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Metabolites Secreted by Fungal Species Isolated from Spoiled Food and their Effect on *Klebsiella Species*

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Abstract: ***Fungi are extremely diverse group of organisms, which produce a wide range of metabolites through various metabolic pathways. This experiment was essentially performed to investigate the antagonistic properties of the fungal isolates (T, S, G, B, I, L) obtained from house hold spoiled food. Agar well diffusion method was used, in which the antibacterial activity of fungal isolates was examined by testing the effect of their culture filtrates on MHA against the growth of the active lab culture of Klebsiella sp. Results revealed that the highest antibacterial properties were possessed by the fungal isolates T and S. An attempt was made to extract the metabolite through solvent extraction and simple distillation methods. Also characterization of the metabolites produced in this process, was done through TLC. The results indicate that the fungal isolates contain antibacterial properties which are evident against bacterial sp. Further research has to be done in order to characterize the metabolites obtained through the fungal isolates. Further identification techniques, such as UV and IR analysis of the metabolites can be performed in order of a clear understanding and biochemistry of the isolated fungal metabolites. Additional studies can lead to the discovery of novel metabolites and their characteristic features which can have a wide range of applications in Pharmaceutical, neutraceutical, agricultural industries, and also many bioremedial properties.***

Keywords: Metabolites, Fungal Species, Spoiled Food, Klebsiella Species

List of abbreviations

sp.	Species
MHA	Muller Hinton Agar
TLC	Thin Layer Chromatography
Rf	Retardation factor
UV	Ultra Violet
IR	Infrared
C	Carbon
N	Nitrogen
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
pH	Power of Hydrogen
PDB	Potato Dextrose Broth
rpm	Revolutions Per Minute
℃	Degree Centigrade
mL	milliliter
mm	millimeter
µm	Micro meter
hrs	hours
min	minutes
Mg/mL	Concentration
ZOI	Zone of Inhibition
PEN	Penicillin
CTR	ceftriaxon

1. Introduction

1.1Metabolites

The metabolism can be defined as the sum of all the biochemical reactions carried out by an organism. Metabolites are the intermediates and products of metabolism and are usually restricted to small molecules. Metabolism is defined as the sum of all the biochemical reactions carried out by an organism. Metabolites are the intermediates and products of metabolism and are usually restricted to small molecules.

- **Primary metabolite** is a kind of metabolite that is directly involved in normal growth, development, and reproduction. It usually performs a physiological function in the organism (i. e. an intrinsic function).
- **Secondary metabolites** are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism.

Though secondary metabolites are derived from primary metabolism, they do not make up the basic molecular skeleton of the organism. Its absence does not immediately curtail the life of an organism, a feature contrary to primary metabolite, but the survival of the organism is impaired to a larger extent. Its presence and synthesis are observed in ecologically disadvantaged species within a phylogenetic group (Tiwari R, Rana CS et al., 2015). The metabolite can serve as a starting material for deriving a product of interest, extended further through chemical or biological transformation. New analogue or templates in which secondary metabolite serve as lead compounds, leads discovery and design of new drugs.

The intermediates of primary and secondary metabolites overlap with each other. Amino acids can be considered to be secondary metabolites as they are the products of primary metabolites (Verpoorte R, van der Heijden R, 2000). The mosaic nature of the intermediate implies that there is a common biochemical pathway being shared by both, the primary and secondary metabolism (Yeoman MM and Yeoman CL, 1996).

The excess of C and N in the primary metabolism are shunted into the secondary metabolites, to form an inactive

form of primary metabolism, which serves as the buffer zone. Through a process of metabolic disintegration of secondary metabolites, the stored C and N can revert to primary metabolites when on demand. For an organism to survive, there should be dynamism and a delicate balance between various activities of both the primary and secondary metabolism, which are strongly influenced by the development of the organism, cell development, tissue differentiation, and also the external pressures such as the utilization of certain growth factor (Collin HA, 2001)

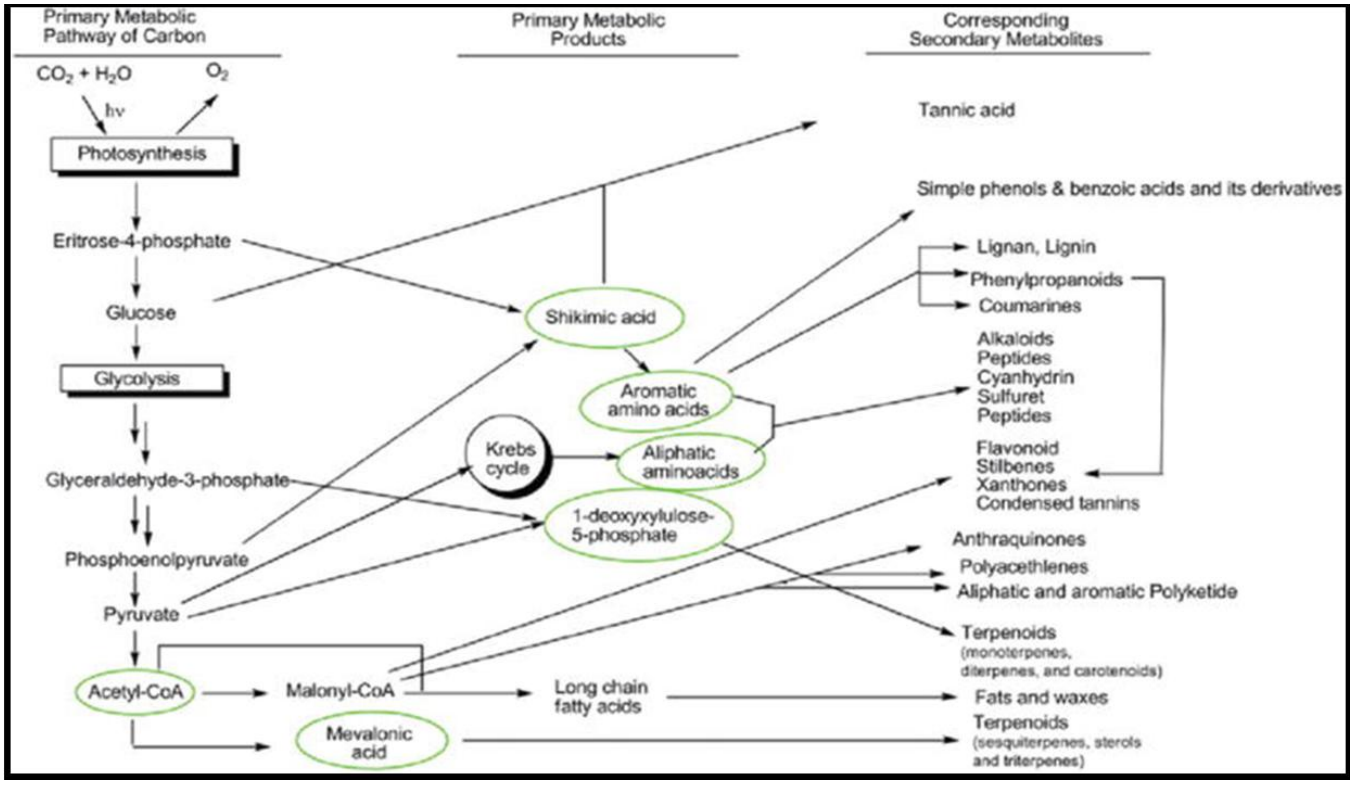

Figure 1: Schematic diagram representing the integration of primary and secondary metabolism (JánosBérdy, Journal Antibiot, 2005)

The principle process of natural fermentation product synthesis can be advantageously scaled up and employed to maximize its application in the field of medicine, agriculture, food, and the environment. Antagonistic strains belonging to the Trichoderma and Fusarium genera were able to produce various secondary metabolites which can play a role in the mechanism of their biological activity. Production of antimicrobial secondary metabolites has been reported in many fungal biocontrol agents (Gottlieb and Shaw 1970). They are produced during the late growth phase of the microorganisms. The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in the logarithmic phase and depressed in stationary growth phases (Demain, 1999).

S.No.	Intermediates from primary metabolic pathway	Secondary metabolites derived
1.	Shikimic acid	Ergot alkaloids, antibiotics: candicidin and chloramphenicol
2.	Amino acids	Antibiotics: penicillin, cephalosporins and cephamycins, and gramicidin, immunosuppressive cyclosporine
3.	Acetyl-CoA and other Kreb's cycle intermediates	Antibiotics: erythromycin, antiparasitic avermectin antitumor doxorubicin, taxol
4.	Sugars	Antibiotics: streptomycin and kanamycin.

Figure 2: Types of secondary metabolites produced from different intermediates of the primary metabolic pathway (JánosBérdy, Journal Antibiot, 2005)

1.2 Fungal Secondary metabolites

Fungi are eukaryotic organisms that are known for their quality of inhabiting almost all ecological niches of the Earth and have the ability to utilize various solid substrates as a consequence of the diversity of their biological and biochemical evolution. Some of the solid substrates utilized by fungi are dead and decaying material, including herbivore dung (saprophytic and coprophilous fungi), live plants (endophytic, parasitic, and mycorrhizal fungi), lichens (lichenicolous and endolithic fungi), and insects (entomopathogenic fungi). A characteristic feature of many of these fungi, especially those that exhibit filamentous growth and have a relatively complex morphology, is their ability to produce secondary metabolites. Soilborne, parasitic, and saprophytic fungal sources are relatively well investigated concerning their secondary metabolites, and currently, there is intense interest in secondary metabolites of symbiotic fungi that live in association with land plants, insects, lichens, and marine organisms. In contrast to primary metabolites such as proteins, DNA, RNA, polysaccharides, and so on, which occur universally, secondary metabolites are small - molecule organic compounds found restricted to a particular species, genus, or family. Thus, the presence or absence of certain secondary metabolites has been used successfully in the classification (chemotaxonomy) of large ascomycete genera (including Alternaria, Aspergillus, Fusarium, Hypoxylon, Penicillium, Stachybotrys, and Xylaria) and in a few genera of basidiomycetes. Many secondary metabolites are not involved directly in the normal growth, development, or reproduction of the fungus in which they occur, but they may play an important role in ecological interactions with other organisms. For this reason, many fungal secondary metabolites exhibit useful biological activities and are of interest to the pharmaceutical, food, and agrochemical industries. Production of secondary metabolites often occurs after fungal growth has ceased as a result of nutrient limitations but with an excess carbon source available, making it possible to manipulate their formation. Intriguingly, some endophytic fungi are capable of producing secondary metabolites previously known from plants. Filamentous fungi produce a diverse array of secondary metabolites. These are often restricted to a narrow set of species within a phylogenetic group (Ellen M Fox and Barbara Howlett, 2008).

Fungi are an extremely diverse group of organisms, with about 230, 000 species distributed widely essentially in every ecosystem. Among them, only limited species are considered to be effective biocontrol agents. The fungal antagonists restrict the growth of plant pathogens by the three suggested mechanisms: antibiosis, competition, and parasitism. Besides, they also induce the defense responses in host plants, termed “induced systemic resistance” (van Loon et al.1998). Among the above - mentioned mechanisms, antibiosis is considered the most important, in which the antagonists produce an array of secondary metabolites such as antibiotics and toxins, which contribute to the antagonistic activity of fungal biocontrol agents against plant pathogens. They are produced during the late growth phase of the microorganisms. The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in the logarithmic phase and depressed in stationary growth phases. The microbial secondary metabolites have a distinctive molecular skeleton which is not found in the chemical libraries and about 40% of the microbial metabolites cannot be chemically synthesized (Feher M, Schmidt JM, 2003).

Table 1: Secondary metabolites produced by fungi

Name of secondary metabolite	Source of secondary metabolite	Biological activity	Reference
Lovastatin	*Monascusruber; Aspergillusterreus*	Enzyme inhibitor	Dewick, 2009
Limonene and guaiol	*Trichodermaviride*	Antimicrobial	Awad et al., 2018
Tubercullariols	*Tubercularia* sp.	Anticancer	Xu et al.2009
Oxaline	*Penicilliumraistricki*	Anti - cell proliferation	Sumarah et al.2011
Benzomalvin C	*Penicilliumraistrickii*, *Penicillium* sp.	Antimalarial	Stierle et al., 2000
Roquefortine C	*Penicilliumroqueforti; Penicilliumcrustosum*	Neurotoxin	Kim et al., 2004; Xu et al.2009
Pravastatin	*Penicilliumcitrinum*	Anticholesterolemics	Gonzalez et al., 2003

1.3 Classification of fungal secondary metabolites

Fungal Secondary Metabolites can be classified into four main classes based on their chemical properties: polyketides, terpenoids, shikimic acid derived compounds, and non - ribosomal peptides. Hybrid metabolites which are composed of moieties from various classes are common, like the meroterpenoids, which are a mix between terpenes and polyketides. Ascomycetes have more genes of secondary metabolism than basidiomycetes, archeo - ascomycetes, and chytridiomycetes, whereas hemi - ascomycetes and zygomycetes have none, the analysis of available fungal genomes revealed has this. (Collemare*et al*., 2008

1.3.1 Terpenoids and steroids

They are major group of substances derived biosynthetically from isopentenyldiphosphate. Currently, over 35, 000 known terpenoid and steroid compounds are identified. Terpenoids have different variety of unrelated structures, while steroids have a common tetracyclic carbon skeleton and are modified terpenoids that are biosynthesized from the triterpenelanosterol.

1.3.2 Alkaloids

There are over 12, 000 known compounds of alkaloids, and their basic structures consist of basic amine group and are derived biosynthetically from amino acids.

1.3.3 Fatty acid - derived substances and polyketides

Around 10, 000 compounds are identified and are biosynthesized from simple acyl precursors such as propionyl CoA, acetyl CoA, and methylmalonyl CoA.

1.3.4 Nonribosomal polypeptides

These amino acids derived compounds are biologically synthesized by a multifunctional enzyme complex without direct RNA transcription.

1.3.5 Enzyme cofactors

Enzyme cofactors are nonprotein, low - molecular enzyme component

1.4 Regulation of Fungal Secondary Metabolites

A high degree of environmental interaction, particularly sources of abiotic stress for either the host or the fungus such as drought or heat stress, also effect on the interactions (e. g., Fountain et al., 2014). Fungal genes involved in stress related responses, especially to oxidative stress, are highly represented in phytopathogenic fungi (Karányi et al., 2013) and fungal Secondary Metabolite toxins often play a role in triggering these responses. Some fungal Secondary Metabolites, such as pigments, polyols and mycosporines, are associated with pathogenicity and/or fungal tolerance to several stress - inducing environmental factors, including temperature and UV light (Sinha et al., 2007). Moreover, environmental factors (e. g., light, temperature, pH, calcium, and nutrients) regulate Secondary Metabolite production in a concerted way.

1.5 Application of secondary Metabolites

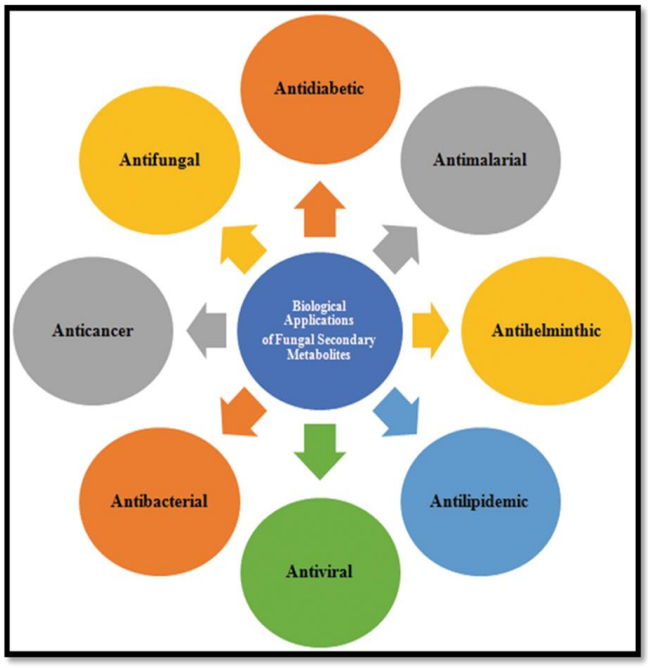

Figure 3: Various Applications of Fungal Secondary Metabolites
Courtesy: Arpita Ray, Shruti, 2021

1.5.1 Antibiotics

The antibiotics are defined as the complex chemical substances, the secondary metabolites which are produced by microorganisms and act against other microorganisms. However, antibiotics produced by microorganisms have been very useful for the cure of certain human diseases caused by bacteria, fungi and protozoa.

1.5.2 Antitumor Agents

Natural product and its derivatives account for more than 60% of anticancer formulations. Actinobacteria derived antineoplastic molecules currently in use are actinomycin D, anthracyclines (daunorubicin, doxorubicin, epirubicin, pirarubicin, and valrubicin), bleomycin, mitosanes (mitomycin C)

1.5.3 Pharmacological and neutraceutical agents

Discovery of the fungal statins was one huge success, including compactin, lovastatin, pravastatin, and others which act as cholesterol - lowering agents. Lovastatin is produced by *A. terreus.* Of great importance in human medicine are the immunosuppressants such as cyclosporinA, sirolimus (rapamycin), tacrolimus, and mycophenolatemofetil. They are used for heart, liver, and kidney transplants and were responsible for the establishment of the organ transplant field.

1.5.4 Agricultural and animal health products

kasugamycin and polyoxins are used as biopesticides. *Bacillus thuringiensis* crystals, nikkomycin, and spinosyns are used as bioinsecticides; bioherbicides (bialaphos) find application as bioherbicides; ivermectin and doramectin as antihelmintics and endectocides against worms, lice, ticks, and mites

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Objectives

- To isolate the fungi from spoiled food samples
- To perform the antibacterial activity of fungal metabolites by agar well/ agar cup bioassay method
- To isolate and characterize fungal metabolites

2. Review of Literature

S. No.	Author	Year	Work
1	Deeksha Sharma, et al.	2016	Evaluation of bioactive secondary metabolites from endophytic fungus Pestalotiopsisneglecta BAB - 5510 isolated from leaves of Cupressustorulosa D. Don
2	ZsoltKaranyi, ImreHolb, *et al.*	2013	FSRD: fungal stress response database. Database (2013) Vol.2013: article ID bat037
3	Abdulwahid B. A. Al - Shaibani, Faiz I. Al -	2013	"Extraction and Characterization of Antibacterial Compound from Aspergillusniger"

	Shakarchi, et al.		
4	Gunatilaka, A. A. Leslie	2010	Fungal Secondary Metabolites
4	Ellen M Fox, Barbara Howlett	2008	"Secondary metabolism: regulation and role in fungal biology"
5	Feher Mand Schmidt JM	2003	Property Distributions: Differences between Drugs, Natural Products, and Molecules from Combinatorial Chemistry
6	Gonzalez et al.	2003	Microbial Secondary Metabolites Production and Strain Improvement
7	Demain L	1999	Pharmaceutically active secondary metabolites of microorganisms. Applied Microbiology and Biotechnology

3. Materials and Methods

3.1 Isolation of fungi from spoiled food samples

Samples were collected from household spoiled foods, such as Tomato (*Licoperciconesculentum*) (7 days old), Kiwi (*Actinidiadeliciosa*) (5 days old), Strawberry (*Fragariaananassa*) (7 days old), Sprouts of Green Gram (8 days old), Bread (7 days after the expiry date), Idli Batter (7 days old), Laddoo; and were inoculated on to CzepekDox Agar by Quadrant Streaking Method in complete sterile conditions. Streptomycin was added in order to prevent any unwanted bacterial growth. The plates were kept for incubation at room temperature for 7 days.

3.2 Identification of fungal isolates

After incubation, the positive cultures were further analyzed by transferring a loopfull of the fungal isolate onto a glass slide, then lactophenol–cotton blue stain was added and the smear was covered with a cover slip before examined under 40× magnification power of the compound light microscope for hyphal and spore morphology.

3.3 Detection of antagonism of the fungal metabolite using *Klebsiella sp.*

The isolated fungi were cultivated on PDB by inoculating selected fungal cultures in 250 mL Erlenmeyer flask containing 100 mL of the medium. The flask was incubated at 28 °C for 2 week with periodical shaking at 150 rpm, as suggested by Abdulwahid B. A. Al - Shaibani, *et al.*, 2013. After incubation, the obtained mycelia were separated by centrifuging at 6000 rpm for 15 min. The supernatant was passed through a Millipore filter (11 µm) to get a spore free culture filtrate.

3.3.1 Agar Well Diffusion

An active culture of laboratory strain (*Klebsiella sp*.) was spread on the nutrient agar plate; wells were made in the plate, 2 cm away from the center. The culture filtrate (antibacterial compound) in a quantity of (100µl) was pipetted in each well, while one well was filled with only PDB medium as a control. The plates were incubated at 37◦C for 24 - 48 hrs. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

3.4 Extraction of the metabolites

To the supernatant, 10% methanol was added. Metabolite was extracted by solvent extraction procedure using ethyl acetate and methanol as organic solvents. To the supernatant obtained, equal volume of solvents were added, mixed well for 10 min and kept for 5 min till the two clear immiscible layers formed. As suggested by (Parisa Azerang, Vahid Khalaj *et al*., 2019), the upper layer of solvent containing the extracted compounds was separated using separating funnel. Solvent was evaporated and the resultant compound was dried with the help of simple distillation process to yield the crude metabolite. The crude extract was then dissolved in Dimethyl Sulphoxide at 1 mg/mL of concentration and kept at 4 °C.

3.5 Characterization of the metabolite

TLC (Thin Layer Chromatography) was performed in order to know the characteristic similarity of the extract to that of the antibiotics itself. The Rf value of the partially purified compound was calculated against the pure compounds, using silica get as the stationary phase and a mixture of hexane and ethyl acetate as the mobile system in a ratio of 1: 1 (Chemistry LibreTexts, TLC). And was kept in a Ninhydrin Chamber for 2 mins. Then the Rf values were calculated.

4. Results and Discussions

4.1 Isolation of fungi from spoiled food samples

The fungal isolates from spoiled food samples were isolated by streaking plate method on Czepekdox agar plates and slants Fig 4 - 7. As texted in (Black and Jacquelyn G, 1999). Since the dye Rose Bengal was added, there was no bacterial contamination seen in any plates or slants

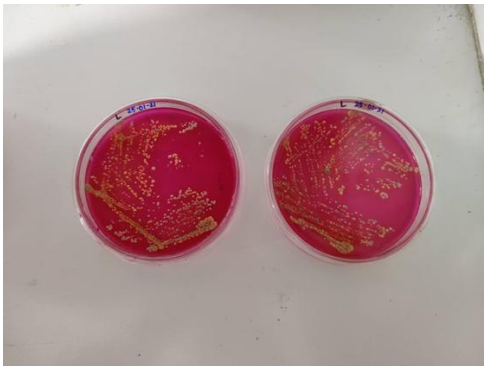

Figure 4: Streak plates of samples G and L

Figure 5: Streak plates of samples T and S

Figure 6: Streak plates of samples I and B

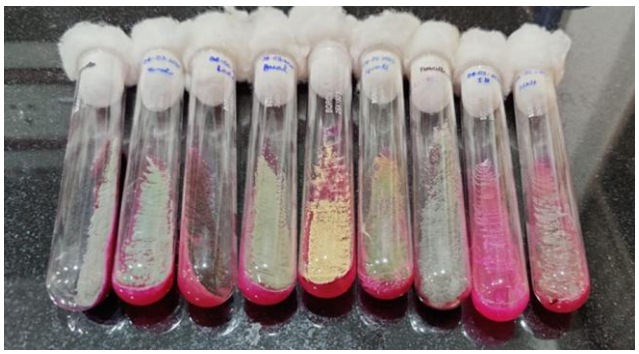

Figure 7: Slants of isolated organisms

4.1 Identification of the isolates

From Table 2, it can be inferred that the isolate T from Spoiled Tomato is *Penicillium sp.* Because of the dense brush - like spore - bearing structures. The conidiophores are branched and are terminated by clusters of flask - shaped phialides, which clearly indicates that the organism is a *Penicillium sp.* In the S isolate, due to the presence of septate and hyaline hyphae, round vesicles with radiate heads and prominent black spores were observed in the colony morphology, which indicate the presence of *Aspergillus sp*. Fungal isolate K did not show any characteristic growth on the Czepekdox Agar. The dense felt like grayish green color colonies and the appearance of spore bearing brush like conidiophores suggested the presence of *Penicillium sp*. in the G isolate. B isolate showed a grayish velvety colony with black sporangia at the tips of the sporangeophores when viewed under microscope, indicating the presence of *Rhizopus sp.* The long sporangeophores with broad hyphae under the microscope and white cottony colonies identified as*Mucor sp*. (isolate I). Many small single and spherical cells with budding like appearance in the field view of the microscope, and creamy white, raised colonies suggest the presence of *Yeast sp.* (isolate L).

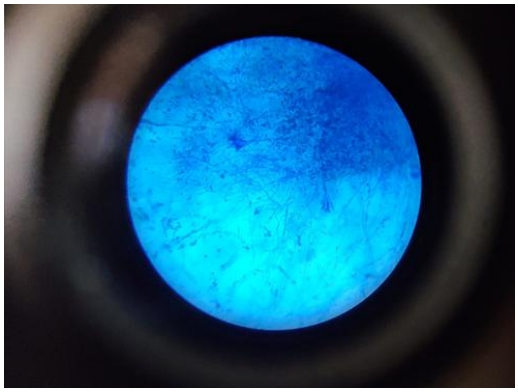

Figure 8: Isolate T

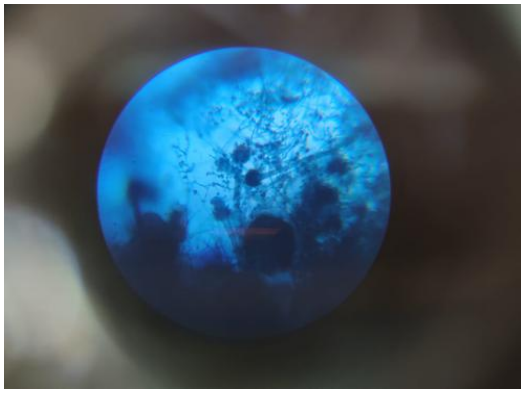

Figure 9: Isolate S

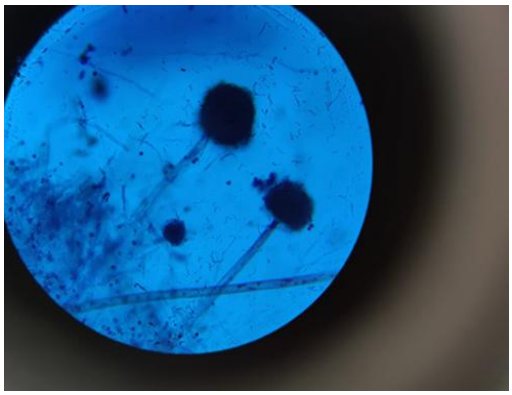

Figure 10: Isolate B

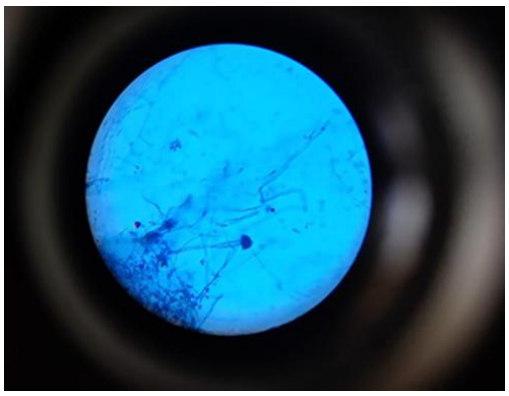

Figure 11: Isolate I

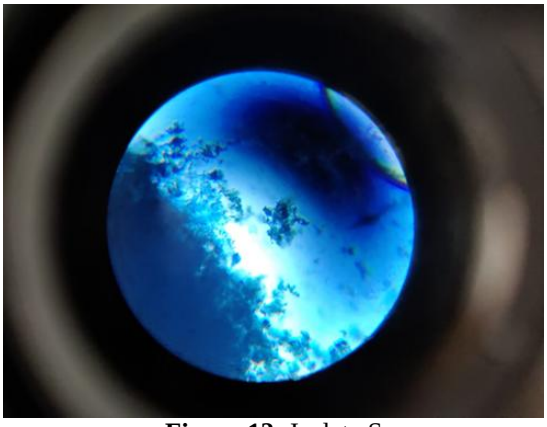

Figure 12: Isolate S

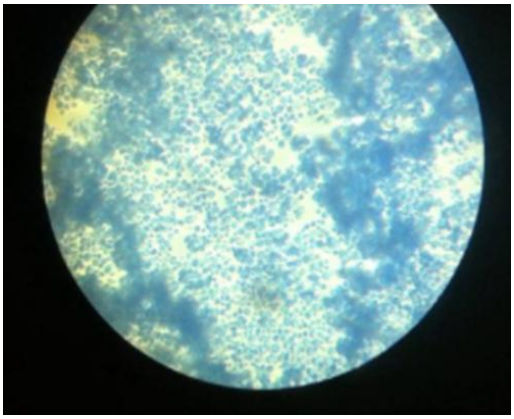

Figure 13: Isolate L

Table 2: Fungal source, colony morphology, microscopic view, and names

Fungal isolate	Spoiled food	Colony Morphology	Microscopic view	Isolated fungi
T	Tomato (Lycopersiconesculentum)	fast growing, in shade of green, consisting of a dense felt of conidiophores	dense brush - like spore - bearing structures, The conidiophores are branched and are terminated by clusters of flask - shaped phialides	*Penicillium sp.*
S	Strawberry (Fragariaananassa)	initially covered with a white, fluffy, aerial mycelium and as colony matures, their surface covered with black spores	Hyphae are septate and hyaline, rough and its Vesicle is Round, radiate head.	*Aspergillus sp.*
K	Kiwi (Actinidiadeliciosa)	-	-	No particular fungal growth
G	Sprouts of Green Gram	fast growing, in shade of green, consisting of a dense felt of conidiophores	spore - bearing dense brush - like structures, The conidiophores are simple, phialides bearing conidiophores	*Penicillium sp.*
B	Bread	Grey, velvety colonies	black sporangia at the tips of the sporangiophores are rounded and produce numerous nonmotile multinucleate spores	*Rhizopus sp.*
I	Idly Batter	Cottony white colonies	Has broad hyphae which are non - septate and scarce. Sporangiophores are long	*Mucor sp.*
L	Laddoo	Creamy whitish raised colonies	single - cell organisms of spherical, elliptical or cylindrical shape, Budding is observed	*Yeast sp.*

4.2 Detection of antagonism of the fungal metabolite using *Klebsiella sp.*

Fig 14 shows the abundant growth of the fungal isolates all over the surface of the PDB after the incubation for 2 weeks and then the broth was filtered using 11µm Whatmann filter paper. The filtrate of these broths were subjected to centrifugation. (Parisa Azerang, Vahid Khalaj *et al*., 2019)

Figure 14: PDB flasks of cultured fungi after 2 weeks of incubation

After the centrifugation process, pellets were settled down at the bottom of the centrifuge tubes which indicated the accumulation of the cell debris of the fungal isolates and the clear broth solutions in the form of supernatant were obtained, which is shown in the Fig 15. The obtained supernatant according to (Abdulwahid, *et al.*, 2013), most likely containes the metabolites.

Figure 15: Centrifuge tubes of PDB

4.2.1 Agar well diffusion

The extractions from isolates T, S, G, B, I, and L have shown antimicrobial activity on active culture of laboratory bacterial strain (*Klebsiella sp.)* by forming the zone of inhibition around the colonies and the zone of inhibition was measured. In Table 3 and Fig 18 a clear description of the measurement and antagonistic character of the extracts of the isolates are depicted

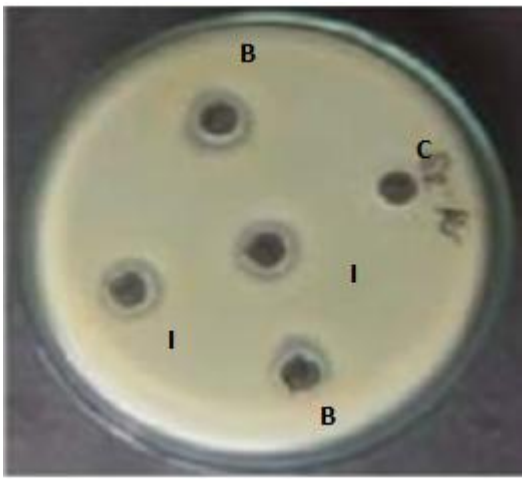

Figure 16: Agar well diffusion of I and B

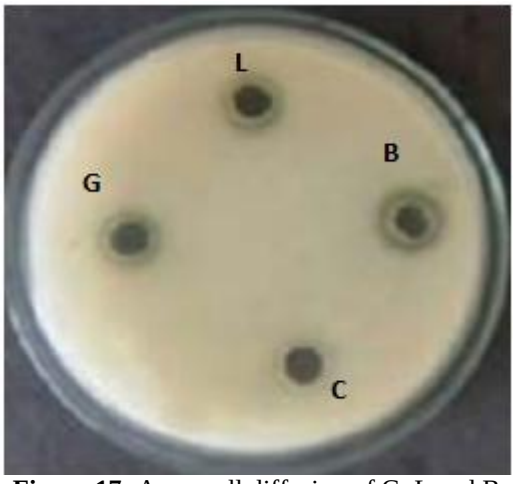

Figure 17: Agar well diffusion of G, L and B

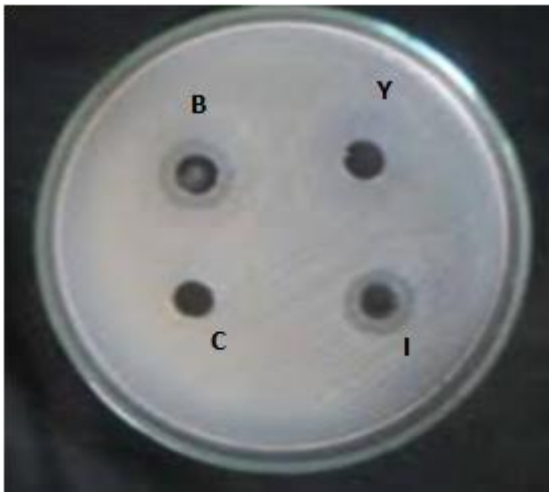

Figure 18: Agar well diffusion of B, Y and I

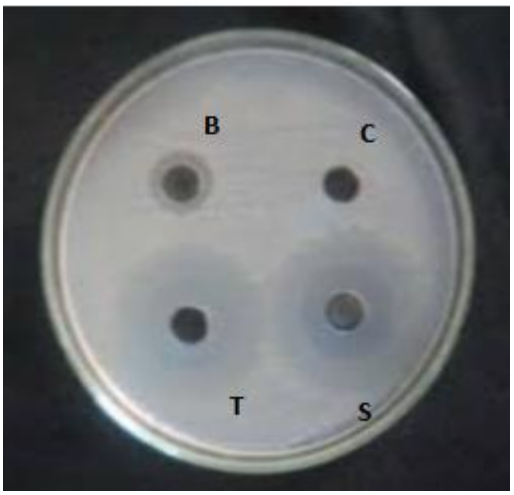

Figure 19: Agar well diffusion of B, T and S

The extractions from isolates T, S, G, B, I, and L have shown antimicrobial activity on active culture of laboratory bacterial strain (*Klebsiella sp.)* by forming the zone of inhibition around the colonies and the zone of inhibition was measured. In Table 3 and Fig 18 a clear description of the measurement and antagonistic character of the extracts of the isolates are depicted

Table 3: Zones of inhibition of the fungal isolates

Isolate	Fungal Isolate	Zone of Inhibition seen (ZOI) (mm)
T	*Penicillium sp.*	20
S	*Aspergillus sp.*	20
G	*Penicillium sp.*	3
B	*Rhizopus sp.*	4
I	*Mucor sp.*	4
L	*Yeast sp.*	2

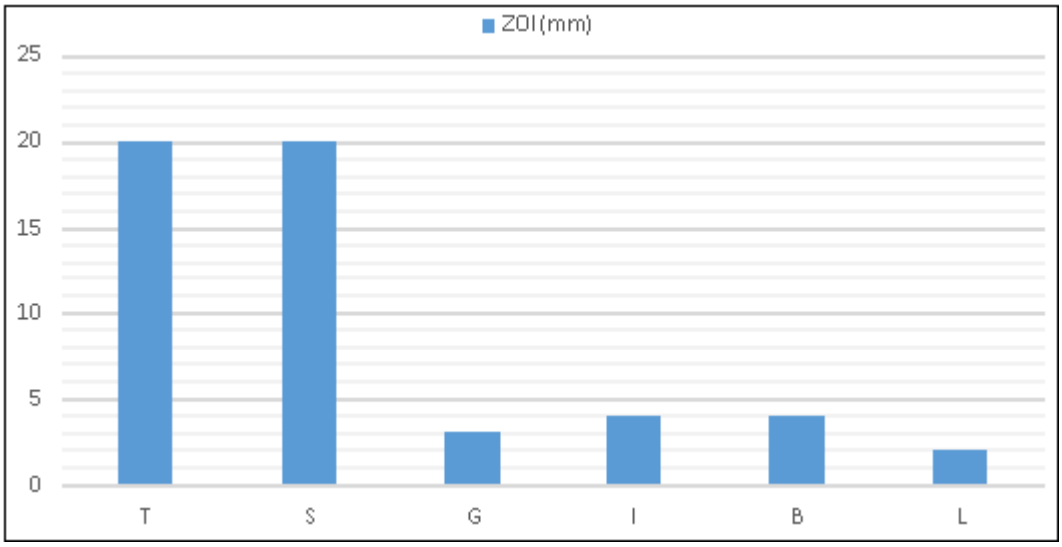

Figure 20: Graphical representation of zones of inhibition of fungal isolates

The above graph derived from the Table 3, mentions that the extracts of isolates T and S are the highest with 20mm and 20mm zones of inhibition respectively. The extracts of isolates G, I, B and L showed the zones of inhibition which were 3mm, 4mm, 4mm and 2mm respectively.

4.3 Extraction of the metabolites

In order to extract the metabolites, the supernatants of the isolates which had the highest measure of Zones of Inhibition were selected, which are T and S; and was subjected to separation through the solvent extraction method in a seperatory funnel as shown in Fig 21. The two immiscible liquids were separated by vigerous shacking of the seperatory funnel for 10 mins. The organic layer, which had a strong alcoholic odour was collected.

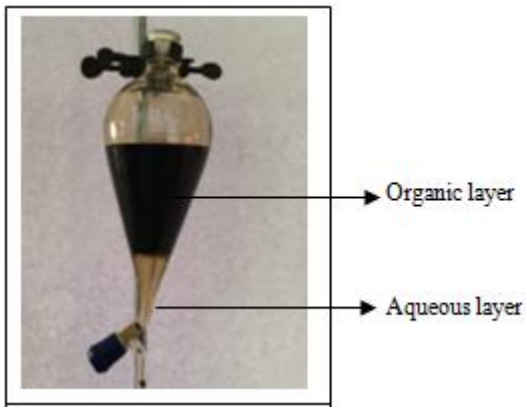

Figure 21: Separation of metabolites from supernatant

Figure 22: Extraction of the metabolite using simple distillation
Courtesy: Simple Distillation, MeitYOLabs
https: //www.youtube. com/watch?v=2XBrj - ZEnEo

After the separation of the organic layer from the solution, the organic layer was subjected to simple distillation as depicted in Fig 22, (Parisa Azerang, VahidKhalaj *et al*., 2019) with the aim to vaporize the organic solvent present in it and extract out the metabolites of the fungal isolates.

4.4 Characterization of the metabolite

The crude extract was spotted, and the solvent front was allowed to be developed. The running lane was then dried to separate the bioactive compounds.

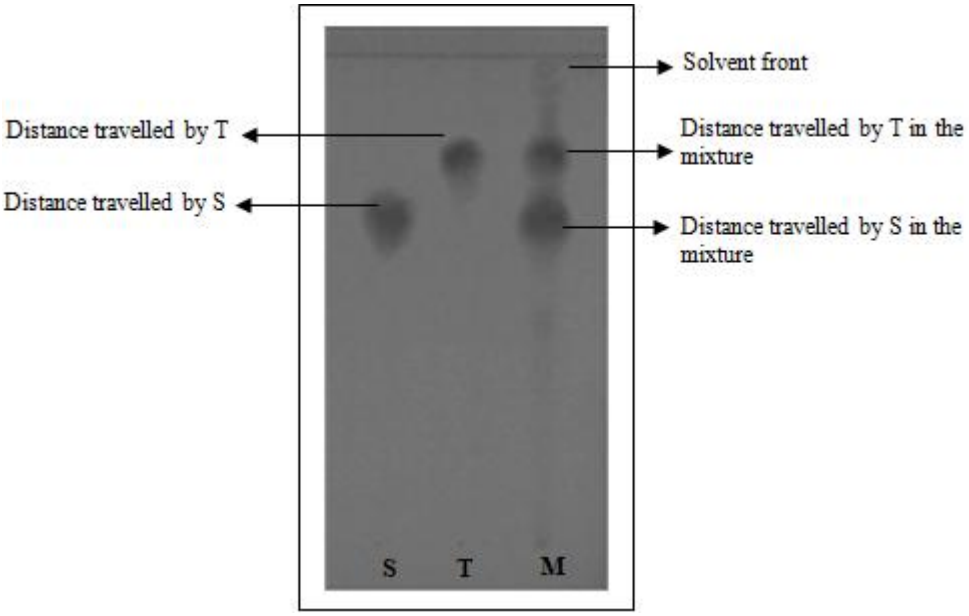

Figure 23: TLC plate of spots S, T and M (T+S)

The TLC plate showing the spots of the extract of isolate S, T and their mixture and the solvent front can be seen in Fig 23.

Table 4: Rf values of T and S

S. No.	Sample	Solvent Front (mm)	Distance Travelled by Solute (mm)	Retardation Factor (Rf)
1	T	55	43	43/55 = 0.7818
2	S	55	35	35/55 = 0.6363
3	Mixture (M): T+S	55	43	43/55 = 0.7818
			35	35/55 = 0.6363

The TLC plate which was ran in the mobile phase, resulted with a solvent front with 55mm and the distance travelled by the extract of isolate T and the extract of isolate S were 43mm and 35mm respectively. The Rf values of T and the S are similar to that of the Rf values of the standard antibiotics Penicillin (PEN) and Cephalotrixin (CTR) respectively, Gabriel Hancu, *et al.*, 2013

5. Conclusion

The antagonistic properties of the fungal isolates (T, S, G, B, I, L) obtained from spoiled food (Tomato, Strawberry, Sprouts of Green Gram, Bread, Idli Batter, Laddoo) were isolated on Czepekdox Agar medium. Agar well method was used, in order to detect the antibacterial activity of fungal isolates by testing the effect of their culture filtrates on MHA against the growth of the active lab culture, *Klebsiella sp*. Results reveal that the lowest antibacterial properties were exhibited by the isolates Y, I, G and B, whose Zones of Inhibitionwere 2mm, 4mm, 3mm and 4mm respectively and the highest antibacterial properties were possessed by the fungal isolates T and S whose Zones of Inhibition were 20mm and 20mm respectively. An attempt was made to extract the metabolite through solvent extraction and simple distillation methods. Also characterization of the metabolites produced in this process, was done through TLC. The results indicate that the fungal isolates contain antibacterial properties which are evident against *Klebsiella sp.* The Rf values of T and the S were similar to that of the Rf values of the standard antibiotics Penicillin (PEN) and Cephalotrixin (CTR) respectively, Gabriel Hancu, *et al.*, 2013

6. Further Studies

Further research has to be done in order to characterize the metabolites obtained through the fungal isolates. Further identification techniques, such as UV and IR analysis of the metabolites can be performed in order of a clear understanding and biochemistry of the isolated fungal metabolites.

Additional studies can lead to the discovery of novel metabolites and their characteristic features which can have a wide range of applications in Pharmaceutical, neutraceutical, agricultural industries, and also many bioremedial properties.

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8. Declaration

The current study **"Metabolites Secreted by Fungal Species Isolated from Spoiled Food and Their Effect on *Klebsiella species*"** has been carried out under supervision of **Dr. B. Aruna, Assistant Professor, Department of Microbiology, St. Francis Degree and P. G College for Women, Begumpet, Hyderabad.** I hereby declare that the present study that has been carried out by me**, Bhattar Supraja Acharya** during the year 2021 is original and no part of it has been carried out prior to this date.

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Appendix

Media Compositions

Czepekdox Agar Composition

- Sucrose – 30mg/ml
- Sodium Nitrate - 2mg/ml
- Dipotassium Phosphate - 1mg/ml
- Magnesium sulphate – 0.5 mg/ml
- Potassium Chloride – 0.5 mg/ml
- Ferrous sulphate – 0.01 mg/ml
- Agar – 15 mg/ml
- pH – 7.3 - 7.5
- temperature - 25˚C

Muller Hinton Agar Composition

- Beef extract – 2 mg/ml
- Acid Hydrolysate of Casien – 17.5 mg/ml

- Starch – 1.50 mg/ml
- Agar – 17.0 mg/ml
- Distilled water – 1000ml

Potato Dextrose Broth Composition

- Potato infusion – 200mg
- Dextrose – 20mg
- Distilled water - 1000ml
- Final pH – 5.1 - 0.2
- Temperature - 25˚C

Antibiotics

- Streptomycin Powder

Dyes

- Rose Bengal