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NOTE

Effect of different substrates on bioactive compounds of Shiitake Mushroom

Dhamini[1], Upma Dutta[1]*, Komal[1], Sachin Gupta[2], Moni Gupta[3] & Julie D Bandral[4]

[1]Division of Microbiology, Faculty of Basic Scienes, SKUAST-J, 180009J & K, India

[2]Division of Plant Pathology, Faculty of Agriculture, SKUAST-J, 180009 J & K, India

[3]Division of Biochemistry, Faculty of Basic Sciences, SKUAST-J, 180009J& K, Indai

[4]Division of PHM, Faculty of Horticulture and Forestry, SKUAST-J, 180009J & K, India

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Lentinula edodes (Shiitake mushroom) is a well-known culinary and medicinal fungus that is prized for its nutritional content and healing properties. This study examined the influence of four substrates—wheat straw, poplar, sheesham (Dalbergia sissoo), and mixed sawdust (1:1 mixture of poplar and sheesham sawdust)—on the antibacterial and antioxidant characteristics of Shiitake mushrooms. Fruiting bodies from each treatment were desiccated, pulverized, and extracted using ethyl acetate. Extracts were subjected to phytochemical analysis, antimicrobial testing against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, as well as antioxidant assays. The agar well diffusion method showed that mushrooms grown on mixed sawdust exhibited the highest antimicrobial activity against *E. coli* (12.00 ± 1.00 mm), *P. aeruginosa* (9.67 ± 0.52 mm), and *K. pneumoniae* (14.33 ± 1.07 mm). followed by poplar and wheat straw and sheesham substrate had the least activity. Mixed sawdust extracts also showed the lowest minimum inhibitory concentration (MIC) values of 100 µg/ml against *E. coli* and *K. pneumoniae* and 125 µg/ml against *P. aeruginosa*. The mixed sawdust extracts demonstrated superior antioxidant potential, indicated by the lowest IC_{50} values in the DPPH (27.32 µg/ml) and β-carotene/linoleic acid (28.43 µg/ml) assays. Additionally, mushrooms grown on mixed sawdust showed the highest total phenolic content (34.77 mg GAE/gdw) and total flavonoid content (11.58 mg QE/gdw), which correlated positively with improved antibacterial and antioxidant activities.The study reveals that substrate composition greatly influences Shiitake mushroom bioactivity, with mixed sawdust being most effective, underscoring its potential for medicinal and nutraceutical applications.

Keywords: Antimicrobial activity, Antioxidant activity, IC_{50}, *Lentinula edodes*, Minimum inhibitory concentration (MIC

The Japanese name for *Lentinula edodes,* shiitake mushroom, comes from the fact that it grows on the shii tree. It serves as an anticarcinogenic, anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral and antithromobotic agent in cardiovascular disorders[1]. They have 58-60% carbs, 20-23% protein (which is 80-87% digestible), 9-10% fiber, 3-4% lipids and 4-5% ash. It is cultivated on sawdust from the woddy plants Babla (*Acacia nilotica L.*), Champa (*Michelia champaca L.*), Jackfruit (*Artocarpus heterophyllus Lam*), Mango (*Mangifera india L.*) or mixtures of sawdust from all the trees with equal amounts or rice straw to see how it grows and bears fruit[2]. In general the yield of shiitake mushroom from mixed substrate ranges from 120.33 to 300.80g per packet[3]. Due to its lignocelluloytic enzymes, shiitake mushrooms have traditionally been grown on logs. Several lignocelluslosic materials and agri-industrial waste help shiitake grow and thrive. Substrates vary in pH, nutrients, water, and other factors that affect mycelium and fruiting body growth. They can affect mushroom quality and quantity because of this. Crop waste like wheat straw, corncobs, and sawdust provides nitrogen and carbon to the mycelium. Coffee grinds and chicken dung add nutrients and increase mushroom production[4]. It has been shown to have strong antioxidant properties, which makes it a functional food that is good for people's health. Antioxidant compounds are thought to shield cells from free radicals, which may lead to cancer, cardiovascular diseases and other health conditions[5]. Its nutritional elements consist in bioactive polysaccharides including b-D-glucan, heteroglucan, xylomannan, lentinan and eritadenine; free sugars including arabinose, mannose and glycerol; vitamins (B2, B12, D2)[6]. Shiitake mushrooms are composed of dietary fiber, a variety of macro and micronutrients, sugars, tocopherols and minimal amount of saturated fatty acids[7]. Consequently, by offering a healthy, high-yielding, low-input and environmentally sustainable source of food and income, shiitake mushroom farming could greatly help to ensure food safety[8].

Several studies have shown that the cultivation substrate has a major impact on the accumulation of bioactive chemicals and the biological activities that are linked to them, in addition to the development and production of shiitake mushrooms[16,17,19]. The

*Correspondence:
E-mail: upmaduttamicro@gmail.com

synthesis of phenolic compounds, flavonoids, polysaccharides, and other secondary metabolites with antibacterial and antioxidant characteristics can be changed by variations in substrate composition[20].

According to earlier research, the total phenolic content, total flavonoid content, antioxidant potential, and antibacterial activity of shiitake mushrooms grown on substrates with different lignocellulosic compositions vary significantly[23]. In order to increase the medicinal and nutraceutical potential of shiitake mushrooms, substrate composition modification is crucial.

The substrates utilized in this study—wheat straw, poplar sawdust, sheesham sawdust, and mixed sawdust—were chosen due to their quantity, affordability, and local availability in the Jammu area. The cellulose, hemicellulose, lignin, and nutritional contents of these lignocellulosic substrates vary significantly, which may have an impact on the metabolic processes involved in the manufacture of phenolics, flavonoids, and other bioactive metabolites in mushrooms. The production of these chemicals may be boosted by a nutritionally balanced substrate, which would improve the fruiting bodies' antibacterial and antioxidant capacity.

While many studies have examined the impact of cultivation substrates on Lentinula edodes growth and yield, little is known about the relative effects of sheesham sawdust, wheat straw, poplar sawdust, and mixed sawdust substrates on the bioactive potential of shiitake mushrooms. Additionally, there are few studies assessing the connection between the type of substrate, phytochemical composition, and biological activity of shiitake mushrooms grown on different substrates. Thus, the goal of this study was to compare how various substrates affected *L. edodes's* antibacterial activity, antioxidant capacity, total phenolic content, and total flavonoid content. The accumulation of bioactive chemicals and, in turn, the biological activities of shiitake mushrooms were thought to be greatly influenced by substrates with different chemical compositions.

The present study was designed to assess how various cultivation substrates—wheat straw, poplar sawdust, sheesham sawdust, and mixed sawdust—affect *Lentinula edodes's* total phenolic content, total flavonoid content, and antibacterial and antioxidant activities.

Material and Methods

Cultivation of Shiitake Mushroom

Shiitake mushroom was cultivated in the Mushroom unit of SKUAST-J on four different substrates i.e. Wheat straw, poplar, sheesham and mixed saw dust (1:1 mixture of poplar and sheesham sawdust). Before the bags were filled, the substrates' moisture level was adjusted to 65% and wheat bran was added to them (Table 1).The substrate bags were allowed to cool to ambient temperature after being pasteurized for two hours at 22 psi. For inoculation, wheat grain-based spawn was utilized at a 3% spawning rate (dry weight basis).The inoculation bags were kept in a dark, high-CO_2 environment at 22–26°C until the spawn run was finished. After complete colonization, the bags were subjected to ice shock treatment by exposing them to low temperature (4–10°C) for 24–48 h to induce pinhead formation. Bags were moved for pinhead initiation after full colonization and kept at 12–25°C, 85% relative humidity, and 800–1000 lux of light. Temperature and relative humidity were kept between 12 and 25°C and 80%, respectively, throughout fruit body maturity. After being harvested and thoroughly cleaned, the mature fruiting bodies were allowed to dry for 9-10 days at room temperature (25°C).The dried samples were subjected to grinding to get fine powder and stored for further use[9].

Table 1 — Yield of shiitake mushroom grow on four different substrates

S. No	Substrates	Yield(g/bag)
1	Poplar (*Populus*)	174.40 g
2	Sheesham (*Dalbergia sissoo*)	182.22 g
3	Wheat straw	152.33 g
4	Mixed	280.26 g

Extraction of secondary metabolites from fruiting bodies

The extracts were prepared by dissolving 20g of dried mushroom with 200ml of ethyl acetate separately in round bottom flasks and incubated at 30°C and 150rpm for 24 hours, followed by filtration using Whtaman No. 4 filter paper. To maximize the recovery of bioactive chemicals, the extraction process was repeated three times with solvent. The combined extract of ethyl acetate was dried by evaporation at 40°C, then redissolved in ethyl acetate to a concentration of 1mg/ml and kept at 4°C for further use[10].

Evaluation of antimicrobial propeties of crude extract of shiitake mushroom

Agar well diffusion method

The antimicrobial activity of crude extract of shiitake mushroom was evaluated by the Agar well diffuesion method against four pathogenic cultured: *E. coli, K. pneumonia, P. aeruginosa*. Nutrient agar

Table 2 — Yield of crude extract of shiitake mushroom grow on four different substrates using ethyl acetate

S. No.	Substrates	Yield (g)
1	Poplar (*Populus*)	10.46
2	Sheesham (*Dalbergia sissoo*)	0.72
3	Wheat straw	0.88
4	Mixed	11.37

(NA) had been melted and poured into petri dishes and left to solidify. A sterile swab is used to spread human pathogens evenly across the nutrient agar medium. After that, a 6mm cork borer was used to make four wells in the agar plates and pipette was used to fill the samples. The plates were kept at 37±2 for 24 hours and zones of inhibition were measured were measured using a scale. Positive control was streptomycin sulphate, and DMSO served as a negative control[11].

Evaluation of minimum inhibitory concentration of shiitake mushroom

Macro broth dilution method

The MIC of ethyl acetate extract from *Lentinula edodes* was determined using the macro broth dilution method (Table 2). Extract concentrations (20–120 µg/mL) were prepared in nutrient broth after dissolution in 1% DMSO. Overnight bacterial cultures were adjusted to 0.5 McFarland standard (≈1 × 10^6 CFU/mL), and 20 µL of inoculum was added to each tube. Following incubation at 37 °C for 24 h, turbidity was visually assessed. The MIC was recorded as the lowest concentration showing no visible growth[12].

Determination of total phenolic content of shiitake mushroom

The Folin-Ciocalteu's technique was used to measure the phenolic content. The different amount of the extract were mixed with 2ml FCR reagent that had been diluted with water and left at room temperature for 10 minutes. After that, 3ml of a 20% sodium carbonate solution was added. The solution was vortexed for 2 minutes and then kept at dark room for 30 minutes to incubate. A UV-Vis spectrophotometer was used to read the absorbance at 760nm. The data for the concentration of total phenols were calculated using the Gallic acid calibration curve as a standard and wre expresses as mg pf GAE's/gdw of extract[13].

Determination of total flavonoid content of shiitake mushroom

The total flavonoid was determined by aluminium chloride colometric assay. 2ml of 2% aluminium chloride in ethyl acetate were mixed with extracts that had been diluted with the same volume of the extract solution (1mg/ml). Five minutes later, 2ml of 5% of sodium nitrate. After that, the mixture was left at room temperature for 30 minutes to incubate. The spectrophotometer measured the absorbance at 415nm against a blank. Using the quercetin calibration curve as a standard, the total flavonoid concentration was measured and expressed as mg of QE's/gdw of extract[14].

Evaluation of antioxidant properties of Shiitake mushroom

DPPH free radical scavenging assay

Radical scavenging activity of extracts against 2,2-diphenyl1-1picrylhydrazyl (DPPH) radicals was determined spectrophotmetrically. In tubes, 2ml of ethyl acetate solution of DPPH radical were added, along wiith extracts of

shiitake at varied concentrations (20, 40, 60 and 80µg). The mixture was mixed vigorously and then kept at room temperature for 30 minutes. The absorbance was measured at 517nm, and the radical scavenging activity was estimated as a percentage of DPPH using the following formula:

$RSA\% = [(A_0\text{-}A_1/A_0)100]$

Where A_1 = absorbance of the control and A_0 = absorbance of the sample. The plot of inhibition (%) against extract concentration was used to figure out the extract concentration that caused 50% inhibition (IC_{50})[15].

β-Carotene/Linoleic acid test

The β-carotene/linoleic acid assay was used to assess the antioxidant activity of extracts of shiitake. make a solution of β-carotene, 2mg of β-carotene was mixed with 10ml of ethyl acetate. Then 2ml of this solution were put into a round bottom flask with a capacity of 100ml. After the ethyl acetate had entirely evaporated, 25µl of linoleic acid, 200mg of Tween 40 emulisifier and 100ml of distilled water were added and shaken vigorously for 30 minutes. 2ml of aliquots of this reaction mixture were transferred into separate test tubes with varying quantites of extracts (20, 40, 60 and 80µg). After that, the test tubes were shaken and left at room temperature for a while. The spectrophotometer assessed the zero time absorbance at 470nm right after the emulsion was added to each test tubes. After 120 minutes of incubation at room temperature, absorbance values were taken. The same process was used with BHT, with the same amount and a blank of ethyl acetate were repeated. The following equation was used to figure the antioxidant activity:

$$\%AA = 100[\ 1- (A_0-A_t) / (A_{00}-A_{0t})]$$

Where A_0 and A_{00} are the absorbance levels for the samples and controls at the start of the incubation. A_t and A_{0t} are the absorbance values for samples and control after 120 minutes of incubation. The graph of antioxidant activity percentage against extract concentration was used to figure out the extract concentration that gave 50% antioxidant activity (IC_{50}). BHT was used as standard[16].

Stastisitcal Analysis

All the experiments were carried out in triplicates and were expressed as Mean ± Standard Deviation.

Results

Yield from cultivation of shiitake mushroom

The fresh weight of fruiting bodies varied significantly with substrate type. The mixed sawdust produced the highest yield (280.26 g), followed by sheesham (182.22 g), poplar (174.40 g), and wheat straw (152.33 g), indicating that substrate composition strongly influences mushroom productivity (Fig 1).

Yield of crude extract from samples

The collected fruiting bodies from all substrates were dried at room temperature for 9-10 days and then subjected to grinding to get a fine powder. The powder from all the substrates were extracted using ethyl acetate as solvent. Ethyl acetate extraction yielded the highest crude extract from mushrooms cultivated on the mixed sawdust (11.37 g), with progressively lower yields from poplar (10.46 g), wheat straw (0.88 g), and sheesham (0.72 g).

Evaluation of antimicrobial activity of crude extract of shiitake mushroom

The agar well diffusion assay demonstrated that crude extracts from mixed sawdust grown mushrooms exhibited the strongest antibacterial activity against all tested pathogens—*E. coli* (12.00 ± 1.00 mm), *P. aeruginosa* (9.67 ± 0.52 mm), and *K. pneumoniae* (14.33 ± 1.07 mm). *P. aeruginosa* was unaffected by poplar extracts, although they had moderate action against *E. coli* (9.67 ± 0.58 mm) and *K. pneumoniae* (12.67 ± 1.01 mm). Extracts from sheesham showed little effect on *E. coli*, but they inhibited *P. aeruginosa* (8.33 ± 0.45 mm) and *K. pneumoniae*

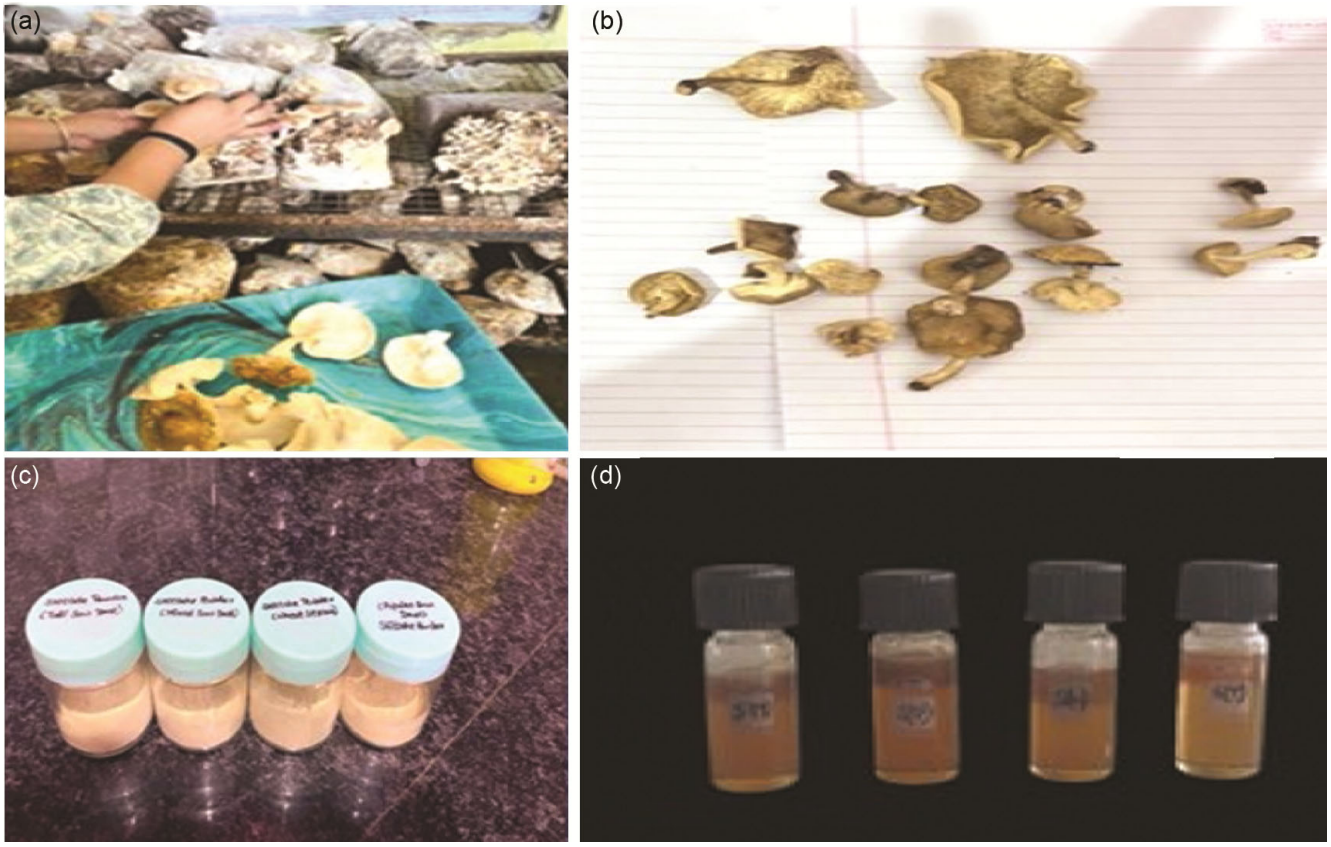

Fig 1 — (A) Cultivation of shiitake mushroom , (B) Drying of fruiting bodies, (C) Fine powder and (D) Extract of fruiting bodies

(8.67 ± 0.46 mm). (Table 3). The inhibition zones of wheat straw extracts ranged from 6.33 mm to 12.33 mm, indicating broad-spectrum but somewhat reduced activity (Fig 2).

Determination of minimum inhibitory concentration (MIC) of shiitake mushroom

MIC determination by the macro broth dilution method revealed that mixed sawdust extracts had the lowest MIC values—100 µg/ml for *E. coli*, 125 µg/ml for *P. aeruginosa*, and 100 µg/ml for *K. pneumoniae*. Poplar extracts ranked second (125–250 µg/ml), while sheesham and wheat straw extracts showed the weakest activity (250–500 µg/ml) (Table 4).

Determination of total phenolic content of Shiitake fruiting bodies

Using Folin–Ciocalteu's method, the highest TPC was recorded for the mixed sawdust extract (34.77 mg GAE/gdw), followed by wheat straw (32.03 mg GAE/gdw), sheesham (27.73 mg GAE/gdw), and poplar (23.23 mg GAE/gdw) (Fig 3).

Determination of total flavonoid content of Shiitake fruiting bodies

Flavonoid estimation via the aluminium chloride method showed the highest content in the mixed sawdust extract (11.58 mg QE/gdw), followed by wheat straw (10.44 mg QE/gdw), sheesham (9.35 mg QE/gdw), and poplar (6.82 mg QE/gdw) (Fig 4).

Evaluation of antioxidant properties of shittake fruiting bodies

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity assessed by DPPH radical scavenging showed the lowest IC_{50} value for the mixed sawdust extract (27.32 µg/ml), indicating maximum activity. Wheat straw (45.07 µg/ml) and sheesham (49.24 µg/ml) extracts showed moderate activity, while the poplar extract exhibited the weakest activity (65.46 µg/ml) (Fig 5).

β-Carotene/Linoleic acid test

In the β-carotene bleaching method, the mixed sawdust again showed superior antioxidant activity

Table 3 — Antimicrobial activity of crude extract of shiitake mushroom grow on four different substrates

Extracts from fruiting bodies	Zone of Inhibition (mm)		
	Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Poplar substrate (*Populus*)	9.67±0.58	0.00±0.00	12.67±1.01
Talli substrate (*Dalbergia sissoo*)	0.00±0.00	8.33±0.45	8.67±0.46
Wheat straw	6.33±0.50	6.67±0.30	12.33±1.04
Mixed substrate	12.00±1.00	9.67±0.52	14.33±1.07
Positive Control	19.00±0.58	22.00±1.00	20.00±1.00
Negative Control	No growth	No growth	No growth

[Data expressed as Mean ± Standard Deviation (n=3); Negative control: DMSO; Positive control: Streptomycin sulphate]

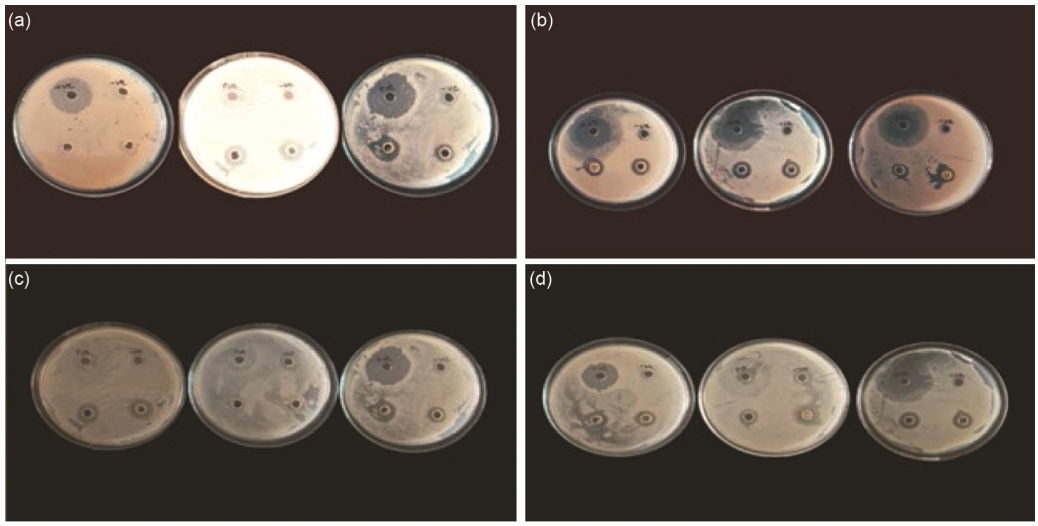

Fig 2 — A[ntimicrobial activity of shiitake mushroom grow on (A) sheesham substrate, (B) mixed substrate, (C) poplar substrate and (D) wheat straw against *E. coli, P. aeruginosa* and *K. pneumoniae*.

Table 4 — Minimum inhibitory concentration of crude extract of shiitake mushroom grow on four different substrates

Substrates	MIC (ug/ml)		
	Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Poplar (*Populus*)	125	250	150
Sheesham (*Dalbergia sissoo*)	250	500	250
Wheat straw	250	500	250
Mixed	100	125	100

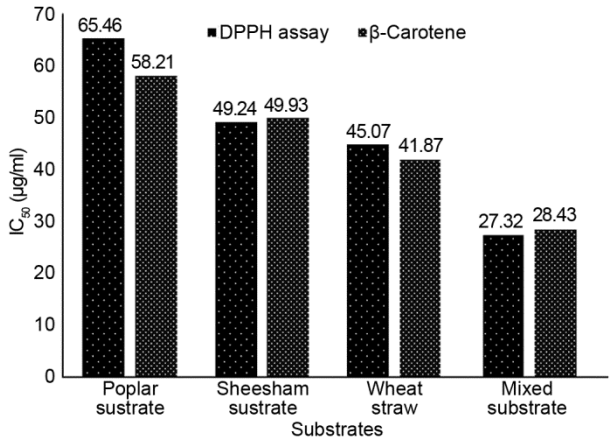

Fig 3 — Graphical representation of DPPH assay and β-Carotene/Linoleic acid test

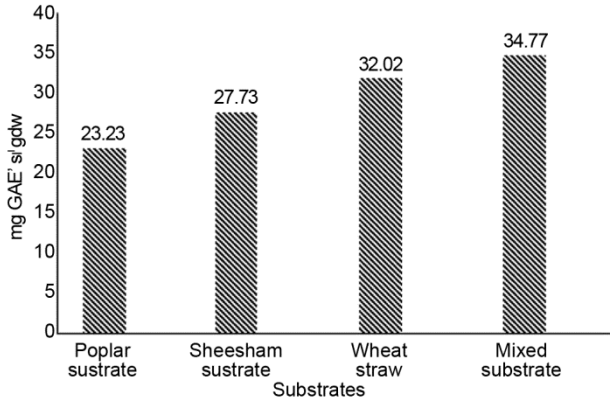

Fig 4 — Graphical representation of Total Phenolic content

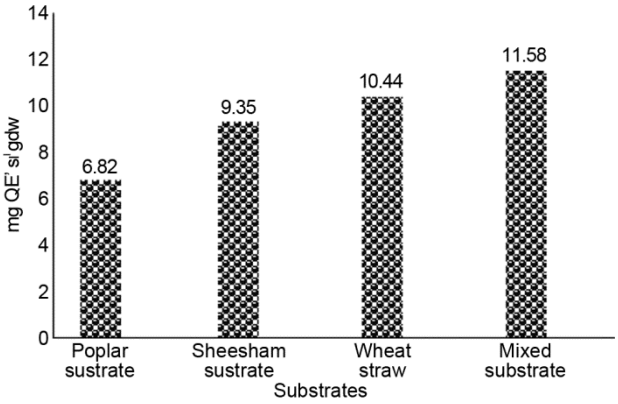

Fig 5 — Graphical representation of Total Flavonoid Content

(IC_{50} = 28.43 µg/ml). Wheat straw (41.87 µg/ml) and sheesham (49.93 µg/ml) displayed moderate effectiveness, whereas poplar had the highest IC_{50} (58.21 µg/ml), indicating the lowest activity.

Discussion

The present study confirmed that *Lentinula edodes* possesses notable medicinal properties and is widely consumed due to its high nutritive value.

In Skuast-J's mushroom unit, shiitake mushrooms were grown on wheat straw, poplar sawdust, sheesham sawdust, and mixed sawdust and harvested. Most fruiting bodies were found on mixed sawdust, followed by talli, poplar, and wheat straw.Combining two lignocellulosic materials improves the mixed sawdust substrate's physical and chemical properties. Mixtures have better aeration, porosity, moisture retention, and nutritional balance. These factors provide the ideal environment for L. edodes to proliferate and distribute mycelium, which speeds up fruiting body production and improves Baktemur *et al*[17]. According to Desisa *et al*[18] substrate combinations increase plant access to nutrients, which promotes growth. To get the most mushrooms with the best quality.

Normal solvent extraction with ethyl acetate extracted the fruiting bodies. Fruiting bodies of mixed sawdust yielded more extract than other substrates. Mixed substrate mushrooms may have had a higher extractive yield due to their better biochemistry. Because they contain more nutrients and lignin, mixed substrates help produce and preserve bioactive substances such phenolics, flavonoids, terpenoids, and polysaccharides.The quality and composition of the substrate considerably affects the quality and production of mushroom crude extracts, according to Imam *et al*[19].

The crude extract from all substrates' fruiting bodies was tested for *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* using agar well diffusion. The crude extract from mushrooms grown on the mixed sawdust substrate inhibited all three pathogens, even though substrate activity varied. A diverse substrate with lignin and nutrients may

produce a large range of bioactive secondary metabolites that kill bacteria according to Baptista *et al*[20]. According to Kumla *et al*[21] mushroom substrate greatly affects extract bioactivity.

All substrate extracts with antibacterial activity were tested for MIC by microdilution against *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Ethyl acetate extract of Lentinula edodes cultivated on a mixed substrate had the highest antibacterial activity against all strains. The rich and varied substrate, which boosts bioactive secondary metabolite production such phenolics and flavonoids, may explain this powerful inhibitory action. The mixed substrate's nutritional and lignocellulosic diversity may increase these chemicals, which inhibit the growth bacterial cells Chun *et al*[22]. Bhattacharjya *et al*[23] found that mushroom extracts, including Lentinula edodes, Ganoderma lucidum were performed best antimicrobial activity.

The study assessed the antioxidant capabilities of crude extracts using DPPH assay and β-Carotene/Linoleic acid test system. Both approaches found that its crude extract from mixed substrate fruiting bodies had more antioxidants than others. Due to its phenolic and flavonoid content, the mixed substrate extract is more antioxidant. According to Prathima *et al*[24], shiitake mushrooms contain high β-carotene and DPPH radical scavenging properties.

Phenolic chemicals were highest in the crude extract of mixed sawdust fruiting bodies, according to the FCR technique. Crude extract of mixed sawdust fruiting bodies had the highest flavonoid content, measured using the aluminium chloride method. Shiitake mushroom phenolic content was studied by Baptista *et al*[20]. Their investigation showed that high-temperature extractions consistently produced increased total phenol activity. Our findings match Prathima and Prabhakar's[24] observation that shiitake mushrooms had the lowest flavonoid concentration.

Conclusion

This study demonstrates that the selection of cultivation substrate significantly affects the production, phytochemical composition, and bioactive qualities of Shiitake mushrooms (*Lentinula edodes*). Mixed sawdust was the best of the four substrates examined. It produced the most mushroom biomass, extract yield, and phenolic and flavonoid levels. These higher quantities of phytochemicals directly improved the extracts' ability to fight bacteria and free radicals, as shown by the lowest MIC values against *Escherichia coli* and *Klebsiella pneumoniae* and the highest radical scavenging and lipid peroxidation inhibition. The tight link between phenolic/flavonoid levels and biological activity shows that these chemicals are very important in determining how mushrooms work. Overall, the results show that small changes to the way Shiitake mushrooms are grown, like choosing the right substrate, can greatly improve their nutritional and therapeutic value. This makes them an even stronger source of natural antioxidants and antimicrobials for functional food and nutraceutical uses.

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Conflict of Interest

Authors declare no competing interest.

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