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In Vitro Cholesterol Esterase Inhibitory Activity of Some Purified Phenolic Acids From *Agaricus bisporus*: An Investigation of Cardioprotective Properties

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Abstract

Background: Mushrooms with significant traditional medicine properties are rich sources of vitamins, proteins, fibres, minerals, and polyphenolic compounds.

Objectives: *Agaricus bisporus* (edible white button mushroom) extracts and their purified polyphenolic content were examined in terms of cholesterol esterase (CEase) inhibitory activity.

Methods: Organic solvent-based extraction methodologies were adopted to prepared the extracts. Two dimensional preparative thin layer chromatography (2D PTLC) technique and direct MS/ESI$^+$ method were also used to purify and elucidate the polyphenolic structures. Standard spectrophotometric analyses were employed to determine the flavonoid and phenolic acid contents as well as CEase inhibitory activity in both extract and purified polyphenols.

Results: The findings supported the strong cholesterol esterase inhibitory activity of aqueous and methanolic extracts (separating funnel: 97.9 $\pm$ 1.1% and 97 $\pm$ 0.8%), and acetone extract (shake flask: 97.6 $\pm$ 0.2%). The investigation also documented the remarkable quantity of flavonoid (32.61 $\pm$ 4.1 mg/g tissue) and phenolic acid (12.38 $\pm$ 1.32 mg/g tissue). A fraction of strong blue colour phenolic acid was purified using 2D PTLC technique, and the direct MS/ESI$^+$ method adopted for the purified eluate also revealed the presence of p-hydroxy benzoic acid (m/z 136), ferulic acid (m/z 194), cinnamic acid (m/z 146), and protocatechuic acid (m/z 154). The purified phenolic acids represent a moderate cholesterol esterase inhibitory activity (24.7 $\pm$ 1.7%; IC_{50} = 2.61 μg).

Conclusions: The study revealed the remarkable CEase inhibitory property, implying the cardioprotective attribute of *A. bisporus*.

Keywords: *Agaricus bisporus*, Cholesterol Esterase, Phenolic Acid, Preparative Thin Layer Chromatography

1. Background

Cardiovascular diseases (CVDs) are considered to be the leading cause of morbidity and mortality among human beings. According to Global Burden of Disease (GBD) report, 9.1 million deaths from CVDs were recorded in 1990s, and about 75% increase is expected by 2020 (1). Similarly, the age-standardized deaths from ischemic heart disease (178 per 1,00,000 persons each year), stroke incidence (154 per 1,00,000 persons each year), cerebrovascular disease (90 per 1,00,000 persons each year) and hypertensive heart disease (18 per 1,00,000 persons each year) were higher in India (2). Risk factors such as smoking, hypertension, hypercholesterolemia, high cholesterol to HDL ratio, type 2 diabetes mellitus (T2DM), and central/ visceral obesity were reported for CVDs (3). Currently, antidyslipidemic- (HMG CoA reductase inhibitors) and antiplatelet/antithrombotic-oriented drugs have been available in the market to control several CVDs (4). the other special strategies such as targeting chemokines, microRNA, and regeneration of cardiomyocytes are new fields of research as it is revealed that they significantly control CVDs (5). On the other hand, the adverse effects of the allopathy-based pharmacotherapy have diverted the attention of scientific community towards herbal- and fungal-based natural products as a treatment for CVDs. Although there is no proof regarding their efficacy and safety, consumption by children and pregnant women, and their specified active ingredients in natural products-based therapy, few medicinal mushrooms (e.g. *Agaricus bisporus*, *Grifola frondosa*, *Lentinus edodes* and *Pleurotus ostreatus*) with some health beneficial properties such as alter-

ation of lipid metabolism, antioxidant status, antihypertension, and antiplaque formation are marketed to control CVDs (6, 7).

Agaricus bisporus (white button mushroom) belongs to higher Basidiomycota and is a highly-cultivated edible mushroom (accounting for 40% of total mushroom species, which is used as an important nutritional and medicinal source worldwide. It contains high protein, low calories, restricted carbohydrate and lipids, essential fatty acids, fibre, minerals, vitamins (B-complex, C and D), essential amino acids, lectins, indole compounds, and phenolic compounds such as gallic, caffeic, ferulic, p-coumaric, cinnamic, protocatechuic acids, myricetin, and ergothioneine (8-10). Furthermore, *A. bisporus* possesses numerous ethno-medicinal properties, including antioxidant, anticarcinogenic, antimicrobial, antiviral, anti-inflammatory, immunomodulatory, anticancer, antinociceptive, antihypercholesterolemic antidiabetic, antiparasitic, and cardioprotective ones (11-15).

2. Objectives

Accordingly, the present study aimed to investigate *A. bisporus* extracts and purified phenolic acids to explore their cardioprotective properties considering in vitro CEase inhibitory activity.

3. Methods

3.1. Sample Collection and Preparation

The fresh white button mushroom (*Agaricus bisporus*) was procured from a local mushroom cultivator (Coimbatore, Tamil Nadu, India), washed with tap water to remove the dust and dirt, and then rinsed with distilled water. The fruiting body was carefully pruned and used for the experimental analysis.

3.2. Hot Water Extraction (HWE) and Pressurized Hot Water Extraction (PHWE)

A slightly modified procedure adopted by Shakthi Deve et al. (16) was employed for HWE. For this purpose, 1 g of white button mushroom was weighed, sliced into small pieces, and gently homogenized with 20 mL of distilled water using a physical homogenizer for 5 minutes. The resultant mixture was made up to 50 mL with distilled water and kept in a boiling water bath at 90°C for 10 minutes. The extract was filtered using Whatman no. 1 filter paper, and 20% ammonium sulphate was then added. The mixture was centrifuged at 10,000 rpm for 10 min to remove the protein content, and the ions were also removed using dialysis. The resultant supernatant was lyophilized, stored at 4°C, and used for the experimental analysis. The same procedure was adopted for PHWE; however, the contents were incubated in an oil bath at 180°C and 1002 kPa for 10 minutes.

3.3. Organic Solvent Extraction (OSE) by Separating Funnel (SeFM) and Shake Flask (ShFM) Methods

Five grams of the mushroom sample was picked, sliced into pieces, gently homogenized with a physical homogenizer, and transferred into a separating funnel containing 25 mL of organic solvent (a mixture of hexane, chloroform, acetone, ethyl acetate, ethanol, methanol, and distilled water). It was shaken thoroughly for 15 minutes and filtered using Whatman no. 1 filter paper. The residue was then re-extracted using the same solvent for at least thrice, and the mixture was centrifuged at 10,000 rpm for 10 min to remove the protein content. The resultant supernatant was lyophilized, stored at 4°C, and used for the experimental analysis (16). The same procedure was adopted for ShFM; however, the content was shaken in an orbital shaker at 100 rpm for 12 - 16 h (17, 18).

3.4. Estimation of Total Flavonoid Content (TFC) and Total Phenolic Acid (TPA)

TFC was estimated spectrophotometrically, as proposed by Zhishen et al. (19) with some slight modifications. TPA was also determined based on the method described by Eskin et al. (20). Quercetin (TFC) and ferulic acid (TPA) were used to construct the calibration curves.

3.5. In Vitro Cholesterol Esterase (CEase) Inhibitory Activity

The method described by (Kumar et al.(21)) was adopted to investigate in vitro cholesterol esterase inhibitory activity, and the CEase inhibition was calculated as follows: CEase inhibition (%) = [(Activity of control - activity of test)/activity of control] $\times$ 100. In order to detect CEase inhibitory activity, standard markers such as gallic acid, quercetin, rutin and ferulic acid were also analysed.

3.6. 1D Thin Layer Chromatography (TLC) and 2D Preparative Thin Layer Chromatography (2D PTLC) Analyses

The slightly modified method proposed by Sathishkumar et al. (22) was adopted for 1D TLC. Pre-coated and activated silica gel sheet was picked, and about 25 μL of the aqueous extract was spotted 0.8 cm far from the edge of the plate. The chromatogram was developed one dimensionally (1D) in an air-tight chamber in the presence of a mobile phase consisting of hexane: acetone (1:2). The plates were developed and visualized under both short and far UV

light (254 nm and 360 nm) to detect the polyphenols. The 2D PTLC technique was performed based on the method described by Shakthi Deve et al. (16), which was slightly modified.

3.7. High Resolution Liquid Chromatogram-Mass Spectrometry (HRLC-MS)

The purified PTLC eluate was subjected for direct HRLC-MS (electron spray ionization (ESI) positive mode; Q-TOF) analysis as per slightly modified procedure proposed by Sathishkumar et al. (23). The mobile phase composed of 0.3% formic acid (pH = 2.2) [phase A] and acetonitrile (Phase B). Gradient elution was performed at 0.9 ml/ min as follows:

1) Phase A: 88% (70 min);

2) Phase B: 9% - 15% (0 - 21 min), 15% - 22% (21 -45 min), 22% - 35% (45 - 60 min), 35% - 90% (60 - 65 min) and 90% (65 - 70 min)

The mass spectra was scanned in the range 100 - 1000 amu from 0 - 49.5 mins to obtain direct peaks.

3.8. Steam Blanching Method and Sensory Analysis

Fresh mushroom soup was prepared using steam blanching method at two different time duration (1 1/2 and 3 1/2 mins, respectively) at the same temperature by sieving and crushing mode of mushrooms. The soup was served by individuals aged between 18 - 40 years (n = 25), and the sensory analysis was performed based on the collected data from a questionnaire addressing variables such as colour, flavour, taste, consistency (physical texture), and overall quality. The questionnaire was scored using a nine-point Likert scale.

3.9. Statistical Analysis

All the numerical findings were expressed as mean ± SD. One way ANOVA, Student's *t*-test (paired), simple regression equation, and Karl Pearson correlation coefficient were also performed with MS excel version 10.

4. Results and Discussion

In the present study, the polyphenolic content (TFC and TPA) of *A. bisporus* was extracted using different methods such as HWE, PHWE, SeFM, and ShFM (Table 1). The results of the paired Student's *t*-test between TFA and TPA are presented in Table 2. TFC and TPA were expressed as quercetin equivalents (QAE) and ferulic acid equivalents (FAE), respectively. The standard calibration curve constructed for QAE (r^2 = 0.979) and FAE (r^2 = 0.956) revealed a strong correlation between the concentration and absorbance of the polyphenolic content. In contrast, Student's *t*-test results in terms of different extraction methodologies were not significant at 5% (P = 0.368). Moreover, ShFM was found to be superior in extracting increased amount of flavonoid (ethanol: 32.61 ± 4.1 mg/g tissue) and phenolic acid (aqueous: 12.38 ± 1.32 mg/g tissue), which, has established the importance of selection of solvents and extraction method to leach polyphenolic contents from the raw material. The aqueous and alcoholic solvent-based system extracted higher phenolic content which has endorsed the presence of appreciable polar polyphenolic moieties in the fruiting body. A similar result indicating the impact of aqueous system (ShFM) followed by alcoholic solvents (methanol and ethanol) on the extraction of polyphenol and flavonoid was reported by another researcher (24). The same result in the present study also proved the presence of ample amount of polar polyphenols.

The presence of pale yellow flavonoids (Rf = 0.92) and dark blue phenolic acids (Rf = 0.97) was noticed using 1D TLC technique (Figure 1). The previous documentations on the flavonoid obtained from various medicinal mushrooms with Rf values between 0.88 to 0.90 were in a similar vein with this finding in the present study (25). The further attempts to purify the phenolic content using 2D PTLC technique revealed a dark blue single spot (Figure 2). The MS analysis of PTLC purified phenolic acid eluate recorded the presence of four different phenolic acids, including p-hydroxy benzoic acid (RT = 0.786; m/z 136; relative abundance: 3.64×10^6 counts), cinnamic acid (RT = 0.945; m/z 146; relative abundance: 3.37×10^5 counts), protocatechuic acid (RT = 1.214; m/z 154; relative abundance: 4.42×10^5 counts), and ferulic acid (RT = 1.486 min; m/z 194; relative abundance: 1.11×10^7 counts) (Figure 3). According to the findings, there is high concentration of ferulic acid in the fruiting body of *A. bisporus*. Previous studies on methanolic and ethanolic extracts also revealed the presence ferulic acid, p-hydroxy benzoic acid, cinnamic acid, and protocatechuic acid. The present findings in terms of aqueous extract supported the previous reports (26-28). Moreover, no scientific community has investigated the aqueous extract-based phenolic acid profile as such our study was the first one purifying and reporting its phenolic content using mass spectrometry analysis.

The study further observed and compared the impact of extracts and purified phenolic acids on the inhibition of CEase. As shown in Figure 4, most of the organic solvent extracts from ShFM (acetone > ethyl acetate > ethanol > hexane > methanol) and SeFM (methanol > distilled water > ethanol > hexane >) revealed similarly high CEase inhibitory activity; however, HWE (15.6 ± 1.1%) and PHWE

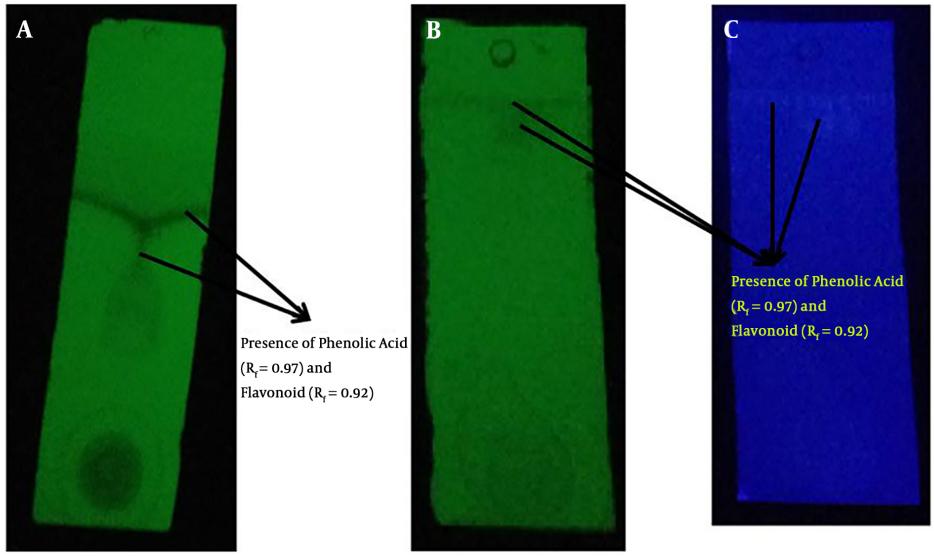

Figure 1. A and B, Visualization of flavonoid and phenolic acid under short UV light; C, visualization of flavonoid and phenolic acid under far UV light

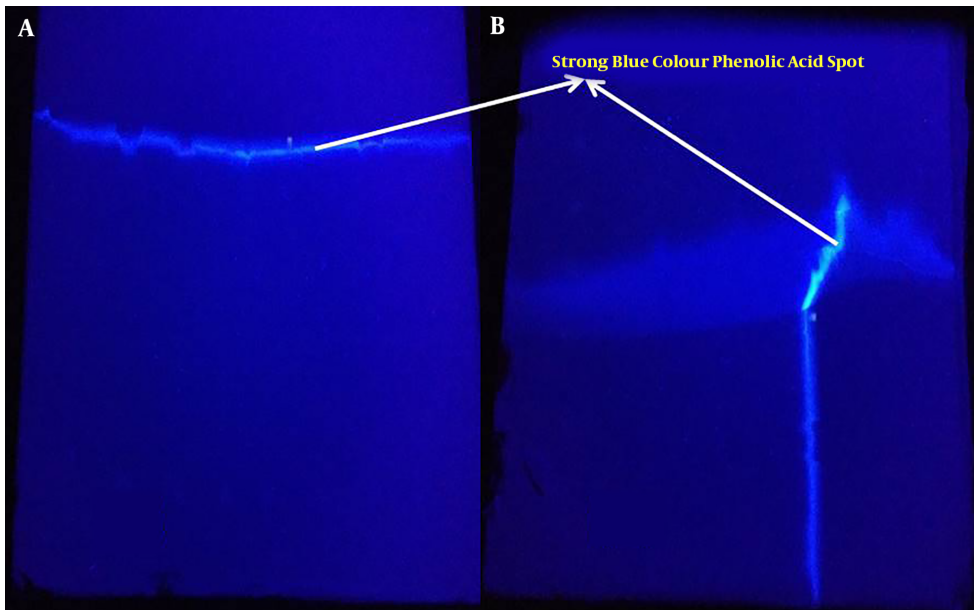

Figure 2. Purification of phenolic acid content using, A, 1D PTLC; B, 2D PTLC technique

(1.5 $\pm$ 2.3%) showed highly poor activity. Interestingly, the standard phenolic markers exhibited a moderate CEase inhibitory activity. Furthermore, the results of one-way ANOVA showed a significant difference at 1% (P = 0.00136), proving the strong efficacy of *A. bisporus* extracts against CEase activity, in comparison to the polyphenolic markers. In a same line, paired Student's *t*-test between ShFM- and SeFM-based extracts revealed not significant difference at 5% (P = 0.128), indicating the similar role of these two methods in inhibiting CEase activity. Moreover, the purified phenolic acids had a moderate cholesterol esterase inhibitory activity (24.7 $\pm$ 1.7%; IC_{50} = 2.61 μg), implying its significant role in controlling the total cholesterol level.

CEase belongs to α/β hydrolase family, and the cat-

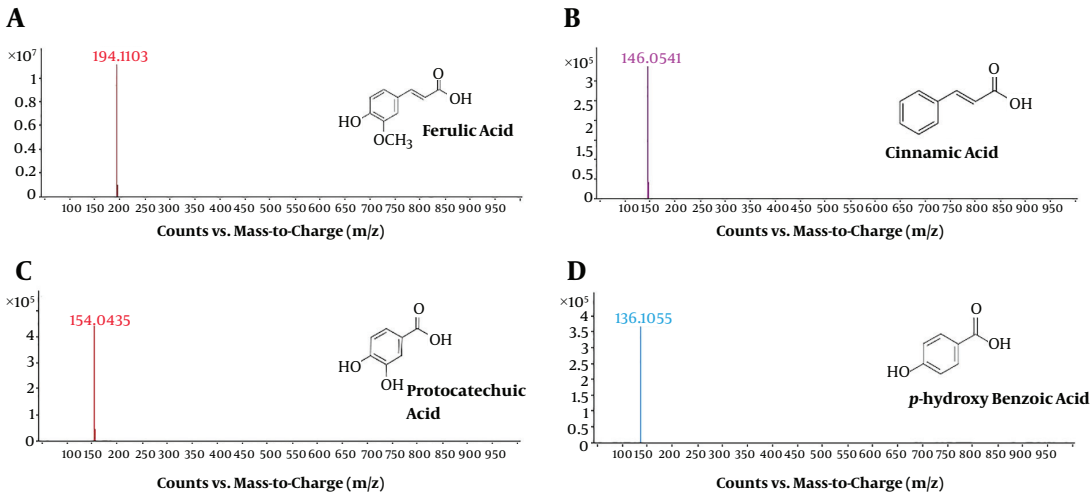

Figure 3. Direct mass spectral (MS) peak of purified phenolic acids

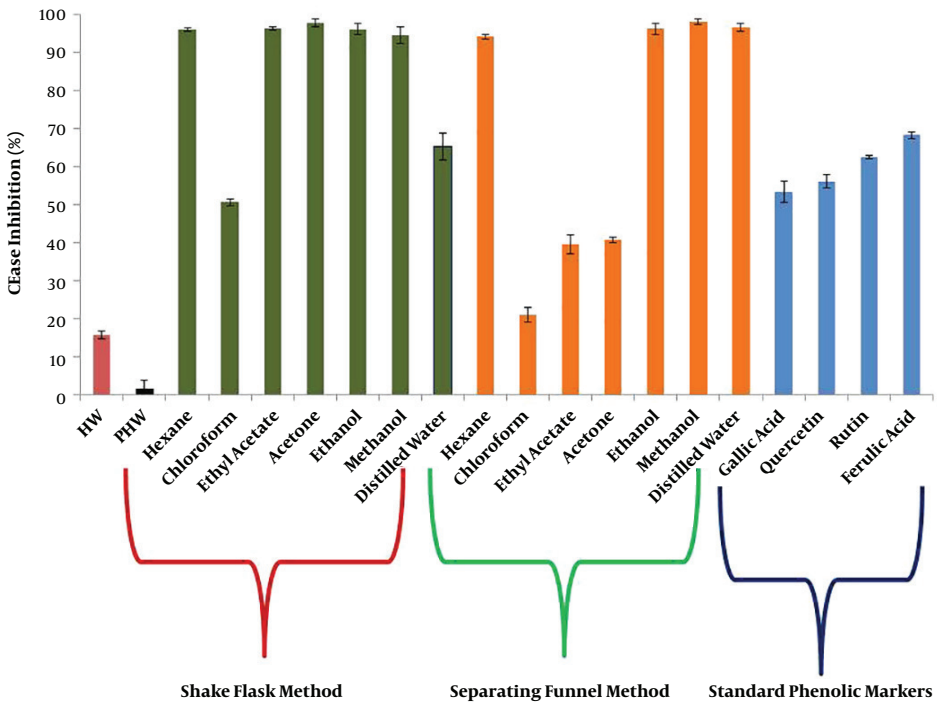

Figure 4. Comparative analysis of CEase inhibition (exhibited by various extraction methods and standard polyphenolic markers)

alytic activity is due to the presence of triad amino acid sequence (Ser194, Asp320 and His435) and oxyanion hole residues (Gly107, Ala108, Ala195). Similarly, residues such as Arg83 and Arg446 are found to be responsible for the activation of bile salt. Ser194 makes a nucleophilic attack on the cholesterol ester bond (carboxyl side), while Asp320 modulates the pKa of the reaction that facilitates the acid base catalysis of His435 upon the carbonyl side of the substrate to form an acyl-enzyme intermediate, and final release of fatty acid and cholesterol (29). Few reports are available, and little is known about the CEase inhibition mechanism, specifically by phenolic acids from mushrooms. Few reports have revealed the inhibitory property of gallic acid, mangiferin, catechin, epicatechin, procyanidins, and saponins from herbal extracts against CEase (30-32). The in silico docking studies on a few flavonoids such as apigenin, curcumin, glycitein, okanin, and rhamnazin also documented the presence of potent CEase inhibitory sites, especially the interaction with the catalytic triad and oxyanion hole residues (33). We assume that an approximately similar mechanism may be adopted by phenolic acids. In this regard, our study successfully proved the potent CEase inhibitory property of *A. bisporus* for the first time.

Table 1. A Comparative Analysis of TFC and TPA of *A. bisporus* Extracts From HWE, PHWE, SeFM and ShFM[a]

Type of Extraction Methodology	Total Flavonoid Content (*QAE mg/g Tissue)	Total Phenolic Acid Content (*FAE mg/g Tissue)
Hot water extraction (HWE)	1.391 ± 0.015^{b}	3.8 ± 0.23^{b}
Pressurized hot water extraction (PHWE)	6.198 ± 0.45^{b}	3.76 ± 0.039^{b}
Separating funnel method		
Hexane	2.436 ± 0.43^{b}	2.85 ± 0.049^{b}
Chloroform	0.713 ± 0.003^{b}	1.103 ± 0.054^{b}
Ethyl acetate	1.327 ± 0.054^{b}	0.343 ± 0.039^{b}
Acetone	0.04 ± 0.003^{b}	$0.971 \pm 0.078a$
Ethanol	0.178 ± 0.035^{b}	0.263 ± 0.056^{b}
Methanol	0.297 ± 0.013^{b}	0.286 ± 0.078^{b}
Distilled water	0.118 ± 0.034^{b}	0.691 ± 0.034^{b}
Pooled fraction	0.21 ± 0.098^{b}	1.333 ± 0.012^{b}
Shake flask method		
Hexane	1.485 ± 0.003^{b}	0.925 ± 0.056^{b}
Chloroform	0.178 ± 0.065^{b}	0.891 ± 0.004^{b}
Ethyl acetate	0.336 ± 0.002^{b}	0.525 ± 0.083^{b}
Acetone	1.287 ± 0.093^{b}	0.685 ± 0.005^{b}
Ethanol	32.61 ± 4.1^{b}	2.182 ± 0.089^{b}
Methanol	0.99 ± 0.005^{b}	6.868 ± 0.8^{b}
Distilled water	0.91 ± 0.034^{b}	12.38 ± 1.32^{b}
Pooled fraction	0.82 ± 0.045^{b}	0.027 ± 0.002^{b}

Abbreviations: FAE, ferulic acid equivalents; QAE, quercetin equivalents.
[a]Values are expressed as mean ± SD.
[b]Non-significant at 5% (Paired Student's *t*-test).

Table 2. Paired Student's *t*-Test Between TFC and TPA

t-Test: Paired Samples	TFC	TPA
Mean	2.8624444	2.216166667
Variance	57.126311	9.456800382
Observations	18	18
Pearson correlation	0.0378624	
Hypothesized mean difference	0	
df	17	
t Stat	0.3405577	
P (T ≤ t) one-tail	0.3688044	
t Critical one-tail	1.7396067	
P (T ≤ t) two-tail	0.7376088	
t Critical two-tail	2.1098156	

Moreover, further studies on the consumer impact of *A. bisporus* soup based sensory qualities ends with good result. The soup (crushed and sieved) prepared at 3 1/2 minutes was preferred by all the consumers as the best recording an average assessment value of 6 for the selected variables. The soup also recorded an appreciable CEase inhibitory property (24.7 ± 0.3%), which was similar to the activity of purified phenolic acids (24.7 ± 1.7%), the retention of phenolic acid (i.e., tolerance of phenolic acids against thermal decomposition) was recorded during the soup preparation process. The present study concludes the significant inhibitory property of *A. bisporus* against CEase as such it can be practised as an appropriate traditional medicine to control/ cure several CVDs.

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Footnotes

Authors' Contribution: Thiyagarajan Sathishkumar was as the supervisor of the project, he planned the study, and

wrote the manuscript. Venkatesan Suriyakala, Muthiah Lakshmanabharathy, and Selvarasu Suganya performed the experimental analysis. Kuppamuthu Kumaresan and Vinohar Stephen Rapheal reviewed the relevant literature of the project (phytochemistry and enzyme inhibitory kinetics). Soundararajan Nithyapriya was an assistant in the mushroom soup analysis process.

Conflict of Interests: The authors declare that there is no conflict of interest.

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References

1. Gupta R. Burden of coronary heart disease in India. *Indian Heart J*. 2005;**57**(6):632–8. [PubMed: 16521628].
2. Prabhakaran D, Jeemon P, Roy A. Cardiovascular diseases in India: Current epidemiology and future directions. *Circulation*. 2016;**133**(16):1605–20. doi: 10.1161/CIRCULATIONAHA.114.008729. [PubMed: 27142605].
3. Guha S, Ghosh A, Chatterjee N, Pal S, Guha S, Chatterjee S, et al. Risk factors for coronary heart disease in Indians: A case-control study from Eastern India. *Indian Heart J*. 2005;**57**(6):738–40. [PubMed: 16521652].
4. Stern CS, Lebowitz J. Latest drug developments in the field of cardiovascular disease. *Int J Angiol*. 2010;**19**(3):e100–5. doi: 10.1055/s-0031-1278379. [PubMed: 22477616]. [PubMed Central: PMC3014595].
5. Dimmeler S. Cardiovascular disease review series. *EMBO Mol Med*. 2011;**3**(12):697. doi: 10.1002/emmm.201100182. [PubMed: 22113984]. [PubMed Central: PMC3377116].
6. Liperoti R, Vetrano DL, Bernabei R, Onder G. Herbal medications in cardiovascular medicine. *J Am Coll Cardiol*. 2017;**69**(9):1188–99. doi: 10.1016/j.jacc.2016.11.078. [PubMed: 28254182].
7. Yeh JY, Hsieh LH, Wu KT, Tsai CF. Antioxidant properties and antioxidant compounds of various extracts from the edible basidiomycete Grifola frondosa (Maitake). *Molecules*. 2011;**16**(4):3197–211. doi: 10.3390/molecules16043197. [PubMed: 21499220]. [PubMed Central: PMC6260640].
8. Muszyńska B, Kala K, Rojowski J, Grzywacz A, Opoka W. Composition and biological properties of agaricus bisporus fruiting bodies- a review. *Polish J Food and Nutr Sci*. 2017;**67**(3):173–81. doi: 10.1515/pjfns-2016-0032.
9. Bhushan A, Kulshreshtha M. The medicinal mushroom Agaricus bisporus: Review of phytopharmacology and potential role in the treatment of various diseases. *J Nat Sci Med*. 2018;**1**(1):4.
10. Owaid MN, Barish A, Ali Shariati M. Cultivation of Agaricus bisporus (button mushroom) and its usages in the biosynthesis of nanoparticles. *Open Agriculture*. 2017;**2**(1). doi: 10.1515/opag-2017-0056.
11. Elmastas M, Isildak O, Turkekul I, Temur N. Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J Food Compos Anal*. 2007;**20**(3-4):337–45. doi: 10.1016/j.jfca.2006.07.003.
12. Ozturk M, Duru ME, Kivrak S, Mercan-Dogan N, Turkoglu A, Ozler MA. In vitro antioxidant, anticholinesterase and antimicrobial activity studies on three Agaricus species with fatty acid compositions and iron contents: A comparative study on the three most edible mushrooms. *Food Chem Toxicol*. 2011;**49**(6):1353–60. doi: 10.1016/j.fct.2011.03.019. [PubMed: 21419821].
13. Komura DL, Carbonero ER, Gracher AH, Baggio CH, Freitas CS, Marcon R, et al. Structure of Agaricus spp. fucogalactans and their anti-inflammatory and antinociceptive properties. *Bioresour Technol*. 2010;**101**(15):6192–9. doi: 10.1016/j.biortech.2010.01.142. [PubMed: 20363124].
14. Jeong SC, Jeong YT, Yang BK, Islam R, Koyyalamudi SR, Pang G, et al. White button mushroom (Agaricus bisporus) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. *Nutr Res*. 2010;**30**(1):49–56. doi: 10.1016/j.nutres.2009.12.003. [PubMed: 20116660].
15. Bhushan A, Kulshreshtha M. Cardioprotective activity of Agaricus bisporus against isoproterenol- induced myocardial infarction in laboratory animals. *Curr Nutr Food Sci*. 2019;**15**(4):401–7. doi: 10.2174/1573401314666180427161119.
16. Shakthi Deve A, Sathish Kumar T, Kumaresan K, Rapheal VS. Extraction process optimization of polyphenols from Indian Citrus sinensis - as novel antiglycative agents in the management of diabetes mellitus. *J Diabetes Metab Disord*. 2014;**13**(1):11. doi: 10.1186/2251-6581-13-11. [PubMed: 24397983]. [PubMed Central: PMC3929254].
17. Sathishkumar T, Baskar R. Screening and quantification of phytochemicals in the leaves and flowers of Tabernaemontana heyneana Wall.-a near threatened medicinal plant. *Res J Indian J Nat Produ Resour*. 2014;**5**(3):237–43.
18. Sathishkumar T, Baskar R. Renoprotective effect of Tabernaemontana heyneana wall. leaves against paracetamol-induced renotoxicity in rats and detection of polyphenols by high-performance liquid chromatography–diode array detector–mass spectrometry analysis. *J Acute Med*. 2014;**4**(2):57–67. doi: 10.1016/j.jacme.2014.02.002.
19. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem Toxicol*. 1999;**64**(4):555–9. doi: 10.1016/s0308-8146(98)00102-2.
20. Eskin NAM, Hoehn E, Frenkel C. A simple and rapid quantitative method for total phenols. *J Agric Food Chem*. 1978;**26**(4):973–5. doi: 10.1021/jf60218a029.
21. Kumar AP, Sivashanmugam AT, Umamaheswari M, Subhadradevi V, Jagannath P. Cholesterol esterase enzyme inhibitory and antioxidant activities of leaves of Camellia sinensis (L.) Kuntze. using in vitro models. *Int J Pharm Sci Res*. 2011;**2**(10):2675.
22. Sathishkumar T, Anitha S, Sharon RE, Santhi V, Sukanya M, Kumaraesan K, et al. Evaluation of in vitroinvertase inhibitory activity of Manilkara zapota seeds - A novel strategy to manage diabetes mellitus. *J Food Biochem*. 2015;**39**(5):517–27. doi: 10.1111/jfbc.12157.
23. Sathishkumar T, Baskar R, Aravind M, Tilak S, Deepthi S, Bharathikumar VM. Simultaneous extraction optimization and analysis of flavonoids from the flowers of Tabernaemontana heyneana by high performance liquid chromatography coupled to diode array detector and electron spray ionization/mass spectrometry. *ISRN Biotechnol*. 2013;**2013**:450948. doi: 10.5402/2013/450948. [PubMed: 25969771]. [PubMed Central: PMC4417552].
24. Abugri DA, McElhenney WH. Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal mushrooms as affected by different solvents. *J Nat Prod Plant Resour*. 2013;**3**(3):37–42.
25. Owusu E, Schwinger G, Dzomeku M, Obodai M, Asante I. Phytochemical, free radical scavenging activity and thin layer chromatography analysis of methanolic extracts of six wild Mushroom species collected from the Shai Hills Reserve of Ghana. *Pharmacogn J*. 2017;**9**(6s):s16–22. doi: 10.5530/pj.2017.6s.152.
26. Barros L, Duenas M, Ferreira IC, Baptista P, Santos-Buelga C. Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species. *Food Chem Toxicol*. 2009;**47**(6):1076–9. doi: 10.1016/j.fct.2009.01.039. [PubMed: 19425182].
27. Palacios I, Lozano M, Moro C, D'Arrigo M, Rostagno MA, Martínez JA, et al. Antioxidant properties of phenolic compounds occurring in edible mushrooms. *Food Chem*. 2011;**128**(3):674–8. doi: 10.1016/j.foodchem.2011.03.085.

28. Gąsecka M, Magdziak Z, Siwulski M, Mleczek M. Profile of phenolic and organic acids, antioxidant properties and ergosterol content in cultivated and wild growing species of Agaricus. *Eur Food Res Tech*. 2017;**244**(2):259–68. doi: 10.1007/s00217-017-2952-9.
29. Hui DY, Howles PN. Carboxyl ester lipase: Structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. *J Lipid Res*. 2002;**43**(12):2017–30. doi: 10.1194/jlr.r200013-jlr200. [PubMed: 12454261].
30. Shah KA, Patel MB, Shah SS, Chauhan KN, Parmar PK, Patel NM. Anti-hyperlipidemic activity of Mangifera indica l. leaf extract on rats fed with high cholesterol diet. *Der Pharmacia Sinica*. 2010;**1**(2):156–61.
31. Ngamukote S, Makynen K, Thilawech T, Adisakwattana S. Cholesterol-lowering activity of the major polyphenols in grape seed. *Molecules*. 2011;**16**(6):5054–61. doi: 10.3390/molecules16065054. [PubMed: 21694670]. [PubMed Central: PMC6264176].
32. Asmaa BH, Ream N. In vitro screening of the pancreatic cholesterol esterase inhibitory activity of some medicinal plants grown in Syria. *Int J Pharmacogn Phytochem Res*. 2016;**8**(8):1432–6.
33. Sivashanmugam T, Muthukrishnan S, Umamaheswari M, Asokkumar K, Subhadradevi V, Jagannath P, et al. Discovery of potential cholesterol esterase inhibitors using in silico docking studies. *Bangladesh Journal of Pharmacology*. 2013;**8**(3). doi: 10.3329/bjp.v8i3.14521.