Y., Kim, J., Choo, H. Y. P., & Kwon, H. J. (2016). Inhibition of 5-lipoxygenase suppresses vascular endothelial growth factor-induced angiogenesis in endothelial cells. *Biochemical and Biophysical Research Communications*, 478, 1117-1122. <https://doi.org/10.1016/j.bbrc.2016.08.078>
- Kunnumakkara, A. B., Banik, K., Bordoloi, D., Harsha, C., Sailo, B. L., Padmavathi, G., Roy, N.K., Gupta, S.C., Aggarwal, B. B. (2018). Googling the guggul (*Commiphora* and *Boswellia*) for prevention of chronic diseases. *Frontiers in Pharmacology*, 9, 686. <https://doi.org/10.3389/fphar.2018.00686>
- Kuttan, G., Pratheeshkumar, P., Manu, K. A., & Kuttan, R. (2011). Inhibition of tumor progression by naturally occurring terpenoids. *Pharmaceutical Biology*, 49, 995-1007. <https://doi.org/10.3109/13880209.2011.559476>
- Lim, H. J., Park, J., Um, J. Y., Lee, S. S., & Kwak, H. J. (2019). Zileuton, a 5-lipoxygenase inhibitor, exerts anti-angiogenic effect by inducing apoptosis of HUVEC via BK channel activation. *Cells*, 8, 1182. <https://doi.org/10.3390/cells8101182>.
- Lokman, N. A., Elder, A. S. F., Ricciardelli, C., & Oehler, M. K. (2012). Chick chorioallantoic membrane (CAM) assay as an *in vivo* model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *International Journal of Molecular Sciences*, 13, 9959-9970. <https://doi.org/10.3390/ijms13089959>
- Longo, E., Morozova, K., Loizzo, M. R., Tundis, R., Savini, S., Foligni, R., ... Boselli, E. (2017). High resolution mass approach to characterize refrigerated black truffles stored under different storage atmospheres. *Food Research International*, 102, 526-535. <https://doi.org/10.1016/j.foodres.2017.09.025>

- Lu, L., Payvandi, F., Wu, L., Zhang, L.H., Hariri, R.J., Man, H.W., Chen, R.S., Muller, G.W., Hughes, C.C.W., Stirling, D.I., Schafer, P.H., Bartlett, J.B. (2009). The anti-cancer drug lenalidomide inhibits angiogenesis and metastasis via multiple inhibitory effects on endothelial cell function in normoxic and hypoxic conditions. *Microvascular Research*, 77, 78-86. <https://doi.org/10.1016/j.mvr.2008.08.003>
- Lyckander, I. M., & Malterud, K. E. (1992). Lipophilic flavonoids from *Orthosiphon spicatus* as inhibitors of 15-lipoxygenase. *Acta Pharmaceutica Nordica*, 4, 159-166.
- Ma, J., Lu, H., Wang, S., Chen, B., Liu, Z., Ke, X., Liu, T., Fu, J. (2015). The anthraquinone derivative emodin inhibits angiogenesis and metastasis through downregulating Runx2 activity in breast cancer. *International Journal of Oncology*, 46, 1619-1628. <https://doi.org/10.3892/ijo.2015.2888>
- Moylan, C., Scanlan, E., & Senge, M. (2015). Chemical synthesis and medicinal applications of glycoporphyrins. *Current Medicinal Chemistry*, 22, 2238-2348. <https://doi.org/10.2174/0929867322666150429113104>
- Mudliyar, D. S., Wallenius, J. H., Bedade, D. K., Singhal, R. S., Madi, N., & Shamekh, S. S. (2019). Ultrasound assisted extraction of the polysaccharide from *Tuber aestivum* and its in vitro anti-hyperglycemic activity. *Bioactive Carbohydrates and Dietary Fibre*, 20, 100198. <https://doi.org/10.1016/j.bcdf.2019.100198>
- Nadim, M., Deshaware, S., Saidi, N., Abd-Elhakeem, M. A., Ojamo, H., & Shamekh, S. (2015). Extracellular Enzymatic Activity of *Tuber maculatum* and *Tuber aestivum* Mycelia. *Advances in Microbiology*, 5, 57817. <https://doi.org/10.4236/aim.2015.57054>
- Nguyen, V. T., Tung, N. T., Cuong, T. D., Hung, T. M., Kim, J. A., Woo, M. H., ... Min, B. S. (2015). Cytotoxic and anti-angiogenic effects of lanostane triterpenoids from *Ganoderma lucidum*. *Phytochemistry Letters*, 12, 69-74. <https://doi.org/10.1016/j.phytol.2015.02.012>

- Pafumi, I., Festa, M., Papacci, F., Lagostena, L., Giunta, C., Gutla, V., Cornara, L., Favia, A., Palombi, F., Gambale, F., Filippini, A., Carpaneto, A. (2017). Naringenin impairs two-pore channel 2 activity and inhibits VEGF-induced angiogenesis. *Scientific Reports*, 7, 1-11. <https://doi.org/10.1038/s41598-017-04974-1>
- Patel, S. (2012). Food, health and agricultural importance of truffles: A review of current scientific literature. *Current Trends in Biotechnology and Pharmacy*, 6, 15-27.
- Patel, S., Rauf, A., Khan, H., Khalid, S., & Mubarak, M. S. (2017). Potential health benefits of natural products derived from truffles: A review. *Trends in Food Science and Technology*, 70, 1-8. <https://doi.org/10.1016/j.tifs.2017.09.009>
- Prinz, H., & Müller, K. (1996). 2-Anthracenonyl acetic acids as 5-lipoxygenase inhibitors. *Archiv Der Pharmazie*, 329, 262-266. <https://doi.org/10.1002/ardp.19963290507>
- Qu, Z., Jiang, C., Wu, J., & Ding, Y. (2016). Lenalidomide induces apoptosis and inhibits angiogenesis via caspase-3 and VEGF in hepatocellular carcinoma cells. *Molecular Medicine Reports*, 14, 4781-4786. <https://doi.org/10.3892/mmr.2016.5797>
- Sagar, S., Yance, D., & Wong, R. K. (2006). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 1. *Current Oncology*, 13, 14-26.
- Seow, L. J., Beh, H. K., Majid, A. M. S. A., Murugaiyah, V., Ismail, N., & Asmawi, M. Z. (2011). Anti-angiogenic activity of *Gynura segetum* leaf extracts and its fractions. *Journal of Ethnopharmacology*, 134, 221-227. <https://doi.org/10.1016/j.jep.2010.12.007>
- Seppänen-Laakso, T., Nygren, H., & Rischer, H. (2017). UPLC-ELSD Analysis of algal lipid classes and derivatization of bound and free fatty acids and sterols for GC-MS methods. In: Spilling K. (Eds) *Biofuels from Algae, Methods in Molecular Biology*. (pp. 223-232) Humana, New York, NY 1980,



1-10. [https://doi.org/10.1007/7651\\_2017\\_109](https://doi.org/10.1007/7651_2017_109)

- Shah, N., Usvalampi, A., Chaudhary, S., Seppänen-Laakso, T., Marathe, S., Bankar, S., Singhal, R., Shamekh, S. (2020). An investigation on changes in composition and antioxidant potential of mature and immature summer truffle (*Tuber aestivum*). *European Food Research and Technology*, 246, 723–731. <https://doi.org/10.1007/s00217-020-03438-7>
- Singh, S., Aggarwal, A., Bhupathiraju, N. V. S. D. K., Arianna, G., Tiwari, K., & Drain, C. M. (2015). Glycosylated porphyrins, phthalocyanines, and other porphyrinoids for diagnostics and therapeutics. *Chemical Reviews*, 115, 10261-10306. <https://doi.org/10.1021/acs.chemrev.5b00244>
- Sun, Y., Zhang, M., & Fang, Z. (2019). Efficient physical extraction of active constituents from edible fungi and their potential bioactivities: A review. *Trends in Food Science and Technology*. <https://doi.org/10.1016/j.tifs.2019.02.026>
- Takahashi, K., Hasegawa, T., Ishii, T., Suzuki, A., Nakajima, M., Uno, K., Yasuda, I., Kishi, A., Sadamoto, K., Abe, F., Tanaka, T. (2013). Antitumor effect of combination of hyperthermotherapy and 5-aminolevulinic acid (ALA). In *Anticancer Research*, 33, 2861-2866.
- Tang, Y., Li, Y. Y., Li, H. M., Wan, D. J., & Tang, Y. J. (2011). Comparison of lipid content and fatty acid composition between *Tuber* fermentation mycelia and natural fruiting bodies. *Journal of Agricultural and Food Chemistry*, 59, 4736-4742. <https://doi.org/10.1021/jf200141s>
- Wang, S., & Marcone, M. F. (2011). The biochemistry and biological properties of the world's most expensive underground edible mushroom: Truffles. *Food Research International*, 44, 2567-2581. <https://doi.org/10.1016/j.foodres.2011.06.008>
- Wang, Z., Dabrosin, C., Yin, X., Fuster, M.M., Arreola, A., Rathmell, W.K., Generali, D., Nagaraju, G.P., El-Rayes, B., Ribatti, D., Chen, Y.C., Honoki, K., Fujii, H., Georgakilas, A.G., Nowsheen, S.,

- Amedei, A., Niccolai, E., Amin, A., Ashraf, S.S., Helferich, B., Yang, X., Guha, G., Bhakta, D., Ciriolo, M.R., Aquilano, K., Chen, S., Halicka, D., Mohammed, S.I., Azmi, A.S., Bilsland, A., Keith, W.N., Jensen, L.D. (2015). Broad targeting of angiogenesis for cancer prevention and therapy. *Seminars in Cancer Biology*, 35, S224-S243. <https://doi.org/10.1016/j.semcancer.2015.01.001>
- Wei, X. Q., Ma, H. Q., Liu, A. H., & Zhang, Y. Z. (2013). Synergistic anticancer activity of 5-aminolevulinic acid photodynamic therapy in combination with low-dose cisplatin on hela cells. *Asian Pacific Journal of Cancer Prevention*, 14, 3023-3028. <https://doi.org/10.7314/APJCP.2013.14.5.3023>
- Wijaya, S., Jin, K. T., Nee, T. K., & Wiart, C. (2012). In vitro 5-LOX inhibitory and antioxidant activities of extracts and compounds from the aerial parts of *Lopholaena coriifolia* (Sond.) E. Phillips & C.A. Sm. *Journal of Complementary & Integrative Medicine*, 9, 11. <https://doi.org/10.1515/1553-3840.1615>
- Xu, J.Y., Meng, Q.H., Chong, Y., Jiao, Y., Zhao, L., Rosen, E. M., & Fan, S. (2013). Sanguinarine is a novel VEGF inhibitor involved in the suppression of angiogenesis and cell migration. *Molecular and Clinical Oncology*, 1, 331-336. <https://doi.org/10.3892/mco.2012.41>
- Yan, X., Wang, Y., Sang, X., & Fan, L. (2017). Nutritional value, chemical composition and antioxidant activity of three *Tuber* species from China. *AMB Express*, 7, 136. <https://doi.org/10.1186/s13568-017-0431-0>
- Yang, S. H., Lin, J. K., Huang, C. J., Chen, W. S., Li, S. Y., & Chiu, J. H. (2005). Silibinin inhibits angiogenesis via Flt-1, but not KDR, receptor up-regulation. *Journal of Surgical Research*, 128, 140-146. <https://doi.org/10.1016/j.jss.2005.04.042>
- Ye, Y. N., Liu, E. S. L., Shin, V. Y., Wu, W. K. K., & Cho, C. H. (2004). Contributory role of 5-

lipoxygenase and its association with angiogenesis in the promotion of inflammation-associated colonic tumorigenesis by cigarette smoking. *Toxicology*, 203, 179-188. <https://doi.org/10.1016/j.tox.2004.06.004>

Yoshikawa, K., Inoue, M., Matsumoto, Y., Sakakibara, C., Miyataka, H., Matsumoto, H., & Arihara, S. (2005). Lanostane triterpenoids and triterpene glycosides from the fruit body of *Fomitopsis pinicola* and their inhibitory activity against COX-1 and COX-2. *Journal of Natural Products*, 68, 69-73. <https://doi.org/10.1021/np040130b>

## Tables

Table 1: Basis for scoring the anti-angiogenic effect

Score	Observation	Effect
0	No change in blood vessels	Inactive
0.5	Minor changes in the blood vessels	Weak
1	Lack of capillaries below the disc area, reduction in the growth of blood vessels and convergence of few micro-vessels	Good
2	Absence of capillaries below the disc area, micro-vessels not visible and convergence of large vessels	Strong

Table 2: Interpretation of the anti-angiogenic effect of the samples based on the mean scores

Elucidation	Anti-angiogenic effect
Mean score < 0.5	no anti-angiogenic effect (inactive)
$0.5 \leq \text{mean score} \leq 1$	weak anti-angiogenic effect
$1 < \text{mean score} < 1.5$	good anti-angiogenic effect
Mean score $\geq 1.5$	strong anti-angiogenic effect

Table 3: List of minor compounds detected in petroleum ether extracts of *T. aestivum* identified using GC-MS analysis

Compound number	Compound name	Retention time (min)	Molecular weight (amu)	Match quality
1	Propanedioic acid, bis(trimethylsilyl) ester	5.835	248.090	76

2	10H-Dibenzo[b,E]thiopyran-10-one, 2-ethyl	5.965	240.061	55
3	3-Methylbenzidine	6.644	198.116	47
4	3-(1-Cyclopentenyl)furan	6.765	134.07	59
5	Butanoic acid, 2-(methoxyimino)-trimethylsilyl ester	7.126	203.098	59
6	Aminolevulinic acid tri-TMS	8.167	347.177	64
7	Nonanoic acid, trimethylsilyl ester	10.661	230.170	68
8	9,10-Anthracenedione, 1-amino-4-hydroxy	11.573	239.058	45
9	Dodecane, 1,1-dimethoxy	14.885	230.225	59
10	9,12,15-Octadecatrienoic acid, methyl ester	24.141	292.240	49
11	6-Amino-2,3-diphenyl-1H-pyrrolo[2,3-b]pyridine	24.381	285.127	64
12	Thymine, 5,6-dihydro-1,3-dimethyl-5,6-bis(trimethylsilyloxymethyl)	24.596	360.190	43
13	Benzylethyl-m-toluidine	24.820	225.152	25
14	Propane, 1,3-bis(dicyclopentylphosphino)	25.534	380.276	27
15	Phenol, 2,6-dimethyl-4-nitroso-	25.629	151.063	62
16	3-(2-Hydroxycyclohexyl)-furan	25.732	166.099	41

17	(3,4-Dihydroxyphenyl)hexanoic acid, tris (O-trimethylsilyl)	26.859	440.223	10
18	Docosanoic acid, methyl ester	27.753	354.350	99
19	4-(2,4,5-Trimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one	30.214	363.147	25
20	Silane, [[(3- $\beta$ )-ergost-8(14)-en-3-yl]oxy]trimethyl	36.527	472.410	90

Table 4: List of minor compounds detected in ethanol extracts of *T. aestivum* identified using GC-MS analysis

Compound number	Compound name	Retention time (min)	Molecular weight (amu)	Match quality
1	Dimethyl 8,13-dibromo-3,7,12,17-tetramethyl-21H,23H-porphine-2,18-dipropionate	6.481	694.079	9
2	1H-Indole-3-acetamide, 5-methyl	7.126	188.095	72
3	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	8.167	291.151	64
4	2,6-Dimethyl-4,4-pentamethylene-1,4-dihydropyridine-3,5-dicarbonitrile	10.042	227.142	47

5	1-Allyl-2-imino-10-oxo-1,10-dihydro-2H-1,9,10a-triazaanthracene-3-carboxylic acid, methyl ester	13.766	310.107	64
6	Lanostane-7,11-dione, (acetyloxy), (13- $\alpha$ ,14- $\beta$ , 17- $\alpha$ )	3- 16.261	500.387	59
7	Acetic acid, [[bis[(trimethylsilyl)oxy]phosphinyl]oxy]-, trimethylsilyl ester	17.362	372.101	53
8	Magnesium, [5,10,15,20-tetrakis(4-chlorophenyl)-21H,23H-porphinato(2-)-N21,N22,N23,N24]	17.612	772.061	4
9	1-(Dimethyl dodecyl silyloxy) octadecane	22.816	496.504	59
10	Salutaridinol	25.715	329.163	45
11	5-Oxo-1-(2-thienylmethyl) pyrrolidine-3-carboxylic acid	26.084	225.046	52
12	Benzothiophene-3-carbonitrile, 4,5,6,7-tetrahydro-2-(1,4-dihydro-2-amino-3-cyano-4,4-ditrifluoromethyl-6-methyl-1-pyridyl)	29.981	432.084	45

<b>13</b>	Friedooleanan-26-oic acid, 3- (acetyloxy), methyl ester, (3- )	30.205	514.402	32
<b>14</b>	Benz[a]phenanthridine, 2,3,10,11-tetramethoxy-8-methyl-	30.274	363.147	59
<b>15</b>	Naringenin-7-O-neohesperidoside, tms	31.383	1156.495	7
<b>16</b>	Heneicosanoic acid, tert-butyltrimethylsilyl ester	32.966	440.405	45

Table 5: Relative amounts of fatty acids detected in the petroleum ether and ethanol extracts of *T. aestivum*

Fatty acid	Relative amount (%)	
	Petroleum ether extract	Ethanol extract
C16:1 (FAME)	0.50	0.72
C16:0 (FAME)	13.57	11.12
C16:0 FFA (TMS)	2.60	5.60
C18:2 (FAME)	18.92	27.44
C18:1 (FAME)	34.28	21.50
C18:0 (FAME)	4.28	3.53
C18:2 FFA (TMS)	9.73	21.92
C18:1 FFA (TMS)	4.97	0.70
C18:0 FFA (TMS)	1.08	2.18



C20:2 (FAME)	0.42	0.65
C20:1 (FAME) + 9-HODE FAME TMS	3.53	1.72
C20:0 (FAME)	0.53	0.87
9-HODE FAME TMS isomer	1.15	0.00
13-HODE FAME TMS	1.09	0.00
9-HODE diTMS	2.09	0.64
9-HODE diTMS isomer	1.26	1.42

FAME = Fatty acid methyl ester; FFA= Free fatty acids; TMS = Trimethylsilyl derivative; HODE = Hydroxyoctadecadienoic acid

Table 6: Score of the anti-angiogenic effect of the truffle extracts and positive controls (silibinin and lenalidomide)

Sample	Dose ( $\mu\text{g}/\text{disc}$ )	Average score $\pm$ S.D.	Anti-angiogenic Effect
<b>Petroleum ether extract</b>	50	0.75 $\pm$ 0.25	Weak
	100	0.81 $\pm$ 0.24	Weak
	200	1.19 $\pm$ 0.50	Good
<b>Ethanol extract</b>	50	0.88 $\pm$ 0.22	Weak
	100	1.13 $\pm$ 0.33	Good
	200	1.31 $\pm$ 0.56	Good
<b>Silibinin</b>	50	0.63 $\pm$ 0.33	Weak
	100	0.88 $\pm$ 0.22	Weak
	200	1.13 $\pm$ 0.33	Good
<b>Lenalidomide</b>	50	0.69 $\pm$ 0.24	Weak

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100	1.06±0.39	Good
200	1.63±0.48	Strong

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Journal Pre-proofs

**Figure captions**

**Figure 1:** Anti-angiogenic effect of the extracts, positive controls, and negative controls on day 7. Shown in the images is angiogenesis in (a) absence of any extracts/drugs (b) presence of blank filter paper disc (c) petroleum ether extract of *T. aestivum* (d) ethanol extract of *T. aestivum* (e) silibinin and (f) lenalidomide. The images are representative of CAMs that were treated with extracts and positive controls with a dose of 200 µg/disc. Arrows show the site on the CAM where the discs were placed.

**Figure 2:** LOX-inhibition (anti-inflammatory) activity of the petroleum ether and ethanol extracts of *T. aestivum*. The experiments were performed in triplicates and the data is represented as mean. Error bars represent standard deviation. Data with the same data labels does not vary significantly ( $P < 0.05$ , Tukey's HSD test).

Figures

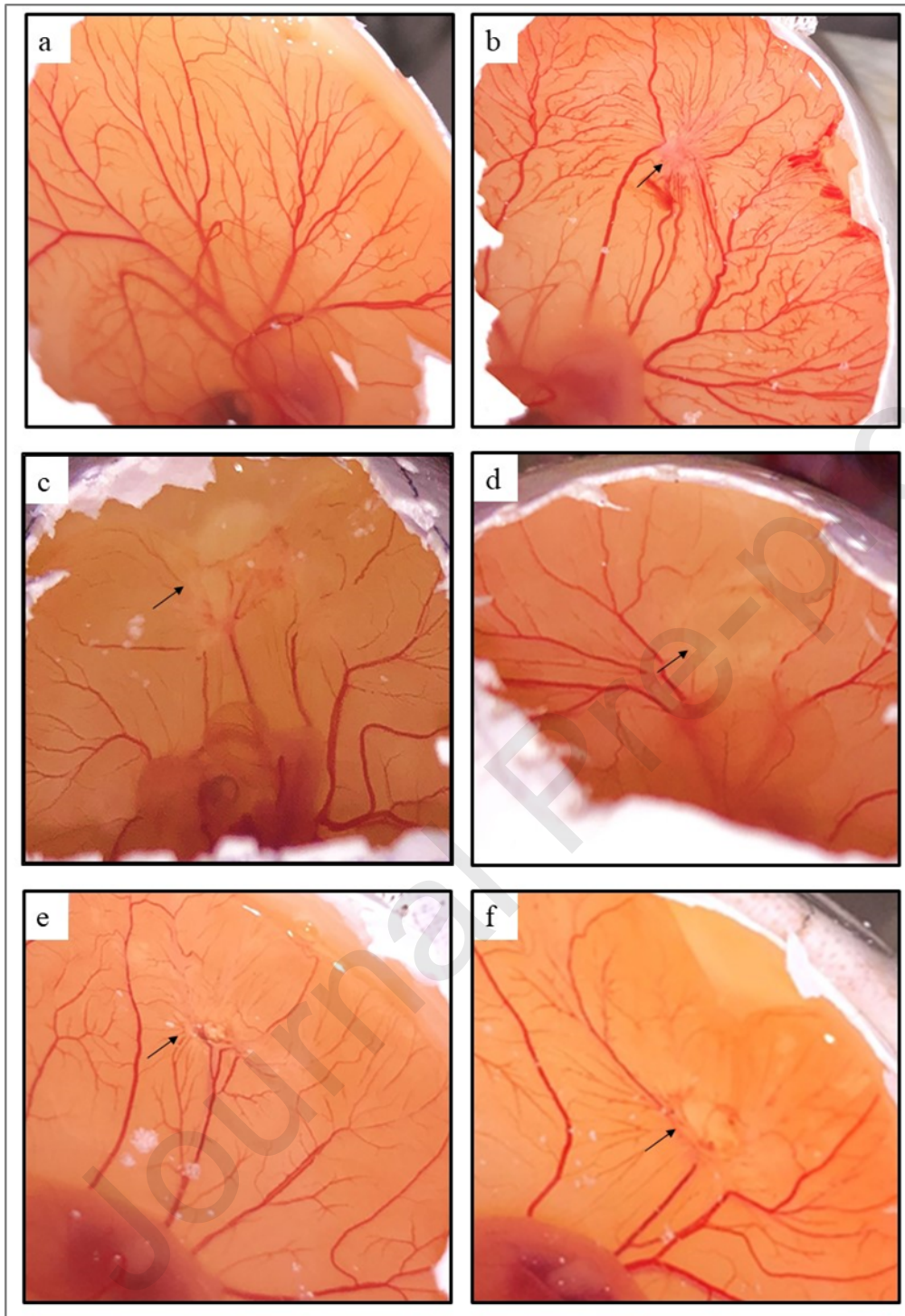
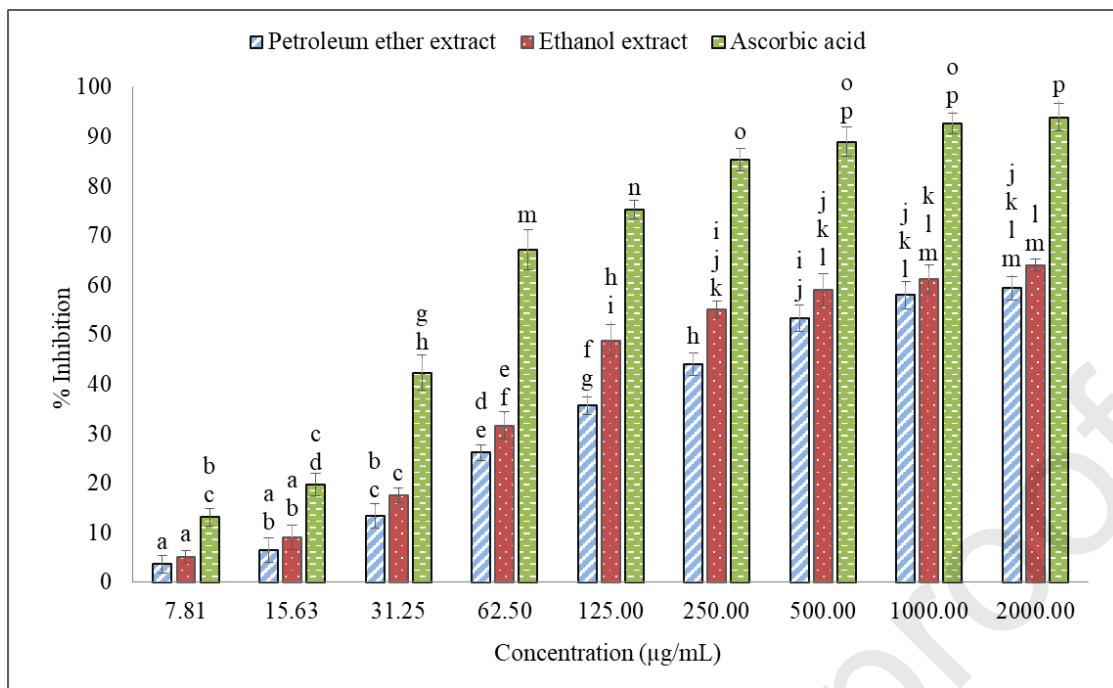


Figure 1

**Figure 2**

## Research highlights

- Extracts of *Tuber aestivum* were evaluated for chemical composition using GC-MS
- Extracts of *Tuber aestivum* displayed potent anti-angiogenic activity
- Extracts of *Tuber aestivum* showed *in vitro* anti-inflammatory activity
- Chemical composition of extracts could be correlated to their biological activities

## Graphical abstract

