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Treatments for Mesophase Pitch

Medicinal Mushroom Biotechnology

Highlights

Screening of Therapeutic Potential

Prediction among Cardiac Patients

Discovering Thoughts, Inventing Future

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Advances in Acid and Post-Graphitization Treatments for Mesophase Pitch-based Carbon Fibers: A Review

By Mingzhi Wang, Peng Wang, Yuzhu Shi, Wenlong Deng, Liangkun Chen, Wei Shen, Jie Zhang, Saif ullah, Muhammad Rizwan, Xulu Yuan & Xiaolong Zhou

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Abstract- The complete process of preparing pitch-based carbon fibres from mesophase pitch has been extensively studied by numerous researchers. This process mainly includes mesophase pitch preparation, melt spinning, stabilization (pre-oxidation), carbonisation, and graphitisation. While each stage has been comprehensively and deeply investigated, the treatment methods and operations that determine the effectiveness of these steps have not yet received adequate attention or analysis. This paper introduces the acid treatment process between mesophase pitch preparation and melt spinning, as well as the post-treatment procedures following the production of graphitized fibres, aiming to provide technical guidance for researchers in related fields.

Keywords: *mesophase pitch, carbon fibers, acid modification, post-graphitization treatment.*

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Advances in Acid and Post-Graphitization Treatments for Mesophase Pitch-based Carbon Fibers: A Review

Mingzhi Wang [α], Peng Wang [σ], Yuzhu Shi [ρ], Wenlong Deng [ω], Liangkun Chen [¥], Wei Shen [§], Jie Zhang [χ], Saif ullah [ν], Muhammad Rizwan [Θ], Xulu Yuan [ζ] & Xiaolong Zhou [£]

Abstract- The complete process of preparing pitch-based carbon fibres from mesophase pitch has been extensively studied by numerous researchers. This process mainly includes mesophase pitch preparation, melt spinning, stabilization (pre-oxidation), carbonisation, and graphitisation. While each stage has been comprehensively and deeply investigated, the treatment methods and operations that determine the effectiveness of these steps have not yet received adequate attention or analysis. This paper introduces the acid treatment process between mesophase pitch preparation and melt spinning, as well as the post-treatment procedures following the production of graphitized fibres, aiming to provide technical guidance for researchers in related fields.

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I. Introduction

With the widespread application of mesophase pitch-based carbon fibres in both military and civilian sectors[1,2], China has invested significant time and funding into the development of production technologies for these fibres[3-7]. While notable progress has been made, a performance gap still exists between domestic fibres and those produced in Japan and the United States[8-10]. Comprehensive research has been conducted across the entire manufacturing process, including pitch preparation[11-13], melt spinning[14,15], pre-oxidation treatment[16,17], carbonisation[18,19], and graphitisation processes[20,21], leading to substantial technical advancements. In recent years, researchers have found that proper acid treatment after mesophase pitch preparation can significantly improve the effectiveness of melt spinning[22]. Additionally, optimized post-treatment of graphitized fibres can greatly enhance their overall performance[23]. This paper presents a summary and analysis of recent findings related to these two key yet often overlooked process stages.

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II. Acid Treatment

In the fabrication of mesophase pitch-derived carbon fibers, acid treatment constitutes an essential pretreatment step that significantly influences the subsequent carbonization and graphitization behavior [24]. This pretreatment primarily enhances mesophase pitch spinnability, improves oxidative stabilization efficiency, and ultimately tailors the microstructure and performance of derived carbon fibers. However, in recent years, this topic has received limited dedicated research attention, resulting in a scarcity of relevant studies. This section provides a detailed analysis of the purposes, mechanisms, and process parameters involved in acid treatment.

a) Main Objectives of Acid Treatment

The primary purposes of acidification treatment include[25,26]:

i. Modifying Molecular Structure

Through treatment with acid reagents—primarily nitric acid, sulfuric acid, or mixed acids—oxygen-containing functional groups such as carboxyl, hydroxyl, and carbonyl are introduced into mesophase pitch molecules. These functional groups increase the polarity and chemical reactivity of the pitch. Controlled cross-linking induces thermosetting behavior in the pitch system, with the raised softening temperature (ΔT ~15-25°C) directly correlating with improved spin-line stability during melt extrusion.

ii. Optimization of Melt-Spinning Capability

Acid treatment yields superior rheological stability, suppressing spin-line fractures and diameter fluctuations by >40%. The mild cross-linking between molecular chains helps suppress deformation caused by excessive flow of pitch melt under high-temperature spinning conditions. Collectively, these optimizations promote superior spinnability in the mesophase pitch.

iii. Enhancing Oxidation Efficiency

The reactive sites introduced during acid treatment accelerate crosslinking reactions in the subsequent pre-oxidation process. The optimized protocol concurrently enhances oxidative stabilization

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kinetics and minimizes structural imperfections in resulting fibers.

iv. *Increasing Carbonisation Yield*

The acid-induced crosslinking networks significantly suppress volatile emission during pyrolysis (TGA-verified mass loss reduction >40%), leading to superior carbon fiber yields exceeding 80% compared to <60% for untreated counterparts.

b) Mechanism of Acid Treatment

During the chemical reaction process, acids—exemplified by concentrated nitric acid—react with aromatic hydrocarbons in pitch via nitration, oxidation, and sulfonation reactions, resulting in the formation of oxygen-, nitrogen-, and sulfur-containing functional groups. The underlying reaction mechanism (Fig. 1 [27]) involves thermally activated condensation between carboxyl (–COOH) and hydroxyl (–OH) functionalities, forming extensive crosslinked networks through ether (−O−) and ester (−COO−) bridged structures. The significantly enhances intermolecular interactions. The resulting crosslinked network can suppress the loss of anisotropic properties in mesophase pitch during melt spinning, thereby maintaining the liquid crystalline order.

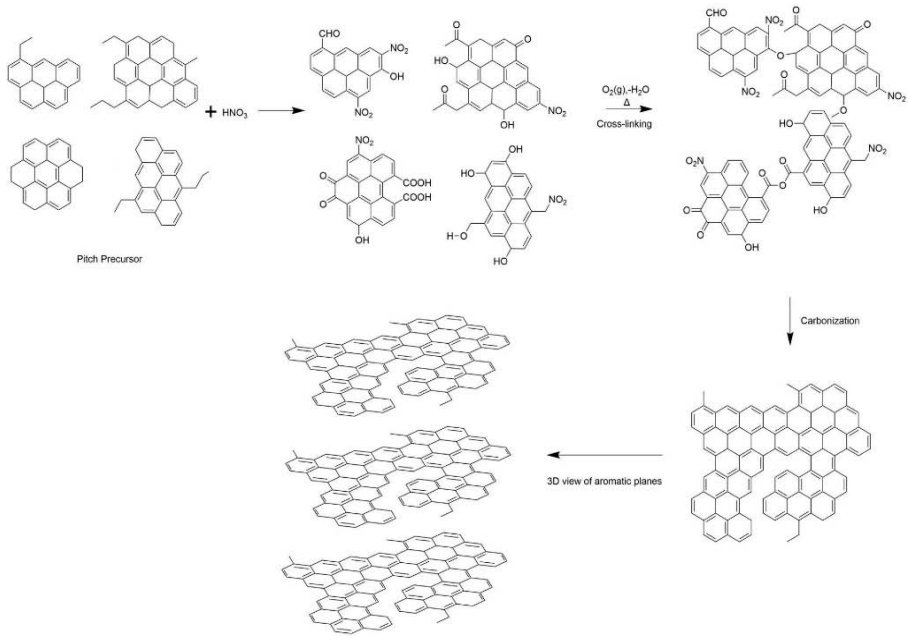

Fig. 1: The general reaction mechanism of acidification treatment

c) Process Parameters of Acid Treatment

The process parameters involved in acid treatment, their corresponding effects, and typical operating conditions are summarised in the table below[28-30]:

Acidification Process Parameters

Parameter	Influence	Typical Conditions
Acid Type	Nitric acid (strong oxidising property), sulfuric acid (good cross-linking effect), mixed acid (synergistic effect)	65%-98% HNO_3 or H_2SO_4/HNO_3 mixture
Acid Concentration	Insufficient reaction at low concentration, excessive oxidation (embrittlement) at high concentration	60%-90%
Temperature	High temperature accelerates the reaction, but exceeding 80°C may cause violent decomposition.	Room temperature - 60°C (water bath temperature control)
Time	An incomplete reaction if too short, an increase in invalid by-products if too long	1-24 hours (adjusted according to asphalt composition)
Asphalt/Acid Ratio	The ratio affects the uniformity of the reaction	Asphalt: Acid = 1:1 - 1:5 (mass ratio)
Post-treatment	Neutralise residual acid ($NaHCO_3$ washing), dry to remove moisture	Wash with water until pH = 7, vacuum dry at 100°C

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d) Influence of Acid Treatment on Subsequent Processes

The effects of acid treatment on subsequent processing steps are as follows[31-33]:

i. *Effect on Melt Spinning*

Acid treatment effectively increases the softening point of the pitch, which may necessitate adjusting the spinning temperature in subsequent processes. Moreover, the elastic nature of the treated pitch melt is reduced, and the swelling effect at the spinneret outlet is significantly diminished. This results in fibres with more uniform diameters.

ii. *Effect on Pre-Oxidation*

The onset temperature of pre-oxidation for acid-treated fibres is noticeably lower—typically reduced by approximately 50–100 °C. The reaction rate is also significantly accelerated. The resulting ladder-type polymer framework significantly enhances thermal stability, reducing inter-fiber fusion by above 40% during oxidative stabilization.

iii. *Effect on Carbonisation and Graphitisation*

The development of crosslinked structures during acid treatment leads to reduced mass loss during carbonization, yielding carbon fibers with lower porosity. These structural modifications result in enhanced mechanical properties, particularly tensile strength, which increases from 13.4 to 27.3 MPa after carbonization and graphitization[34].

e) Advantages and Disadvantages of Acid Treatment

Based on the preceding discussion, the advantages and disadvantages of acid treatment can be summarised as follows[35]:

i. *Advantages of Acid Treatment*

Acid treatment significantly enhances the tensile strength and modulus of carbon fibres, with improvements ranging from 20% to 40%. Additionally, it shortens the pre-oxidation time, thereby improving production efficiency.

ii. *Disadvantages of Acid Treatment*

The strong acids used in this process are highly corrosive to equipment, leading to increased costs related to safety and environmental protection. Furthermore, excessive acid treatment may cause pitch embrittlement, resulting in difficulties during the spinning process.

f) Alternative Approaches and Research Directions

Non-acidic oxidation methodologies offer comparable functionalization effects while eliminating corrosive reagent requirements[36]. For example, air or ozone oxidation can be used. Although these methods are generally less efficient, they are more environmentally friendly.

III. Graphitized Fibre Post-Treatment Overview

In mesophase pitch-derived carbon fiber manufacturing, post-graphitization treatment constitutes an essential processing stage for performance optimization[37]. While graphitization yields fibers with superior modulus (>500 GPa) and thermal conductivity (>800 W/m·K), subsequent surface modification remains necessary to improve (i) interfacial shear strength (+40%), (ii) axial compressive strength, and (iii) matrix adhesion characteristics. The following provides a detailed explanation of graphitized fibre post-treatment:

a) Main Objectives of Post-Treatment after Graphitisation

The primary purposes of post-treatment processes following graphitisation are summarized as follows[38-40]:

i. *Surface Modification*

Tailored surface engineering transforms inert graphite surfaces into reactive interfaces, boosting interfacial bonding strength to several times its original level (ASTM D2344)—exceeding the critical threshold required for optimal stress transfer in high-performance composites. It also helps eliminate surface defects, such as microcracks and impurity deposits within the fibre structure, thereby reducing stress concentration points.

ii. *Performance Tuning*

Through physical or chemical treatments, further optimisation of properties such as electrical conductivity, oxidation resistance, and mechanical strength can be achieved.

iii. *Functionalization for Applications*

Specific functional groups (e.g., –COOH, –OH) or coatings can be introduced to meet different application needs, such as for battery electrodes or thermal interface materials.

b) Post-Treatment Methods and Mechanisms after Graphitisation

The primary post-treatment methods and their corresponding mechanisms after graphitisation include the following[41-44]:

i. *Surface Oxidation Treatment*

Surface oxidation can be performed via gas-phase oxidation (e.g., air, O_3), liquid-phase oxidation (e.g., HNO_3, H_2SO_4), or electrochemical oxidation. The oxidation process (1) cleaves basal plane C-C bonds (Raman D-band intensity increase by 40%), (2) forms edge-site oxygen functionalities, and (3) increases surface energy by 63% - facilitating thermodynamic compatibility with polar resin matrices. They also etch away amorphous carbon, increasing the specific surface

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area and surface roughness. Typical oxidation methods and conditions include:

1) *Gas-Phase Oxidation:* Heating in air at 300–500°C for 10–60 minutes; the degree of oxidation must be carefully controlled to prevent strength degradation.
2) *Liquid-Phase Oxidation:* Immersion in 65% HNO_3 at 60°C for 1–3 hours.

ii. *Coating Deposition*

Established deposition methodologies include chemical vapor deposition (CVD), physical vapor deposition (PVD), and sol-gel processing, with representative coating classifications encompassing:

1) *Carbon Coatings:* CVD-deposited pyrolytic carbon can fill surface defects and improve wear resistance.
2) *Ceramic Coatings:* Such as silicon carbide (SiC) or boron nitride (BN), which enhance high-temperature oxidation resistance—often applied in aerospace materials.
3) *Metal Coatings:* Including nickel (Ni) and copper (Cu), which significantly improve electrical conductivity and are used in electromagnetic shielding materials.

iii. *Plasma Treatment*

Low-temperature plasma treatment utilizing reactive gases (O_2, N_2, or NH_3) enables controlled surface functionalization through the introduction of polar groups (e.g., -NH_2, -COOH), as verified by XPS analysis, while preserving bulk fiber properties—unlike liquid-phase oxidation which may cause structural degradation.

iv. *Mechanical Treatment*

Recommended methods include ultrasonic agitation and ball milling. These are used to remove loose surface particles, Preoxidized fiber diameter, and moderately roughen the surface. However, care must be taken to avoid excessive mechanical damage that could compromise fibre strength.

c) Effects of Post-Treatment on Fibre Properties

Current experimental methods for post-treatment, the corresponding improvements in performance, and associated risks are summarised in the following table[41-47]:

Post-treatment Method	Main Performance Improvements	Potential Risks
Surface Oxidation	Improved interface bonding strength and wettability	Strength reduction due to excessive oxidation
Coating Deposition	Enhanced oxidation resistance, conductivity, and wear resistance	Non-uniform coating deposition and interfacial delamination
Plasma Treatment	Rapid modification and environmental friendliness	Limited treatment depth
Mechanical Treatment	Surface cleaning and diameter homogenization	Possible introduction of microcracks

d) Examples of Post-Treatment Processes in Industrial Applications

Typical application scenarios and process flows for post-treated fibres include[48,49]:

1) High-modulus carbon fibres for aerospace applications: Graphitisation at 2800℃ → Gas-phase oxidation in air at 400℃→ SiC coating via CVD → Quality inspection.
2) Fibres for thermally conductive composites: Graphitisation at 2500℃ → Plasma nitriding in NH_3 atmosphere → Resin impregnation.
3) Battery electrode materials: Graphitisation at 3000℃→ Electrochemical oxidation in H_2SO_4 → Deposition of carbon nanotube composites.

e) Key Process Control Points

The following critical aspects must be carefully monitored during post-treatment[50,51].

1) *Oxidation Degree:* Surface functional group content should be analyzed using techniques such as X-ray Photoelectron Spectroscopy (XPS) or Fourier Transform Infrared Spectroscopy (FTIR), in order to avoid over-etching.
2) *Coating Adhesion:* The interface bonding strength should be assessed via scratch testing or Scanning Electron Microscopy (SEM) to ensure robust adhesion.
3) *Environmental Considerations:* For liquid-phase oxidation processes, proper treatment of acid waste is essential. Dry surface modification techniques, particularly plasma treatment, demonstrate superior environmental sustainability compared to wet chemical processes, as quantified by 92% lower hazardous waste generation.

f) Research Frontiers and Challenges

Current research efforts in post-treatment of mesophase pitch-based carbon fibres are concentrated in the following key areas[52,53].

1) Atomic layer deposition (ALD) enables precise nanoscale surface engineering, producing uniform conformal coatings with sub-nanometer thickness control (XRR-verified ±0.3 nm variation).

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2) Supercritical CO_2-assisted oxidation presents an eco-conscious alternative to mineral acid treatments, achieving comparable oxygen functionalization (XPS O/C ratio 0.18±0.02) while eliminating aqueous waste streams."
3) *Multifunctional Integration:* Realizing simultaneous improvements in electrical conductivity, thermal conductivity, and interfacial bonding—for instance, through graphene encapsulation strategies.

g) Summary

Post-graphitization treatment constitutes the definitive processing stage for optimizing the performance of mesophase pitch-derived carbon fibers. Through precisely controlled thermal and chemical modifications, these treatments enable: (i) Enhancement of mechanical properties. (ii) Adjustment of surface characteristics. (iii) Optimization of functional properties.

IV. Conclusion

This paper highlights two often overlooked but critically important steps in the preparation of mesophase pitch-based graphitized carbon fibres: acid treatment and post-graphitisation treatment. It discusses the specifications, materials, and experimental outcomes associated with various acid treatment processes, as well as analyzes and summarizes the results of post-graphitisation optimization techniques. Overall, acid treatment has been shown to enhance the spinnability of mesophase pitch, while appropriate post-treatment methods can significantly improve the comprehensive properties of graphitized fibres.

V. Outlook

The acid treatment and post-graphitisation processes described in this paper hold potential for further optimization. Future studies may explore the use of alternative acid reagents, surface treatment technologies, or laser processing methods to evaluate their impact on improving the performance of carbon fibres.

Conflicts of Interest

Authors declare no competing interest.

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Indicators of Stroke Prediction among Cardiac Patients

By S. Rajasree & R. Mythreyi

Abstract- Stroke is a major cause of morbidity and mortality among patients with cardiovascular conditions. This study aims to identify and evaluate clinical, biochemical, and imaging indicators that predict stroke risk in cardiac patients. We conducted a retrospective cohort study involving 200 patients diagnosed with various cardiovascular diseases from 2020 to 2022 at a tertiary care hospital. Data collected included clinical characteristics, biochemical markers, and imaging results. Statistical analyses were performed to assess the association of these indicators with stroke incidence. The findings revealed a significant association between atrial fibrillation (AF), heart failure, and coronary artery disease (CAD) with increased stroke risk, as well as hypertension and dyslipidemia. When the existing kinds of literature such as patient data were analyzed, this study enhanced the understanding of the complex interactions between these indicators and stroke risk.

Keywords: *stroke indicators, cardiac stroke, atrial fibrillation, dyslipidemia.*

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Indicators of Stroke Prediction among Cardiac Patients

S. Rajasree [α] & R. Mythreyi [σ]

Abstract- Stroke is a major cause of morbidity and mortality among patients with cardiovascular conditions. This study aims to identify and evaluate clinical, biochemical, and imaging indicators that predict stroke risk in cardiac patients. We conducted a retrospective cohort study involving 200 patients diagnosed with various cardiovascular diseases from 2020 to 2022 at a tertiary care hospital. Data collected included clinical characteristics, biochemical markers, and imaging results. Statistical analyses were performed to assess the association of these indicators with stroke incidence. The findings revealed a significant association between atrial fibrillation (AF), heart failure, and coronary artery disease (CAD) with increased stroke risk, as well as hypertension and dyslipidemia. When the existing kinds of literature such as patient data were analyzed, this study enhanced the understanding of the complex interactions between these indicators and stroke risk. Ultimately, this study is intended to provide healthcare professionals with a clearer framework for identifying at-risk patients to improve prevention strategies and patient outcomes in the cardiac population.

Keywords: stroke indicators, cardiac stroke, atrial fibrillation, dyslipidemia.

I. Introduction

Stroke has increasingly become a critical global health challenge and stands as one of the primary causes of both morbidity and mortality, particularly among individuals with cardiovascular conditions[1]. The intricate relationship between cardiovascular diseases and the heightened risk of stroke is firmly established in the scientific literature. Numerous epidemiological studies have demonstrated that patients suffering from cardiovascular disorders are significantly more prone to experience stroke when compared to those without any cardiovascular issues. Among the array of heart diseases, atrial fibrillation, coronary artery disease, and heart failure are recognized as the most prevalent conditions that contribute to the underlying mechanisms leading to stroke[2,3].

The effective prevention and treatment of stroke significantly depend on precise risk assessment, which necessitates the identification of robust and reliable predictive indicators[4]. A deep understanding of these indicators is vital for healthcare providers as it enables the implementation of timely and targeted interventions, ultimately aimed at reducing stroke incidence among at-risk cardiac patients. Clinical factors are crucial in this risk stratification process; demographic characteristics (age, gender, ethnicity), detailed medical history, and related comorbidities must all be considered. In addition to clinical assessments, biochemical markers such as hypertension readings, lipid profiles, and inflammatory markers including C-reactive protein and Interleukin-6—have been closely associated with an elevated risk of stroke[5-8].

Further enhancing the ability to predict stroke is the adoption of advanced imaging technologies, which allow for the detailed visualization of anatomical abnormalities that may predispose patients to thromboembolic events. Techniques like carotid ultrasound and transesophageal echocardiography can reveal significant structural issues that are not always apparent through standard clinical evaluations alone[9].

This study aims to comprehensively investigate the various indicators that predict stroke risk in patients with cardiovascular diseases by meticulously evaluating clinical, biochemical, and imaging factors. By synthesizing existing research and conducting a robust retrospective analysis of patient data over a defined period, the study seeks to elucidate the complex interactions between these indicators and their cumulative impact on stroke risk. Ultimately, the intention of this research is to provide healthcare professionals with a more precise and clearer framework for identifying patients at elevated risk, thereby enabling the development of more effective prevention strategies and leading to improved patient outcomes. Understanding the multifaceted interplay between heart disease and stroke is imperative, as such knowledge will contribute to more accurate risk classification and the formulation of targeted preventive measures[10].

II. Methodology

a) Study Design

This research employed a comprehensive retrospective cohort design aimed at identifying stroke prediction indicators among patients with cardiovascular diseases. The study encompassed a diverse group of patients treated at a tertiary care hospital from January 2020 to December 2022. Ethical considerations were paramount; thus, the study protocol received approval from the institutional review board to ensure the

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protection of patient rights and confidentiality throughout the research process.

b) Study Population[9,10]

The study population consisted of 200 patients diagnosed with various cardiovascular conditions, with a mean age of 65 years, reflecting a geriatric predisposition commonly seen in cardiovascular disease. Inclusion criteria for the cohort were strictly defined, encompassing patients with confirmed diagnoses of coronary artery disease (CAD), heart failure, or atrial fibrillation (AF). Patients were required to have established medical records and recent evaluations to ensure accurate data collection. Notably, those with a documented history of stroke prior to enrollment were rigorously excluded from the analysis to prevent confounding variables that could skew the study results. This careful selection aimed to create a clear focus on the relationship between existing cardiovascular diseases and their role in stroke risk.

c) Data Collection

Data collection was conducted meticulously, leveraging the hospital's medical records. Clinical information gathered included demographic variables such as age, gender, and ethnicity, as well as detailed medical histories that included previous cardiovascular events and comorbid conditions influencing stroke risk. Risk factor assessments incorporated essential clinical parameters such as blood pressure measurements, which were taken at multiple points to ensure accuracy, and lipid profiles that provided insights into the cholesterol levels of patients[11].

Biochemical markers were a key focus of the study; inflammatory markers, particularly C-reactive protein and Interleukin-6, were carefully measured to assess their correlation with heightened stroke risk. This biochemical analysis was performed using established laboratory protocols to ensure reliability and reproducibility of results[12].

In addition to clinical and biochemical data, advanced imaging modalities were utilized to enhance stroke risk assessment[13]. Imaging studies such as carotid ultrasound and transesophageal echocardiography were employed to identify any structural abnormalities in the heart and blood vessels, which might predispose patients to thromboembolic events[14]. These imaging results were crucial in differentiating patients with significant anatomical risk factors from those without observable issues[15,16].

By integrating clinical, biochemical, and imaging data, this study aimed to create a robust risk profiling framework, ultimately enhancing the understanding of stroke predictors in the cardiac patient cohort.

d) Statistical Analysis

Data were analyzed using SPSS software. Descriptive statistics were calculated for baseline characteristics. Logistic regression models were employed to assess the association between potential stroke indicators and the incidence of stroke, adjusting for confounding variables.

III. Results

a) Participant Characteristics

The cohort comprised 200 patients, with 58% male and 42% female. The prevalence of conditions was as follows: CAD (40%), heart failure (30%), and AF (30%). The average follow-up duration was 24 months, during which 45 patients (9%) experienced a stroke.

Table

Gender	Number of Participants	Percentage
Male	116	58%
Female	84	42%

b) Clinical Indicators

Condition	Association with Stroke	Odds Ratio (OR)	95% CI	Interpretation
Atrial Fibrillation (AF)	Present in 30%of stroke cases: CHA2DS2-VASc $\geq$2 increases risk	3.45	2.10-5.68	Significant association with stroke risk
Heart Failure	Higher Incidence of Stroke (12% Vs. 6% in non heart failure	-	-	P<0.05
Coronary Artery Diseases (CAD)	Two fold increased risk of stroke	2.01	1.25-3.23	-

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c) *Biochemical Indicators*

Parameters	Association with stroke	Odds Ratio (OR)	95% CI	Interpretation
Hypertension	Present in 65 % of stroke cases	2.89	1.65-5.08	Significant association with stroke risk
High LDL Profile (>130mg/dL)	*Higher Incidence of Stroke with 70% of Patients	-	-	P<0.01
Coronary Artery Diseases (CAD)	Two fold increased risk of stroke	2.01	1.25-3.23	-

**Statin use was associated with a 40% reduction in stroke incidence.*

d) *Inflammatory Markers*

Parameters	Association with stroke	Odds Ratio (OR)	95% CI	Interpretation
Elevated CRP levels	(>3 mg/L) were significantly correlated with stroke risk	1.75	1.10-2.79	Significant association with stroke risk

e) *Imaging Indicators*

Parameters	Association with stroke	Odds Ratio (OR)	95% CI	Interpretation
Carotid Ultrasound	Significant carotid stenosis (>50%) was identified in 25% of stroke patients	3.67	1.82-7.42	Significant association with stroke risk
Transesophageal Echocardiography	Detection of left atrial thrombus in AF patients	4.23	1.88–9.48	Correlated with an increased risk of stroke

IV. Discussion

The findings from this study underscore the critical interplay between various cardiovascular conditions and the heightened risk of stroke among cardiac patients. The retrospective analysis involving 200 patients has revealed significant associations of atrial fibrillation (AF), coronary artery disease (CAD), heart failure, hypertension, and dyslipidemia with increased stroke incidence. These results align with existing literature that suggests patients with underlying cardiovascular issues face a notably higher risk of experiencing a stroke compared to the general population[14].

Atrial fibrillation emerged as a prominent risk factor in our study, corroborating previous research that has consistently highlighted its role in thromboembolic events. AF increases the likelihood of blood clots forming in the heart, which can subsequently lead to ischemic strokes. Therefore, early identification and management of AF in at-risk populations are paramount. Similarly, coronary artery disease and heart failure were closely linked with elevated stroke risk. The pathophysiological mechanisms underlying these conditions often involve reduced cardiac output and impaired blood flow, both of which can contribute to the conditions conducive to stroke occurrence.

Hypertension and dyslipidemia were also identified as critical risk factors, reinforcing their recognized roles in cardiovascular health. The combination of elevated blood pressure and abnormal lipid levels can accelerate atherosclerosis, leading to plaque build-up in arteries, which in turn can obstruct blood flow and provoke stroke[15]. This highlights the importance of regular monitoring and management of these risk factors within the cardiac patient population.

Furthermore, the incorporation of biochemical markers and advanced imaging techniques in our study provided a comprehensive approach to stroke risk assessment. The measurement of inflammatory markers such as C-reactive protein and Interleukin-6, known to be associated with systemic inflammation, offered additional insight into cardiovascular health and potential stroke risk. Similarly, imaging modalities like carotid ultrasound allowed for the identification of anatomical abnormalities that might predispose patients to thromboembolic events, which may not be evident through standard clinical assessments alone[16,17].

This study's results contribute significantly to the existing body of knowledge regarding stroke risk

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stratification in cardiac patients. By identifying and evaluating these predictive indicators, healthcare providers can better understand the complexities of stroke risk within this vulnerable population. Ultimately, the findings advocate for a multidimensional approach to risk assessment that encompasses clinical evaluation, biochemical analysis, and advanced imaging[18].

In conclusion, effective stroke prevention in cardiac patients relies heavily on the ability to identify those at heightened risk through a thorough understanding of associated indicators. The insights garnered from this research may facilitate the development of targeted prevention strategies, enabling healthcare professionals to implement timely and appropriate interventions aimed at reducing stroke incidence and improving overall patient outcomes in the cardiac population. Future studies should aim to further refine these indicators and explore additional factors that may influence stroke risk to enhance the predictive capabilities for healthcare providers.

V. Conclusion

The findings highlight the need for thorough risk assessments in cardiac patients to implement timely stroke prevention strategies. Integrating clinical data, biochemical markers, and advanced imaging techniques allows healthcare professionals to more effectively identify at-risk individuals.

By enhancing our understanding of the interplay between cardiovascular diseases and stroke risk, this research equips healthcare providers with insights to refine patient management strategies. The ultimate goal is to reduce stroke incidence and improve outcomes for cardiovascular patients through targeted interventions. Future studies should further validate these findings and explore the connections between cardiovascular diseases and stroke risk.

Analyzing key clinical, biochemical, and imaging markers is crucial for estimating stroke risk in cardiac patients. By incorporating these indicators into standard clinical practice, healthcare professionals can enhance risk stratification and identify effective preventive interventions. This approach will ultimately help reduce the burden of stroke among this vulnerable group.

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Screening of Therapeutic Potential and Compounds of Endemic *Nepeta pilinux* P.H. Davis in Kew Bull. from Şanlıurfa

By Fatma Seker, Gulsum Yildiz, Emine Akyüz Turumtay, Adem Demir, Nimet Baltaş Vagif Atamov & Mine Kurkcuoglu

Van Yuzuncu Yil University

Abstract- *Nepeta pilinux* P.H. Davis in Kew Bull. was recently recorded as an endemic species in Birecik, Şanlıurfa. The essential oils were obtained from air-dried aerial parts by hydrodistillation and their composition was investigated using GC-FID and GC/MS. Determination of antioxidant capacity, and urease and xanthine oxidase inhibitions of the methanolic extracts were performed with HPLC-DAD and spectrophotometry. 34 compounds were identified constituting 89.2% of the total essential oil compounds. The major components were determined as T-cadinol (31.1%), γ-muurolene (14.4%) and 14-nor-cadin-5-en-4-one isomer A (11.0%) in the oil. Mainly rosmarinic acid, chlorogenic acid, and caffeic acid derivatives were quantified together with apigenin, luteolin and tangeretin derivatives in the extracts by HPLC-DAD. The total phenolics of the extract from leaf and flower parts, 50.81 mg GAE.g-1, was higher than the extract from stem part, and the radical scavenging activity of this extract was also stronger. While, the leaf and flower extract had significant urease and xanthine oxidase inhibitory activities (62.47 and 48.48 μg.mL-1), stem extract had low inhibition on both enzymes.

Keywords: *nepeta pilinux, essential oil, rosmarinic acid, GC-FID, GC/MS, HPLC-DAD.*

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Screening of Therapeutic Potential and Compounds of Endemic *Nepeta pilinux* P.H. Davis in Kew Bull. from Şanlıurfa

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Abstract- ***Nepeta pilinux*** **P.H. Davis in Kew Bull. was recently recorded as an endemic species in Birecik, Şanlıurfa. The essential oils were obtained from air-dried aerial parts by hydrodistillation and their composition was investigated using GC-FID and GC/MS. Determination of antioxidant capacity, and urease and xanthine oxidase inhibitions of the methanolic extracts were performed with HPLC-DAD and spectrophotometry. 34 compounds were identified constituting 89.2% of the total essential oil compounds. The major components were determined as T-cadinol (31.1%), γ-muurolene (14.4%) and 14-nor-cadin-5-en-4-one isomer A (11.0%) in the oil. Mainly rosmarinic acid, chlorogenic acid, and caffeic acid derivatives were quantified together with apigenin, luteolin and tangeretin derivatives in the extracts by HPLC-DAD. The total phenolics of the extract from leaf and flower parts, 50.81 mg GAE.g^{-1}, was higher than the extract from stem part, and the radical scavenging activity of this extract was also stronger. While, the leaf and flower extract had significant urease and xanthine oxidase inhibitory activities (62.47 and 48.48 μg.mL^{-1}), stem extract had low inhibition on both enzymes.**

Keywords: nepeta pilinux, essential oil, rosmarinic acid, GC-FID, GC/MS, HPLC-DAD.

I. Introduction

The genus *Nepeta* is distributed over a large part of Central and Southern Europe, and West, Central, and Southern Asia as a multi-regional genus of the Lamiaceae (labiatae or mint) family, consisting of approximately 300 taxa (1, 2). 34 species (40 taxa) of the genus Nepeta were recorded in the Flora of Turkey (3, 4). According to the revision of this genus, it is represented by 39 species (46 taxa) in Turkey with recent studies. Endemism rate on the basis of this species is 44% (5). *Nepeta* species are usually named as catmint or catnip due to the sedative effects on cats and they are commonly used as diuretic, spasmolytic, diaphoretic, bronchodilator, antitussive, anti-asthmatic and sedative agents in Turkey. Due to their antiseptic properties, they are used topically in the treatment of children with skin rashes, and in snake and scorpion bites as well. *Nepeta caes area,* an endemic species in Turkey, has folkloric uses in southern Anatolia and is used as a herbal tea to treat gastric disorders (6,7). Some *Nepeta* species have been known their feline attractant activity since they have nepetalactone and its derivatives which are responsible for attractant properties (1,7). Nepetalactones have been reported to effect on insects compare to DEET (N, N-diethyl-m-toluamide) (8-10). Nepetoideae is essential oil-rich genera of the Lamiaceae, therefore has potential economic interest (11).

According to the various studies on the essential oil composition of *Nepeta* species the essential oil composition depends on the species, place of cultivation, climatic conditions and method of analysis (12-26). The most comprehensive study on 22 *Nepeta* species was performed by Baser et al. (2000). They were classified into two groups according to composition of essential oil of these species; nepetalactone-containing and nepetalactone-less. Nepetalactone-containing species have 4aα-7α-7aα nepetalactone as the most frequently contained nepetalactone. *Nepeta cadmea* Boiss., *Nepeta cataria* L., *Nepeta caesarea* Boiss. and *Nepeta pilinux* P.H. Davis contained 4aα-7α-7aα nepetalactone while *Nepeta racemosa* Lam. contained 4aα-7α-7a*β*-nepetalactone as major compound in their oils. Caryophyllene oxide or 1,8-cineole/linalool were identified in the essential oils of Nepetalactone-less species as the major components. As main compounds, *β*-pinene, *a*-terpineol, germacrene-D, and spathulenol were respectively determined in the oil of *Nepeta phyllochlamys* P.H. Davis, *Nepeta viscida* Boiss., *Nepeta sorgerae* Hedge et Lamond, and *Nepeta trachonitica* Post which are out of the two groups (1).

Being a type of the most popular antioxidant secondary metabolites, phenolic compounds were investigated in the *Nepeta* species as well. Rosmarinic

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acid, epicatechin, chlorogenic acid, caffeic acids, quercetin, rutin, ellagic acid, thymusin, luteolin, and apigenin which are well-known as antioxidant compounds were found in the extracts of *Nepeta cadmea* Boiss., *Nepeta nuda* subsp. *albiflora, Nepeta asterotricha* Rech., *Nepeta rtanjensis* Diklic & Milojević (27-30).

Ureases, a nickel-dependent metalloenzymes, are synthesized by plants, some bacteria, algae and fungi. The jack bean urease (urea amidohydrolase EC 3.3.1.5) catalyzes the hydrolysis of urea to form ammonia and carbon dioxide (31). *Helicobacter pylori*, a gram-negative microaerophilic pathogen survivable in a limited pH (4.0–8.2) range. This pathogen can successfully colonize and persist in the mucous layer of the human stomach since its urease activity which produces ammonia to reduce stomach acidity. Since antibiotic-resistant strains of H. pylori can emerge against antibiotics, it is believed that plant-derived urease inhibitors would be more beneficial against gastroduodenal disease associated with this pathogen (32, 33).

Xanthine oxidase (EC 1.2.3.2.) produces hydrogen peroxide and superoxide anion, which are reactive oxygen species (ROS) during the oxidation of hypoxanthine and xanthine to uric acid. Under physiological conditions, ROS is kept at a low level by the antioxidant system. Disruption of the balance of ROS and antioxidants due to some diseases causes tissue and DNA damage due to the increase in metabolism of ROS. Inhibition of xanthine oxidase reduces the amount of uric acid and ROS in the bloodstream to prevent both hyperuricemia and oxidation stress (34). Inhibitory effects of some flavonoids such as diosmetin, luteolin, chrysoeriol, apigenin, kaempferol on xanthine oxidase have been reported in various in vitro studies (35-37).

Nepeta pilinux P. H. Davis in Kew Bull. is an endemic species of *Nepeta* genus growing in the Southwestern Anatolia (Antalya: Alanya) (3). However, in recent flora research, *Nepeta pilinux* was encountered in Şanlıurfa, Birecik district and was recorded as new endemic species for Şanlıurfa flora. *Nepeta pilinux* is named as 'top pisik otu'. In Şanlıurfa, the fresh aerial parts of the plant are used to heal mouth sores (38).

There is no knowledge about the essential oil and phenolic composition of *Nepeta pilinux* endemic species from Şanlıurfa. Chemical compositions of the polar and apolar extracts from *Nepeta pilinux* were determined by HPLC-DAD and GC/MS respectively for the first time in this study. Radical scavenging activities against DPPH and ABTS radicals and enzyme inhibition activities on urease and xanthine oxidase were investigated in vitro to elucidate the bioactivities which may have developed by this species depending on its chemical composition.

II. Experimental

a) Materials

Nepeta pilinux was collected from Şanlıurfa: Birecik, Kelaynak area in Turkey in 5 May 2018. The voucher specimen has been deposited at the Herbarium in the Recep Tayyip Erdoğan University (RTEUB 6079), Rize, Turkey (Voucher specimen no: FABAK 1702). The plant material was identified by Prof. Dr. Vagif ATAMOV (Recep Tayyip Erdogan University, Faculty of Science and Literature, Department of Biology, Rize, Turkey). All standards of phenolic compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA) but quercetin from Fluka Chemie GmbH (Switzerland). Na_2CO_3 and $K_2S_2O_8$ were provided from Sigma-Aldrich (St. Louis, MO, USA. HPLC grade acetonitrile, methanol, acetic acid, Folin ciocalteau, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied by Merck (Darmstadt, Germany).

b) Isolation of the Essential Oil

Aerial parts of the plant were water distilled for 3 h using a Clevenger-type apparatus. The essential oil was stored at 4°C in the dark until analyzed.

c) Extraction of Phenolic Compounds

The aerial parts of the plant were divided into two parts. The leaves and flowers were combined in one part and the stems were separated into the other part. The part consisting of leaves and flowers was called NP-LF for short, and the part consisting of stem is called NP-S. These parts were finely ground and 0.5 grams of each were defatted by using 10 mL of hexane. The plant residues were dried at 40 °C for 30 min after removing the hexane extract. 20 mL methanol were added to these residues for the extraction of phenolic compounds. Extraction was continued overnight at 37 °C in the dry thermo-shaker cabinet at 352 rpm, then the extracts were centrifuged at 5000 rpm and supernatants transferred into the falcon tube. This procedure was repeated by adding 10 mL of methanol in the residue. All extract was concentrated until 5 mL by using rotary evaporator at 35 °C. 500 and 1500 µL of these extracts were stored at -20 °C for HPLC-DAD analysis, antioxidant and enzyme inhibition tests. Remaining 3 mL of extract was evaporated to calculate the concentration of the extracts. NP-LF and NP-S extracts concentration were 23.17 and 23.50 $mg.mL^{-1}$ respectively.

d) Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC/MS) analysis of essential oil

The oil was analyzed by capillary GC and GC/MS using an Agilent GC-MSD system.

i. *GC/MS analysis Conditions of Essential Oils*

The oil was analyzed by capillary GC/MS using an Agilent GC-MSD system (Agilent Technologies Inc.,

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Santa Clara, CA). HP-Innowax FSC column (Hewlett-Packard-HP, U.S.A.) (60 m × 0.25 mm i.d., with 0.25 μm film thickness) was used for separation of components in the oil and helium as a carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The split flow was adjusted at 40 mL min^{-1} with 40:1 split ratio. The injector temperature was set at 250 °C. Mass spectra were taken at 70 eV with the mass range *m/z* 35-450.

ii. *GC Analysis Conditions of Essential Oils*

The GC analysis was done with Agilent 6890N GC system fitted with a FID detector set at a temperature of 300°C. To obtain the same elution order with GC/MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatogram.

iii. *Identification of Essential Oils*

Identification of essential oil components were performed by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/MS Library, Adams Library, MassFinder Library (39-41) and confirmed by comparison of their retention indices. A homologous series of *n*-alkanes were used as the reference points in calculation of relative retention indices (RRI) (42). The relative percentages of the separated compounds were calculated from FID chromatograms. The analysis results are expressed as mean percentage as listed in Table 1.

e) HPLC-DAD Analysis Conditions of Methanol Extracts

The chromatographic analyses were performed using a Dionex (Thermo scientific, Germering, Germany) Ultimate 3000 high performance liquid chromatography (HPLC) system equipped with an Ultimate 3000 diode array detector (DAD).

A Thermo acclaim C30 column (150mm. 3mm id. 3μm pd) was used with Macherey Nagel (3mm id) guard column. Gradient elution was used with mobil phases; A: 2% acetic acid in water and B: 70%acetonitrile-30% water. Flow rate was 0.37 mL/min and injection volume was 10 μL. Column temperature was 25°C. Following 24 phenolic standards were used to calibration and validation of HPLC-DAD analysis method: Gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid (*p*-OH benzoic acid), vanillic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, vanillin, epigallocatechin gallate (EGCG), epicatechin, *p*-coumaric acid, ferulic acid, chicoric acid, rutin, luteolin-7-glycoside, hesperidin, apigenin-7-glycoside, rosmarinic acid, luteolin, quercetin, hesperetin, apigenin, and tangeretin. They were diluted from their stock solution into nine different concentration at 0.3125; 0.625; 1.25; 5.0; 10.0; 25.0; 40.0 $mg.L^{-1}$ in 1:1 methanol-water solution. External calibration method was used and their regression coefficient were found at least 0.999. Repeatability of the retention time and peak areas were measured as coefficient of variation (CV) which was under 0.93 for retention times and 6.02 for areas of the peaks. Limit of detection and quantification values of the peaks were under 0.11 and 0.37 $\mu g.mL^{-1}$ for all standards. Chromatograms were processed at 254, 280, 315, and 370 nm with DAD which operated 200-400 nm. The identification of the peaks was carried out by comparing the retention times and UV spectra with those of standard phenolic compounds. Some peaks had the same or very similar UV spectra as some standards, but with different retention times. They were defined as derivatives of standards with similar UV spectrum and quantified as equivalent of those standards.

f) Determination of Total Phenolic Content

Total phenolic content was determined by using the yellow colored Folin–Ciocalteu's phenol reagent, which was reduced to its blue complex in the presence of reducing agent such as phenolic compounds (43). Gallic acid and quercetin were used as phenolic standards to generate standard curves in a range of 0.0156 and 0.500 mg/mL at 6 concentration levels (r^2 = 0.998). The optical density of the extracts with phenol reagent in the alkaline solution was measured at 760 nm with a UV–Vis detector (Thermo Scientific Multiskan Go, USA). The results were expressed in mg of gallic acid (GAE) and quercetin equivalent (QE) per gram of extracts. All concentration point of the extracts was analyzed in triplicate.

g) DPPH Free-Radical Scavenging Activity Assay

The free-radical scavenging activity was determined based on the reduction of the purple colored 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical to the yellow colored DPPH-H form by the effect of an antioxidant species such as phenolic compounds in the extracts. It was spectrophometrically performed at 517 nm (44). Briefly, 0.15 mL of plant extract was mixed with 0.15 mL 0.1 mM daily prepared DPPH in methanol and incubated for 30 min in the dark. Gallic acid and quercetin were used as standards to compare with the methanol extracts. Results are reported as SC_{50} values, demonstrating the concentration of extract (μg extract per mL methanol) necessary to scavenge 50% of DPPH•. All concentration point of the extracts was analyzed in triplicate.

h) ABTS Radical Scavenging Assay

7 mM of ABTS solution and 2.4 mM of potassium persulfate solution were mixed in equal quantities and allowing to oxidation reaction of ABTS by $K_2S_2O_8$ for 18 h at room temperature in the dark to form

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the $ABTS^{\bullet+}$ radical. Obtained radical solution was then diluted with methanol 25 times to obtain an $ABTS^{\bullet+}$ solution has optical density of 0.700 ± 0.01 at 734 nm (45). 50 µL plant extracts were allowed to react with 250 µL of the $ABTS^{\bullet+}$ radical solution and the absorbance was measured at 734 nm after 30 min using a spectrophotometer. The $ABTS^{\bullet+}$ scavenging capacity of the extracts were compared with that of gallic acid and quercetin and reported with SC_{50} values (µg extract/ mL methanol). All concentration point of the extracts was analyzed in triplicate.

i) Urease Inhibitory Assay

Urease inhibition of the extracts were performed according to the phenol-hypochlorite method developed by Weatherburn (1967) (46). Jack bean urease was used as a model enzyme. Optical density of the resulting blue-navy colored mixture at 625 nm were recorded on a spectrophotometer (1601UV-Shimadzu, Australia). To calculate the IC_{50} values of the polar extracts, different concentrations of the extracts or inhibitory compounds were prepared. Acetohydroxamic acid, well-known inhibitor of urease, were used as positive control.

j) In Vitro Anti-Xanthine Oxidase Assay

The inhibition of xanthine oxidase was measured by UV spectroscopy technique at 295 nm which is attributed to the released uric acid from xanthine. The inhibitory activity of the extract was determined using a slight modification of the reference methods (34). Briefly, the reaction mixture consisted of 500 mL of the extract solution, (diluted in DMSO), 770 mL of phosphate buffer (pH 7.8) and 70 mL of bovine milk xanthine oxidase (0.4 U/mL, Sigma Aldrich, St. Louis, USA) was prepared. The reaction was initiated by the addition 660 mL of xanthine solution (0.4 mM) into the mixture after incubation at 25°C for 15 min. The assay mixture was incubated at 25°C for 15 min again. The reaction was stopped by adding 200 mL of 0.5 N HCl and the absorbance was measured at 295 nm using UV/vis spectrophotometer (1601UVShimadzu, Australia). A well-known XO inhibitor (XOI), allopurinol (Sigma Aldrich, St. Louis, USA) was used as a positive control. XO activity was expressed as percent inhibition of xanthine oxidase, calculate as (1-B/A) x 100, where A is the change in absorbance of the assay without the test samples. (Δ abs with enzyme - Δ abs without enzyme), and B is the change in absorbance of the assay with the test sample (Δ abs with enzyme - Δ abs without enzyme). The assay was done in triplicate. The IC_{50} value was determined as the concentration of the extract that gave 50% inhibition of maximal activity.

III. Results and Discussion

a) GC And GC/MS Analysis of Essential Oils

Essential oil yield in the sample was calculated as 0.26%. Thirty-four compounds comprising about 89.2% of the essential oil were identified. Identified essential oil components were compared with literature polar column retention times. The major components were determined as T-cadinol (31.1%), γ-muurolene (14.4%) and 14-nor-cadin-5-en-4-one isomer A (11.0%) in the oil. *Nepeta pilinux* essential oil has oxygenated sesquiterpenes (44.6%), sesquiterpenes hydrocarbons (17.3%), oxygenated monoterpenes (10.1%), monoterpene hydrocarbons (2.3%) and others (14.9%). The analysis results were shown in Table 1.

Our results were not similar to the classification of *Nepeta* species reported by Baser et al. (2000) (1). *Nepeta pilinux* which was collected from Antalya, Alanya district was in the group containing nepetalactone, an iridoid monoterpene according to their report. Although 89.2% of the essential oil was determined in *Nepeta pilinux* from Şanlıurfa, nepetalactone was not detected. The determination of different essential oil compositions from *Nepeta pilinux* from different regions may have resulted from the difference in locality.

The major constituent of water-distilled essential oils of *Nepeta heliotropifolia* and *Nepeta congesta* subsp. *cryptantha* was determined by GC/MS and GC-FID and found to be germacrene D (36.7% and 38.5%, respectively). Their main aroma component was determined as eucalyptol (48.0% and 24.7%, respectively) (47). Although the major essential oil and aroma compounds of these two species were quite different from the main essential oils of *Nepeta pilinux,* the compositions of these three species had lots of common compounds such as α and β-pinene, γ−muurolene, myrtenal, pinocarveol, caryophyllene oxide, cubenol, T and α-cadinol ect. The presence of more or less components may be due to differences between species, as well as environmental conditions and the harvesting time of the plant.

Table 1: Composition of the Essential Oil of *Nepeta Pilinux*

RRI[a]	RRI[b]	Components	%	IM
1032	1032[c]	α-Pinene	2.3	t_R, MS
1118	1118[c]	β-Pinene	tr	t_R, MS
1213	1213[d]	1,8-Cineole	tr	t_R, MS
1376		*trans*-Muurola-3,5-diene	0.3	MS
1499	1499[c]	α-Campholenal	0.7	MS
1535	1535[e]	Pinocamphone	0.1	MS

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1553	1553[d]	Linalool	2.3	t_R, MS
1577	1577[e,g]	α-Cedrene	0.3	MS
1586	1586[c]	Pinocarvone	0.7	MS
1617	1613[g]	β-Cedrene	tr	MS
1648	1648[e]	Myrtenal	0.6	MS
1670	1670[d]	*trans*-Pinocarveol	1.3	t_R, MS
1684	1684[e]	*trans*-Verbenol	4.4	MS
1694	1693[g]	β-Acoradiene	tr	MS
1704	1704[c,d]	γ-Muurolene	**14.4**	MS
1706	1706[d]	α-Terpineol	tr	t_R, MS
1726	1725[e]	Verbenone	tr	t_R, MS
1747	1740[e]	*p*-Mentha-1,5-dien-8-ol	tr	MS
1751	1751[d]	Carvone	tr	t_R, MS
1797	1804[d]	Myrtenol	tr	MS
1845	1845[d]	*trans*-Carveol	tr	t_R, MS
1853	1849[d]	*cis*-Calamenene	1.8	MS
2008	2008[c]	Caryophyllene oxide	0.6	t_R, MS
2050	2050[e]	(E)-Nerolidol	0.4	t_R, MS
2080	2080[d]	Cubenol	3.7	MS
2089		6-Methyl-5 (3-methyl phenyl)-2-heptanone	0.8	MS
2187	2187[d]	**T-Cadinol**	**31.3**	MS
2256		*epi*-α-Bisabolol	0.3	t_R, MS
2257	2233[f] 2256[e]	Cadalene	0.5	MS
2258	2219[c] 2255[d]	α-Cadinol	1.4	t_R, MS
2264	2264[e]	4,7-dimethyl-1-tetralone	0.8	MS
2320	2324[c]	**14-Nor-cadin-5-en-4-one** isomer A	**11.0**	MS
2349	2349[e]	Cadina-4, 10 (15)-dien-3-one	6.1	MS
2931	2931[c] 2913[f]	Hexadecanoic acid	3.1	MS
		Monoterpene hydrocarbons	2.3	
		Oxygenated monoterpenes	10.1	
		Sesquiterpenes hydrocarbons	17.3	
		Oxygenated sesquiterpenes	44.6	
		Others	14.9	
		Total %	**89.2**	

RRI[a]: RRI Relative retention indices experimentally calculated against *n*-alkanes; RRI[b]: RRI from literature [c (48); d (49); e (50); f (51); g (52)] for polar column values; % calculated from FID data; tr; Trace (<0.1 %); Identification Method (IM): t_R, Identification based on comparison with co-injected with standards on a HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the libraries.

b) HPLC-DAD Analysis of Phenolic Compounds in the Methanol Extracts

The list of standard phenolic compounds has a wide range of phenolic standards such as 5 benzoic acids (gallic, protocatechuic, *p*-OH benzoic, vanilic, and syringic acid), 1 hydroxybenzaldehyde (vanillin), 5 cinnamic acids (caffeic, *p*-coumaric, ferulic, chicoric and chlorogenic acid), 3 flavanols (catechin EGCG, epicatechin), 1 flavonol with its 1 glycoside (quercetin and rutin), 3 flavones with 2 sugar attached derivatives, (luteolin, apigenin, tangeretin, luteolin-7-glucoside, apigenin-7-glucoside), 1 flavanone with its 1 glycoside (hesperidin and hesperetin), and rosmarinic acid which is a caffeic acid ester *(supp. Table 1)*.

A total 6.9 g and 2.1 g of phenolic compounds per 100 g of NP-LF and NP-S, respectively, were quantified by HPLC-DAD *(Table 2).* The stem part of the plant had at least 3 times less amount of phenolic compounds than the leaf and flower parts (NP-LF). Rosmarinic acid (RA) was found to be the major compound at 3.1 g per 100 g of NP-LF (3.1%) as expected from Lamiaceae family members. The peaks which had same UV spectrum as RA but eluted earlier were identified as RA derivatives (der.) and quantified as equivalent of RA *(Fig. 1).* They could be the sugar or other functional groups attached to the RA causing this early elution. Total ratio of RA derivatives in the extracts

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were 4.3% for NP-LF and 0.3% for NP-S *(Table 2)*. Caffeic acid and derivatives including RA covered 4.5% of the NP-LF.

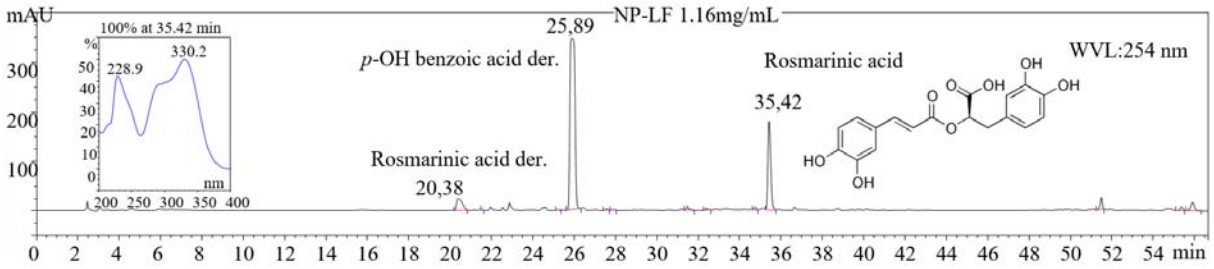

Figure 1: HPLC-DAD chromatogram of the extract from leaf and flower parts of *Nepeta pilinux*

Protocatechuic, chlorogenic, caffeic, *p*-coumaric, chicoric acids and luteolin-7-glycoside were also determined in the extracts by comparing the retention time and UV spectrum of the peaks with those of the standard phenolics. There was extremely large peak among others in the chromatograms at 254 nm which was eluted at around 25.9 min and had overlaying two compounds with 243 and 257 nm maximum wavelengths. These peaks were identified as *p*-OH benzoic acid der. and quantified as its equivalent *(Fig. 1 and Table 2)*. The peak eluting at 21.4 min. and having max. absorbance at 293 nm was identified as *p*-OH benzaldehyde (4-hydroxybenzaldehyde) since its elution order and UV spectrum were consistent with this phenolic aldehyde (53). The regression equation of vanillin which is the 4-hydroxy-3-methoxybenzaldehyde was used to quantified *p*-OH-benzaldehyde due to their structural and spectral similarity. Four tangeretin derivatives were identified tentatively by comparing UV spectrum of these peaks with that of tangeretin. Since tangeretin has 5 metoxy groups without hydroxyl group, it is eluting in the last part of the chromatogram from

Table 2: The amount and spectral details of phenolic compounds determined in the extracts by HPLC-DAD

	RT		Maximum Wavelength		mg/100g extract	
	NP-LF	NP-S	λ max	Compounds	NP-LF	NP-S
1	ND	8.6	259-292	protocatechuic acid	ND	5.5
2	15.8	15.3	232-284	catechin der.	83.8	57.7
3	20.4	20.3	330-300sh-232	rosmarinic acid der.	1055.9	164.9
4	21.4	21.2	293	*p*-OH benzaldehyde	2.8	16.2
6	22.0	21.7	326-295sh-237	chlorogenic acid	86.9	6.7
7	22.9	ND	328-295-242	caffeic acid	75.9	ND
8	ND	22.9	315-230	*p*-coumaric acid der.	ND	14.2
9	23.7	ND	333-243	chicoric acid der.	2.8	ND
10	ND	23.9	281-229	syringic acid der.	ND	5.4
11	24.5	24.3	329-302sh-249	rosmarinic acid der.	84.3	31.9
12	25.2	25.0	330-300sh-243	rosmarinic acid der.	29.0	6.7
13	25.9	25.7	243 and 257 mix	*p*-OH benzoic acid equivalent	2204.7	1605.3
14	26.8	26.6	229-310	*p*-coumaric acid	3.7	2.4
15	27.1	26.8	352-255	luteolin-7-gycoside der.	12.7	5.7
16	28.9	ND	312-295-228	p-coumaric acid der.	11.6	ND
17	29.2	29.0	329-305sh-243	chicoric acid	3.7	2.1
18	ND	30.8	329-233	rosmarinic acid der.	ND	3.7
19	31.5	31.2	338-253-225	apigenin-glycoside der.	54.4	3.9
20	ND	31.4	342-246-226	luteolin-glycoside der.	ND	2.5
21	32.4	ND	327-228 and 347-270	ferulic acid der. and luteolin-7-glycoside	mix	ND
22	ND	32.4	320-244	caffeic acid der.	ND	1.2
23	33.2	33.0	228-278	syringic acid der.	31.4	20.1

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24	33.4	33.2	228-278	syringic acid der.	17.8	17.3
25	ND	33.4	331-275	apigenin-glycoside der.	ND	3.2
26	ND	34.1	338-275	apigenin-glycoside der.	ND	5.6
27	35.4	35.3	330-290sh-229	rosmarinic acid	3099.7	59.1
28	ND	35.7	314-230	*p*-coumaric acid der.	ND	2.7
29	36.6	ND	328-300sh-234	caffeic acid der.	21.6	ND
30	ND	38.3	319-230-295sh	ferulic acid der.	ND	6.8
31	38.7	38.6	328-277-226	tangeretine der.	10.7	43.6
32	39.4	39.2	329-277	chicoric acid der.	6.3	1.6
33	ND	39.4	338-276	apigenin der.	ND	5.6
34	39.8	ND	327-300sh-239	chlorogenic acid der.	8.4	ND
35	ND	41.6	267-340	apigenin der.	ND	6.4
36	ND	42.0	324-270	tangeretin der.	ND	1.6
38	ND	45.1	328-277-214	tangeretin der.	ND	1.4
39	ND	49.8	327-275-223	tangeretin der.	ND	7.4
40	ND	50.3	316	*p*-coumaric acid der.	ND	16.2
41	50.4	50.6	315-214	*p*-coumaric acid der.	11.5	0.9
				total	6919.7	2135.2
				total rosmarinic acids	4269.0	266.3

Since *Nepeta pilinux* is an endemic species and newly recorded in Şanlıurfa flora, there is not any scientific report for its phenoreverse phase chromatography *(Table 2).* Tangeretin derivatives may differ from each other in the number of hydroxyl and methoxyl groups and the presence of sugar or other functional groups. Thymusin which is the 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (5,6,4' demethyl-tangeretin) and isothymusin were determined in *Nepeta asterotricha* Rech. (29). Therefore, two of the tangeretin der. could be thymusin and isothymusin. Apigenin and luteolin derivatives, determined in the extracts based on their UV spectra, were identified and quantified as apigenin, apigenin glycoside der. or luteolin glycoside der. based on their elution orders.

lic composition. On the other hand, there are some reports for chemical composition of the other species of *Nepeta* genus to compare with. For instance, epicatechin, caffeic acids, chlorogenic acid, quercetin and ellagic acid, well-known as antioxidant compounds were found in the ethanol extract of *Nepeta cadmea* Boiss. by HPLC-DAD (27). They couldn't have determined RA because it was not in their standard compound list. Unlike their results, epicatechin, quercetin and ellagic acid were not detected in *Nepeta pilinux* extracts *(Table 2).* Observation of similar but different phenolic compounds in species shows that differences between species also cause differences in metabolic product synthesis. In addition, different derivatives of phenolic compounds can be observed in samples of the same species grown in different geographical conditions. RA, apigenin, and quercetin were determined as major compounds in the ethanol extract of *Nepeta nuda* subsp. *albiflora* (28). Isolation of some iridoid glycosides such as nepetamoside, nepetaracemoside B, nepetonic acid and some polyphenol and flavonoid components such as RA and its methyle ester, thymusin, luteolin and apigenin from *Nepeta asterotricha* Rech. were performed by Goldansaz et al. (2019) (29). Methanolic extracts from *Nepeta rtanjensis* Diklic & Milojević which is an endemic perennial plant, in a very limited area in Southeast Serbia were investigated in a scientific study (30). The presence of high levels of chlorogenic acid, RA and rutin in these extracts was thought to be the reason for their antigenotoxicity.

RA, which is the main component in these extracts, is synthesized by the phenylpropanoid pathway starting with L-phenylalanine and L-tyrosine. From L-phenylalanine, *t*-cinnamic acid, *p*-coumaric acid and *p*-coumaroyl-CoA are successively produced, while p-hydroxyphenylpyruvic acid, p-hydroxyphenyllactic acid are successively produced from L-tyrosine. Then, the production of p-coumaroyl-p-hydroxy-phenyllactic acid by condensation of p-coumaroyl-CoA and p-hydroxyphenyllactic acid is followed by RA synthesis (54). Chlorogenic acid is synthesized by the condensation of *p*-coumaroyl-CoA with quinic acid (55). These metabolites which were found in the *Nepeta pilinux* extracts, support the survival of plants against harsh environmental conditions, therefore they have been seen by humans as a remedy for various diseases for many years. It has been reported that RA, chlorogenic acid and its metabolite caffeic acid have a neuroprotective effect due to their antioxidant capacity (56, 57).

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c) *Total Antioxidant Characterization of the Methanol Extracts*

Total phenolic content (TPC) and radical scavenging activities (RSA) against DPPH and ABTS radical were evaluated to characterize the antioxidant capacity of the extracts and results were presented in Table 3. TPC of the NP-LF and NP-S extract from *Nepeta pilinux* was 50.81 and 13.37 mg GAE/g respectively. Consistent with the TPC of NP-LF which was found around fourfold of NP-S, RSA of NP-LF were higher (433.18 and 82.29 µg / mL for DPPH and ABTS respectively) than NP-S as expected (Table 3). The antioxidant capacity of this species was found to be quite consistent comparing with other species in the literature reports. Antioxidant activity of methanolic extracts from flower and leaf parts of *Nepeta rtanjensis* were reported by Bošnjak-Neumüller et al. (2017). They have found that the leaf extract had higher antioxidant capacity with TPC of 62.73 mg of GAE/g and IC_{50} value of RSA against DPPH as 112.59 µg/mL than those of flower extract. The antioxidant activities of ethanol, methanol, acetone, and water extracts from *Nepeta cadmea* were presented by Kaska et al. (2018). They have found that the water extract had the highest RSA (IC_{50} value of DPPH as 25.54 µg/mL and ABTS, 14.51 µg/mL) in these four extracts. Highest TPC with 79.84 mg GAE/g, was found in the methanol extract while the highest total flavonoids with 77.09 mgQE/g was in the acetone extract. It was reported by Teber and Bursal (2020) that ethanol and water extracts of *Nepeta nuda* subsp. *albiflora* had strong antioxidant effects with IC_{50} values of DPPH as 54.4 and 113.0 µg/mL, respectively. The TPC of flowers, leaves and roots methanol extracts of *Nepeta humulis* were found as 123.18, 66.20 and 54.77 mg GAE/g extract, respectively. Flower extract which had the highest TPC displayed best RCA with IC_{50} of 1290 and 350 $\mu g.mL^{-1}$ against DPPH and ABTS respectively (58).

Table 3: Total phenolic content, radical scavenging activity and enzyme inhibition of the extracts

Samples and standards	TPC[a]		RSA[b]		Enzyme inhibition	
	mgGAE/g	mgQE/g	DPPH SC_{50}	ABTS SC_{50}	urease IC_{50}	XO IC_{50}
NP-LF	50.81±1.50	35.40±1.06	433.18±12.74	82.29±0.98	62.47±0.10	48.48±0.10
NP-S	13.37±0.34	9.03±0.24	523.49±5.82	381.58±5.15	230.59±0.23	222.67±0.13
Gallic Acid			1.52±0.06	3.29±0.09		
Quercetin			5.87±0.13	8.52±0.16		
Acetohydroxamic acid					24.56±0.29	
Allopurinol						0.54±0.04

GAE, Gallic acid equivalent; QE, quercetin equivalent; SC_{50}, value of the concentration of extract required to scavenge 50% of DPPH and ABTS radicals (µg extract per mL methanol); IC_{50}, value of the concentration of extract required to inhibite 50% of *Jack bean* urease and bovine milk xanthine oxidase enzymes (µg extract per mL methanol). [a]Total phenolic contents are expressed in mg GAE/g extract and mg QE/g extract. [b]Radical Scavenging Activity

d) *Urease and Xanthine oxidase inhibitions of the methanol extracts*

The urease enzyme inhibition of the NP-LF with IC_{50} value of 62.47 $\mu g.mL^{-1}$ was only three times lower than the inhibition of acetohydroxamic acid (24.56 $\mu g.mL^{-1}$) which is standard medicine *(Table 3)*. NP-S had low inhibition against to this enzyme with IC_{50} value of 230.59 $\mu g.mL^{-1}$. The xanthine oxidase inhibition of the NP-LF and NP-S extracts, 48.48 and 222.67 $\mu g.mL^{-1}$ respectively, were quite lower comparing to the inhibition of allopurinol (0.54 $\mu g.mL^{-1}$) which is standard medicine reducing the production of uric acid in the body caused by certain cancer medications and kidney stones. Akdeniz et al. (2020) were screened urease inhibition effect of the essential oils and ethanolic extracts of *Nepeta heliotropifolia and Nepeta congesta* subsp. *cryptantha* comparing with the standard thiourea. They reported that none of them exhibited urease inhibitory activity (47). In another study, ethyl acetate sub fraction of *Nepeta praetervisa* showed significant urease inhibitory activity (68%) (59). The structure-activity relationship revealed that the planar flavones and flavonols with a 7-hydroxyl group such as chrysin, luteolin, kaempferol, quercetin, myricetin, and isorhamnetin inhibited xanthine oxidase activity at low concentrations, while the nonplanar flavonoids, isoflavones and anthocyanidins were less inhibitory (35). Although the inhibitory effects of the extracts cannot compete with acetohydroxamic acid and allopurinol, the fact that they are a natural herbal inhibitor source shows that these extracts are more suitable for use.

IV. Conclusion

This was the first investigation on the chemical composition and bioactivities of the *Nepeta pilinux.* The polar and apolar extracts of aerial parts had high amount of phenolic and essential oil compounds and demonstrated the potential antioxidant capacities. The polar extract with 3.1% rosmarinic acid had urease and

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xanthine oxidase inhibition as well. This comprehensive evaluation of *Nepeta pilinux* revealed that this endemic plant could be the source of valuable therapeutic compounds. Besides, this report would be the incentive for further works on this plant's metabolites.

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Disclosure Statement

No potential conflict of interest was reported by the authors

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Medicinal Mushroom Biotechnology

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Abstract- Since most of medicinal mushrooms are rare in nature production of fungal fruiting bodies using artificial cultivation in a form of farming has been intensively established during the last 40 years. Solid state cultivation of various medicinal mushroom mycelia in various types of bioreactors, suitable for veterinary use, appears slightly in last few decades. Developing submerged technologies, using stirred tank and air lift bioreactors, are the most promising technologies for fast and large cultivation of medicinal pharmaceutically active products for human need. This potential initiates the development of new drugs and some of the most attractive over the counter human and veterinary remedies. This article is an overview of the engineering achievements in comprehensive medicinal mushroom mycelia cultivation.

Keywords: *medicinal mushrooms, cultivation techniques, bioreactors.*

GJSFR-B Classification: *LCC: QK617, RS431.M8*

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Medicinal Mushroom Biotechnology

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Abstract- Since most of medicinal mushrooms are rare in nature production of fungal fruiting bodies using artificial cultivation in a form of farming has been intensively established during the last 40 years. Solid state cultivation of various medicinal mushroom mycelia in various types of bioreactors, suitable for veterinary use, appears slightly in last few decades. Developing submerged technologies, using stirred tank and air lift bioreactors, are the most promising technologies for fast and large cultivation of medicinal pharmaceutically active products for human need. This potential initiates the development of new drugs and some of the most attractive over the counter human and veterinary remedies. This article is an overview of the engineering achievements in comprehensive medicinal mushroom mycelia cultivation.

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I. Introduction

A total of more than 200 medicinal functions are thought to be produced by medicinal mushrooms (MM) and fungi including antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, cholesterol-lowering, antiviral, antibacterial, anti-parasitic, antifungal, detoxification, hepatoprotective, anti-diabetic, anti-obesity, neuroprotective, neuroregenerative, and other effects. Also, substances derived from MM can be used as painkillers or analgetics [4, 5, 6]. The best implementation of MM drugs and dietary supplements has been in preventing immune disorders and maintaining a good quality of life, especially in immunodeficient and immuno-depressed patients, patients under chemotherapy or radiotherapy, patients with different types of cancers, chronic blood-borne viral infections of Hepatitis B, C, and D, different types of anemia, the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), herpes simplex virus (HSV), Epstein Bar virus, Influenza viruses A and B, H5N1 [7], COVID-19 [8-10], West Nile virus, chronic fatigue syndrome, patients with chronic gastritis and gastric ulcers caused by *Helicobacter pylori*, and people suffering from dementia (especially Alzheimer's disease) [1, 2, 3, 5].

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To combat these threats, humankind is focusing more and more attention on mushrooms and mushroom products. Mushrooms themselves are consumed regularly as part of the human diet and are treated as healthy or functional foods. On the other hand, the term mushroom nutriceuticals or dietary supplements has been applied to products derived from medicinal mushrooms that are taken to enhance general health and fitness but are not a regular food but a dietary food supplements [1].

II. Main Pharmaceutically Active Compounds

The main components of these supplements are polysaccharides, triterpenes and immunomodulatory proteins. Polysaccharide components, in particular, have been widely investigated as a source of anti-tumor and immunostimulating agents. They are widely distributed in mushrooms, with over 660 species from 183 genera reported to contain pharmacologically active polysaccharides. About 77% of all medicinal mushroom products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild, 21% from culture mycelium and 2% from culture broths. Precisely how these products work is still a matter of conjecture, but numerous laboratory animal tests as well as human clinical trials have shown them to be effective. In some cases, attention has focused on a single bioactive mushroom component and its effectiveness in treating specific disease conditions, much like a pharmaceutical. In the case of nutriceuticals/dietary supplements, emphasis has been placed on a combination of components that collectively impact on an individual's overall health and quality of life [2].

Many such products are currently available, and their market value worldwide increased from 1.2 billion in 1991 to 3.6 billion USD in 1994. The combined market value of medicinal mushrooms, mushroom extracts and derived products in 1999 was estimated to be 6.0 billion USD. That year, the United States nutraceutical market alone was valued at 35 million USD. Since then, demand has increased between 20% and 40% annually depending on the species, with *Ganoderma*-based dietary supplements alone valued at 1.6 billion USD. The MM industry has grown from small-scale (cottage-based) operations aimed at supplementing household incomes, to medium and mega-sized industrial

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ventures. This review examines the past, present and future of MM development, and includes a pyramid model addressing key issues [3].

They are of different chemical composition, such as polysaccharides, glycopeptide-protein complexes, proteoglycans, proteins and triterpenoids, with most scientific attention focussed to the group of non-cellulosic β-glucans with β-(1-3) linkages in the main chain of the glucan, and additional β-(1-6) branch points, that are characteristic for the antitumor and immuno-stimulating action. Mushroom polysaccharides do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. Their mechanisms of action involve them being recognized by several immune cells receptors as non-self molecules, so the immune system is stimulated by their presence. Structurally different β-glucans have different affinities toward receptors and thus generate different host responses [12].

Immunomodulating and antitumor activities of these metabolites are related to immune cells such as hematpoietic stem cells, lymphocytes, macrophages, T cells, dendritic cells, and natural killer cells, involved in the innate and adaptive immunity, resulting in the production of biologic response modifiers [13]. Clinical evidence for antitumor and other medicinal activities come primarily from some commercialised purified polysaccharides, such as lentinan from Shiitake - *Lentinula edodes,* krestin from *Coriolus versicolor*, grifolan from *Grifola frondosa*, and schizophyllan from *Schizophyllum commune* [12,14], but polysaccharide preparations of some other medicinal mushrooms also show promising results.

III. Wood Degrading *Basidiomycetes* with Pharmacological Effects

Almost unknown in Western Scientific Research only three decades ago, some of the wood degrading *Basidiomycetes* became intensely and systematically studied due to their promising pharmacological effects (Figures 1 A-E). Among them, *Ganoderma lucidum* – Ling zhi or Reishi, *Grifola frondosa* – Maitake, *Hericium erinaceus* – Lion mane and *Cordyceps militaris* have been known from the traditional Asian medicine, and *Trametes versicolor* Turkey tale, (previous *Coriolus versicolor*) are the subject of this review.



Figure 1: *Ganoderma lucidum* (A), *Trametes versicolor* (B) (previous *Coriolus versicolor*), *Grifola frondosa* (C), *Hericium erinaceus* (D) and *Cordyceps militaris* (E)

Technological possibilities for commercial cultivation gave rise to the number of patents, which are protecting inventions related to new methods and technologies for cultivation of fruit bodies and/or mycelium biomass, methods of active compounds isolation, and development of new commercial formulations and products. *Ganoderma lucidum* has been identified as a medicinal mushroom with the largest number of patented inventions [11].

a) Ganoderma Lucidum

Ganoderma (W. Curt.: Fr.) Lloyd is a white rot wood *Basidiomycete* that degrades lignin and possess hard fruiting body. *G. lucidum* is from *Ganoderma sp.* the most often reported as a source of various medicinal compounds. In Asian traditional medicine, the fruiting body of *G. lucidum,* named Ling-zhi in Chinese and Reishi in Japanese language, has been used for treatment of several diseases for thousands of years, as

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reported in Shen Nong's Materia Medica (Leung et.al., 2002; Kim and Kim, 2002; Lin, 2009).

Modern uses of *Ganoderma* include treatment of coronary heart diseases, arteriosclerosis, hepatitis, arthritis, nephritis, bronchitis, asthma, hypertension, cancer and gastric ulcer [15,16] Publications also report on *Ganoderma* antiallergenic constituents [17], immunomodulatory action [18-20], antitumor activity [21-23] cardiovascular effects [24], liver protection and detoxification, and effects on nervous system [22,25]. New reports emphasize its potential in treatment of viral, especially HIV infections [26, 27].

Pharmaceutically active compounds from *Ganoderma lucidum* include triterpenoids, polysaccharides (1,6-β-D-glucans and 1,3-β-D-glucans), proteins, proteoglycans, steroids, alkaloids, nucleotides, lactones and fatty acids, amino acids, nucleotides, alkaloids, steroids, lactones and enzymes. Over 100 triterpenoids were found in *Ganoderma* spp., such as ganoderic (highly oxygenated C_{30} lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic and ganolucidic acids, lucidones, ganoderals and ganoderols [11].

A large and diverse spectrum of chemical compounds with a pharmacological activity has been isolated from the mycelium, fruiting bodies and sclerotia of *Ganoderma* mushrooms: triterpenoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes [28, 29]. There is abundant evidence that polysaccharides isolated from *G. lucidum* are immunomodulatory effective [4, 30-34,]. Studies have shown that the most active immunomodulatory polysaccharides are (1→3)-β-D and (1→6)-β-D glucans, that can be precipitated by ethanol. Their prevailing structure is β-1,3 D-glucopyronan with 1–15 units of β-1,6 monoglucosyl side chains. Their 1,3-linked backbone, relatively small side chains, and an organized helical structure are beneficial for the immunostimulation. Although they are chemically heterogeneous, these polysaccharides are usually termed as *β*-glucans [30-32, 36, 37].

Bioactive polysaccharides have been isolated from different sources of *G. lucidum*: basidiocarps, spores and from the mycelial biomass cultivated in liquid culture. Few have been isolated from the culture medium [37]. Though different models of the fungal cell wall differ somewhat, scientists agree that β-glucan is not located on the surface of the wall but is more or less immersed in the wall material. Generally, the cell wall of most fungi contains five main components: (1→3)-β-D glucan, (1→6)-β-D glucan, chitin, and glycoproteins. β-glucan forms 9–46 % of cell wall mass [36, 37]. A large number of studies have shown that polysaccharides of *G. lucidum*, especially β-D glucan, can modulate the functions of many components of the immune system such as the antigen-presenting cells, T and B lymphocytes, NK cells, neutrophil granulocytes, dendritic cells and, on cytokine production [30, 32, 38, 39].

b) Grifola Frondosa

Grifola frondosa also known as Maitake is white rot lignin degrading *Basidiomycete* with excellent nutritional and medicinal properties. *Grifola frondosa* active compounds primarilly belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins, and proteins. This products have been used for treatment of a series of diseases, including hepatitis, arthritis, nephritis, bronchitis, coronary heart diseases, asthma, arteriosclerosis, hypertension, cancer and gastric ulcer. Newer investigations report on *Grifola frondosa* antiallergenic constituents, immunomodulatory action and treatment of HIV infections, antitumor and cardiovascular effects, liver protection and detoxification and effects on nervous system [40].

G. frondosa has gained in popularity among consumers, not only because as a gastronomic delight of its taste and flavor, but also because of its reported medicinal value. Its active compounds primarily belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins, and proteins. Medicinal effects of *G. frondosa* are numerous, including anti-cancer activity [41, 42], immune system stimulation [43,44], effects on angiogenesis [45], reduction of benign prostatic hyperplasia [46], antibacterial [47] , and antiviral effects [48], effects on lipid metabolism and hypertension [41], antidiabetic activity [49], vitality and performance enhancement [50], antioxidant effects, and beneficial cosmetic effects on skin [51]. According to Shen (2001), more than 20 anti-tumor polysaccharides have been isolated and purified from *G. frondosa* [52].

c) Hericium Erinaceus

In *H. erinaceus* various pharmaceutically active substances were found. Phytosterols (β-sitosterol and ergosterol), lower the content of low density lipoproteins (LDL) and triglycerides that operate anticarcinogenic as well they reduce the metabolism of fats [53]. In *H. erinaceus* fruitbody numerous constituents such as are polysaccharides, proteins, lectins, phenols, hericenones, erinacines and terpenoids were identified. They strengthen the immune system, relieve gastritis and gastrointestinal infections, reflux and upset stomach due to stress [54].

H. erinaceus water-soluble polysaccharides increased activity of macrophages and other immune cells in the fight against cancer cells, but also demonstrated the reduction of formation of metastases. The most outstanding activity of the extract of *H. erinaceus* is that it strengthens the immune system and

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activate the synthesis of nerve growth factor [55]. Due to the increased proliferation of T and B-lymphocytes it strengthens the immune system and strengthens the body's natural defences Thus, *H. erinaceus* expresses very positive effects on prolongation of quality of life of the cancer patients [53].

Among the compounds isolated from fruiting bodies and cultured mycelia of *H. erinaceum*, most interesting are the low-molecular-weight compounds belonging to a group of cyathin diterpenoids (erinacines A–K, P, and Q). Several of them, i.e. erinacines A–H, are known to have a potent stimulating effect on nerve growth factor (NGF) synthesis *in vitro* [56-61].

H. erinaceus polysaccharides (HEP) derived from fruiting bodies and mycelium severed as effective therapeutic agents in liver damage-associated diseases. A study demonstrated that the serum levels of aspartate aminotransferase and glutamic pyruvic transaminase activities in carbon tetrachloride-induced hepatic injury were decreased by administration with extracellular and intracellular HEP (200, 400, and 800 mg/kg/day) from mycelium, but the blood lipid levels in the serum of mice were enhanced [62]. Moreover, Kim *et al*. found that 10 mg/kg/day HEP markedly alleviated *Salmonella typhimurium*-induced liver damage and reduced infected mice mortality [63]. Zhang *et al*. revealed that endo-HEP potent hepatoprotective effect *in vivo*, which may be due to its powerful antioxidant capacity. Taken together, the HEP could be exploited as a supplement in the prevention of hepatic diseases [64].

d) Trametes Versicolor

Trametes versicolor, previous *Coriolus versicolor,* also known as Turkey tail mushroom is one of the most attractive medical fungi. It is known for its use and success as a remedy in Asian traditional medicine [65-67]. *T. versicolor* pharmaceutical activities include immunomodulation, antibody production, activation of apoptosis etc.

The two most prominent products of *T. versicolor* are polysaccharide Krestin (PSK) and polysaccharide peptide (PSP) both potentially highly active pharmaceutical substances in complementary cancer treatments. PSP has a variety of physiological effects, such as immunological enhancement, antitumor, liver protecting, oxidation resistance, and reducing blood fat. PSP has been clinically used in treating cancers, hepatitis, hyperlipidemia, chronic bronchitis, and other diseases. The clinical data also demonstrate that PSP has diverse functions such as improving the quality of patients' life, enhancing learning and memory, and antiulcer effects [70-73].

Current studies support PSP as an immunotherapeutic. PSP activates and enhances the function and recognition ability of immune cells, strengthens the phagocytosis of macrophages, increases the expressions of cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukins (IL-1β and IL-6), histamine, and prostaglandin E, stimulates the filtration of both dendritic cells and T-cells into tumors, and ameliorates the adverse events associated with chemotherapy. In recent years, immunotherapy has been widely used in cancer treatment. However, to use PSP as an immunotherapeutic at world stage, further chemical, biochemical and pharmacological studies of PSP are needed [66].

In vitro and *in vivo* studies have shown that the mixture of PSP and PSK has a synergistic action that highly affects immune cell proliferation and highly expresses antitumor activities [65, 67, 68, 74-76].

e) Cordyceps Militaris

The usage of natural/herbal medicines over the synthetic ones has seen an upward trend in the recent past. *Cordyceps* being an ancient medicinal mushroom used as a crude drug for the welfare of mankind in old civilization is now a matter of great concern because of its unexplored potentials obtained by various culture techniques and being an excellent source of bioactive metabolites with more than 21 clinically approved benefits on human health [77-78].

The studies by many researchers in the past on *Cordyceps* have demonstrated that it has anti-bacterial, anti-fungal, larvicidal, anti-inflammatory, anti-diabetic, anti-oxidant, anti-tumor, pro-sexual, apoptotic, immunomodulatory, anti-HIV, remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous, besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities [79-82].

Cordycepin has received much attention due to its broad-spectrum biological activity. It is known to interfere with various biochemical and molecular processes including purine biosynthesis [83, 84], DNA/RNA synthesis [85] and mTOR (mammalian target of rapamycin) signaling transduction [86]. *Cordyceps* has been included as one of the growing numbers of fungal traditional Chinese medicine (FTCM) used as cures for modern diseases with many products available commercially.

Great potential of pharmaceutical active compounds and *Cordyceps militaris* extract contains many biological bioactive materials, such as the terpenoids cordycepin and cordycepic acid, polysaccharides, sterols and other compounds [87]. *Cordyceps militaris* main active component is terpenoid cordycepin that inhibit the development of cancer cells including antitumor, anti-metastatic, insecticidal, anti-proliferative, anti-bacterial properties, anti-fungal, larvicidal, anti-inflammatory, anti-diabetic, anti-oxidant, pro-sexual, apoptotic, immunomodulatory, anti-HIV, remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous,

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besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities, anti-leukemia and antimalarial activities [77-82].

The second main active components are polysaccharides, which research have shown to be effective in regulating blood sugar and also have anti-metastatic and antitumor properties [87-89]. The most outstanding active substance of *C. militaris* is Cordycepin. The structure of Cordycepin is very much similar with cellular nucleoside, adenosine. Cordycepin, i.e., 3′-deoxyadenosine, is the main active constituent which is most widely studied for its medicinal value having a broad spectrum biological activity and acts like a nucleoside analogue [90].

Cordycepin alone has been widely explored for its anti-cancer/anti-oxidant activities, thus, holding a strong pharmacological and therapeutic potential to cure many dreadful diseases in future. Further investigations need to be focused on to study the mechanistic insight into the mysterious potential of this medicinal mushroom on human health and promoting its cultivation strategies for commercialization and ethno-pharmacological use of this wonderful herb [91, 92].

IV. Cultivation Technologies

Since medicinal mushrooms are scarce in nature, cultivation of fruit bodies on artificial media has been introduced. Traditional cultivation of fruit bodies on wood logs has been known for centuries. With time, cultivation methods have diversified, modified and developed (Figure 2). [11].

Besides on wood logs, fruit bodies are being produced on sawdust substrates in trays or beds, and in sterilised plastic bags or in bottles. In addition, production of fungal mycelia has been developed in bioreactors, utilizing submerged cultivation in liquid media, or solid state cultivation on various secondary wastes substrates from wood and agricultural industry [93].

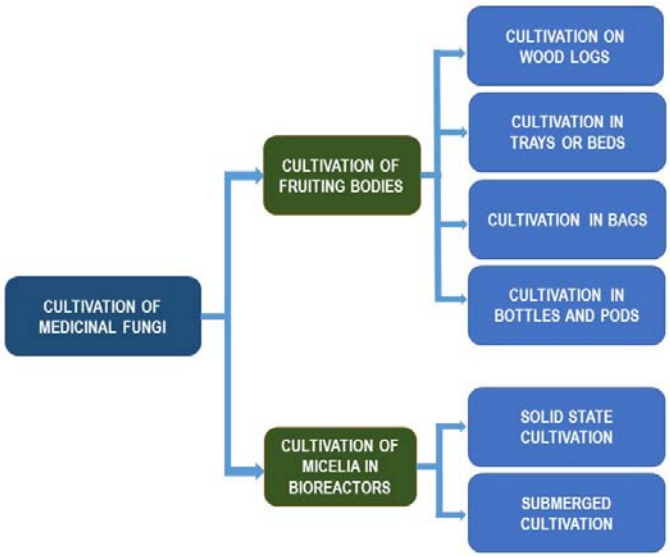

Figure 2: Technological possibilities of cultivating medicinal fungi fruit bodies or fungal biomass on a commercial scale adapted from [11]

a) Farming Fruit Bodies Cultivation

In the wild, wood degrading mushrooms grow primarily on hardwood of trees. However, under artificial cultivation conditions, they thrive on various other substrates containing lignin and cellulose, and therefore have a high potential for recycling different types of organic waste materials from wood and agriculture industry. As lignocellulose containing wastes are produced worldwide in large quantities, and in many instances they pose a threat to the environment. Cultivation of edible and medicinal wood degrading fungi on lignocellulosic substrates offers almost unlimited possibilities and economically viable potentials for large scale commercial cultivation on a World scale [94].

In farming fruit bodies cultivation substrate cultivation methods are divided mostly into bottle cultivation and bag cultivation, sometimes also called synthetic logs. Bag cultivation has more advantages, such as the use of more substrate, a strong body and convenient manipulation, so it is more widely used. Both production processes include the following main steps: raw material preparation, mixing, bagging (bottling) and sterilization, inoculation, spawning, embedding in soil (or transfer to mushroom house), fruiting body development, management and harvesting [94].

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Figure 3: Farming production of *Ganoderma lucidum* fruit boddies (Photo A.Gregori)

For a production of *G. lucidum* fruit bodies supplemented sawdust is performed in heat-resistant polypropylene bottles or bags. Sawdust can be supplemented with rice bran (10%) and $CaCO_3$ (3%), moistened with water and filled (700 g) into plastic bags. A plastic collar is then fitted onto each bag and stopped with a cotton plug. After 5h of heat treatment (95-100°C) and cooling, substrate is inoculated with grain or sawdust spawn, and incubated for 3 to 4 weeks (or until the spawn fully colonizes the substrate) [95].

b) Solid State Cultivation

Solid state cultivation (SSC) is a three-phase heterogeneous process taking place in various bioreactors, comprising solid, liquid and gaseous phases, which offers potential benefits for the microbial cultivation for bioprocesses and products development.

Microbial growth on solid state substrate particles is very close to the growth of fungi in the natural environment. Main source of water, carbohydrates, phosphorous, nitrogen and sulphur are intrapartically bounded, therefore the microbial culture applied have to posses the abilities to access the water and essential element sources out the solid matrix Concerning that on the tips of young growing hiphae fungal polysaccharides are produced. Polysaccharide gel has actually two functions. Primary it serve as a gel media where from lignocellulitic enzymes are secreted in the solid wooden matrix and secondary as a sticky material used for anchoraging of hyphae and for moving on the solid surface. Produced fungal polysaccharides have also a whole palette of their pharmaceutical activities that are used in traditional Eastern medicine already for centuries [96].

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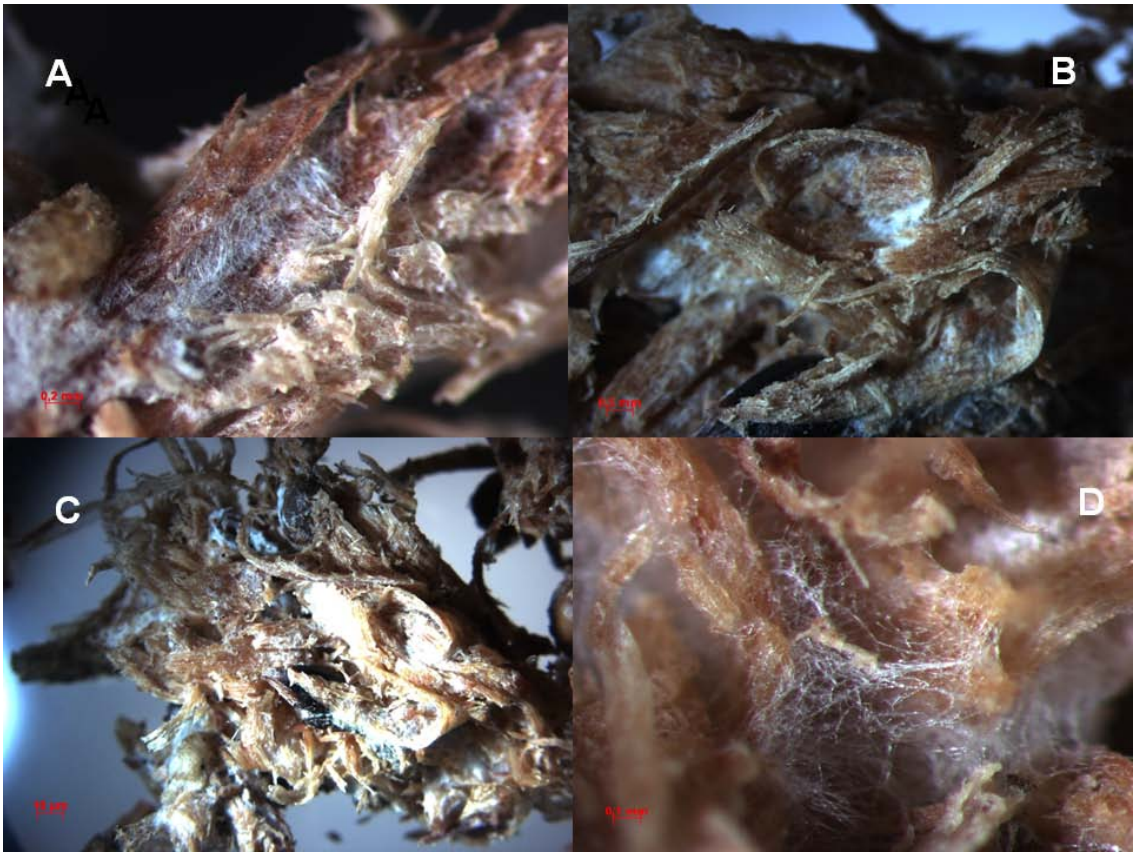

Figure 4: Growth of *Hericium erinaceus* on solid substrate 124 h (D), 145 h (A), 234h (B) and 280 h (C), (Photo M. Vittori)

SSC in bioreactors involves the growth and metabolism of microorganisms in fully control environment. Mycrobial growth in takes place in aerated beds of moist solid materials in which the interparticle spaces contain a continuous gas phase and little or no free water. The upper limit of moisture content for solid state cultivations is determined by the absorbancy of the solid, which varies between substrates, although for most substrates a free water becomes apparent before 80% moisture level is reached. Although fungal mycelia growth in SSC is very close to the growth in nature habitats fungal fruit boddies are not produced in this technology [93]. An example of SSC mycelia growth in presented in Figure 4.

Over the last two decades, SSC has gained significant attention for the development of industrial bioprocesses, particularly due to lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination.

An important advantage of solid state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as wood and agricultural secondary residue with little pretreatment or enrichment. For this reason, solid state cultivation seems to be most appropriate for the production of pharmaceutically active animal feed supplements, for which the whole fermented substrate can be used as the product [97]. Results of solid state medicinal mushroom cultivation on various substrates are presented in Table 1.

Table 1: Solid state cultivation of various medicinal mushroom in 15 L horizontal stirred tank bioreactor (HSTR). Own results

Fungus	Substrate	Biomass (mg/g)	Intracellular IPS (mg/g)
*Ganoderma lucidum**	Beech saw-dust	68	7.45
Grifola frondosa	Beech saw-dust	54	4.70
Trametes versicolor	Corn straw	83.5	5.95
Hericium erinaceus	Husked and paddy millet	350	3.07
Cordyceps militaris	Husked paddy millet+ rice	236	10.42

**European strain MZKI G93*

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An important advantage of solid state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as an agricultural residue with little pretreatment or enrichment. On the other hand, the use of an undefined medium, such as sawdust, might make the product purification more difficult. For this reason, solid state cultivation seems to be most appropriate for the production of pharmaceutically active animal feed supplements, for which the whole fermented substrate can be used as the product [98].

In recent years, substantial credibility in employing solid state cultivation (SSC) technique has been witnessed owing to its numerous advantages over submerged bioprocessing (SC). In spite of enormous advantages, true potential of SSC technology has not yet been fully realized at industrial scale [96].

c) Submeged Cultivation

Submerged cultivation of mushrooms represent the best and the fastest technology for a large scale production of medicinal mushroom mycelia and their products for a human use. In recent years, its submerged cultivation has received great interest in Asian countries as a promising alternative for efficient production of medicinal mushroom mycelia and its valuable metabolites [99].

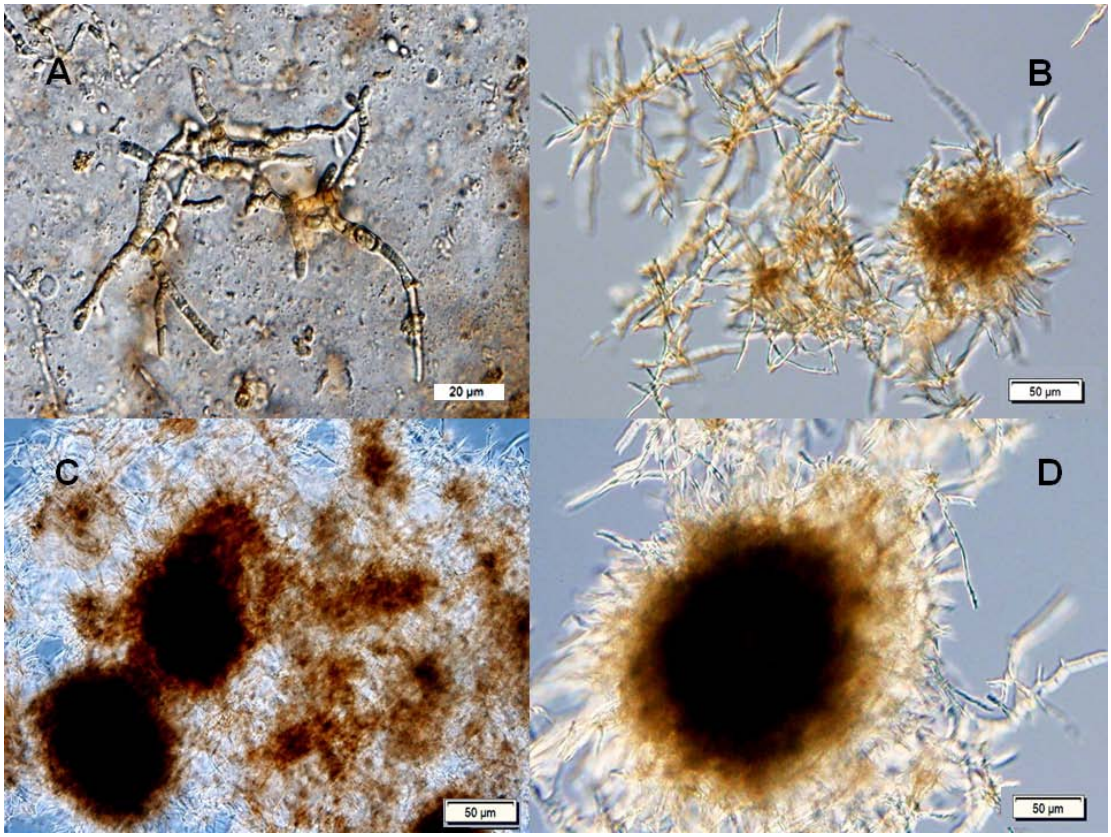

Figure 5: Growth of *Hericium erinaceus* in submerged cultivation. (A) fungal mycelia (14 h)(magnification 1000x); (B) mycelia clumps (218 h) (400x), (C) mycelia pellets (262 h) (100x), (D) mycelia pellets (346 h) (100x) (Photo M.Vittori)

Mycelial growth and the results of submerged medicinal mushroom cultivation of five species on various substrates are presented in Figure 5 and Table 2.

The problems in submerged cultivation of fungal biomass increase with increase the broth viscosity during cultivation because of changes in the morphology, fungal biomass and extracellular polysaccharide (EPS) production. Therefore one of the most important factors of large-scale submerged cultures in bioreactors is related to the heath and mass transfer liquid phase oxygen supply. It is necessary to characterize the variations that occur during the submerged cultivation in bioreactors and their effects on growth and product formation [100,101].

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Table 2: Results of submerged cultivation of five species of medicinal mushrooms in bioreactors

Species	Substrate	Bioreactor	Products	
Grifola frondosa	Optimized medium for mycelial biomass: 45.2 gL^{-1} glucose, 2.97 gL^{-1} KH_2PO_4, 6.58 gL^{-1} peptone Optimized medium for extracellular polysaccharides 58.6 gL^{-1} glucose, 4.06 gL^{-1} KH_2PO_4 3.79 gL^{-1}peptone	15 L STR bioreactor innoculum 10% (v/v), T 25 °C, initial pH 5.5, aeration rate 8.0 vvm, agitation speed 80 rpm	Biomass 22.50 gL^{-1} Extracellular polysaccarhides 1.32 gL^{-1}	[103]
Ganoderma lucidum	Potato dextrose 101.2 gL^{-1} glucose 2 % olive oil	10 L STR bioreactor innoculum 10% (v/v) T 30 °C, initial pH 5.8, aeration rate 1.0 vvm, agitation speed 300 rpm	Biomass 15.9 gL^{-1} Extracellular polysaccarhides 9.6 gL^{-1} Intracellular polysaccarhides 6.3 gL^{-1}	[104]
Hericium erinaceus	30 gL^{-1} corn flour 10 gL^{-1} glucose 3.0 gL^{-1} yeast extract 1.0 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $CaCO_3$ 15 mL of corn steep liquor	15 L STR bioreactor innoculum 10% (v/v), T 28 °C initial pH 5.7 aeration rate 0.8 vvm, agitation speed 80 rpm	Biomass 20.5 gL^{-1} Extracellular polysaccarhides 4.25 gL^{-1}	[105]
Trametes versicolor	35 gL^{-1} glucose 0.5 gL^{-1} yeast extract, 5.0 gL^{-1} pepton 1.0 gL^{-1} KH_2PO_4 0.5 gL^{-1} $MgSO_4 \times 7 H_2O$ 0,05 gL^{-1} tiamin	10 L STR inoculum 10% (v/v) , T 28 °C initial pH 5.7 aeration rate 1.0 vvm, agitation speed 400 rpm	Biomass 18,5 gL^{-1} Extracellular polysaccharide 3.8 gL^{-1}	[106]
Cordyceps militaris	80 gL^{-1} glucose, 10 gL^{-1} yeast extract, 0.5 gL^{-1} $MgSO_4 \cdot 7H_2O$ 0.5 gL^{-1} KH_2PO_4	5 L STR bioreactor T 24 °C, pH 5.8, agitation 200 rpm, aeration 1.5 vvm,	Biomass 40.60 gL^{-1} Extracellular polysaccharide 6.74 gL^{-1}	[107]

d) Submerged Cultivation of the other Species in Bioreactors

Besides the cultures present in this chapter, there are also some other medicinal mushrooms that were submerge cultivated in mostly in lab scale bioreactors.

Inonotus levis and *Agaricus nevoi* cultivation was proceed in a 10 L stirred-tank bioreactor using substrate based on glucose and corn steep liquor at pH 5.5. Agitation speed gradually increased from 50 to 300 rpm and T 28 °C. *I.levis* developed very rapidly and after 5 days of cultivation the culture reached the stationary phase of growth with a high level of mycelia biomass of 16 g/L at level of EPS concentration 4.2 g/L. *A. nevoi* was distinguished by a much lower growth rate and entered the stationary growth phase on day 10 with mushroom biomass 12 g/L and EPS of 3.9 g/L [102].

Submerged cultivation of *Agaricus brasiliensis* was studied in 1 L stirred tank reactor. Sucrose was found to be most effective for EPS production. Yeast extract was the best for EPS among the inorganic and organic nitrogen sources tested. The factorial experiment demonstrated that a temperature of 30 °C and a pH of 6.1 were the best for the EPS production. Glucose 10 g/L, yeast extract 3 g/L , K_2HPO_4 0.6 g/L and $MgSO_4$ 0.3 g/L [108].

Armillaria mellea was cultivated on glucose 40 g/L and yeast extract based substrate in 5 L stirred tank reactor at 22°C; the two-stage aeration rate strategy (1.2→0.6 vvm); 150 rpm, controlled pH 4.0, 6.65 g/L fungal biomass and 233.2 m g/L of extracellular polysaccharides with antioxidant properties were obtained [109].

Pleurotus pulmonarius was studied in submerged cultivation in a 2 L stirred-tank reactor. Substrate was composed by 20 g/L of brown sugar, 4 g/L rice bran, 4 g/L malt extract, and 4 g/L of yeast extract with initial pH of 5.5 Incubated at 28◦C with agitation speed of 250 rpm and oxygen partial pressure of 30–40%. Maximum *P. pulmonarius* dry biomass production of 11.8 g/L was achieved after 3 days of cultivation [110].

Pleurotus saca was submerged cultivated on substrate consist of beer worth substrate (batch mode in 10-L stirred tank reactor. Agitation speed was 500 rpm and aeration 5 $Lmin^{-1}$ and pH 6.2, up to 48.5 g/L of dry biomass was obtained [111].

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Pleurotus ostreatus was cultivated in a 20 L stirred tank bioreactor in a submerged process with enhanced glucan and dietary fibres content, using 57 g/L xylose and 37 g/L corn steep liquor. High yields 39.2 g/L of dry biomass was obtained [112,113].

Ganoderma lingzhi were studied in 5 L stirred bioreactor. The optimum conditions were an initial pH of 5.9, 20.0% DO and T 29° C. These conditions resulted in a triterpene acids (TA) of 0.31 g/L. Furthermore this optimized conditions were then successfully scaled up to a production scale of 200 L, and maximum TA production and productivity of 0.29 g/L and 0.05 g/L day^{-1} were achieved. [114].

e) *Differences between Solid State and Submerged Cultivation*

Main difference and benefits of solid state and submerged medicinal mushroom cultivation are presented in Table 3.

Table 3: Comparison of solid-state and submerged cultivation [96]

Solid state cultivation	Submerged cultivation
Some products can only be produced well under low moisture conditions. For other products, if the producing organisms require free water, solid state cultivation cannot be used.	A wide range of products can be produced, from a wide range of micoorganisms and fungi. Many products are produced best under submerged cultivation.
The medium is relatively simple (eg. grain) and unrefined. It may contain all nutrients necessary for growth, or only require wetting with a mineral solution. Pretreatment can be as simple as cooking or grinding. However, the substrate composition and characteristics can be variable.	The medium often contains more highly processed ingredients and is therefore more expensive. Unprocessed ingredients may need processing to extract and solubilize the nutrients. With defined media good reproducibility is possible
The low water availability helps to select and protect against growth of contaminants.	The water activity is usually very high and many contaminants can grow well.
Media are concentrated and smaller bioreactors can be used, leading to higher volumetric productivities, even when growth rates and yields are lower.	Media are dilute and therefore occupy larger volumes, leading to lower volumetric productivities.
High substrate concentrations can enable high product concentrations.	High substrate concentrations can cause rheological problems. Substrate feeding systems may be required.
Aeration requires less power since pressures are lower. Gas transfer is easier since the particles have a large surface area.	High air pressures can be required. Gas transfer from the gas to liquid phase is slow and can be limiting.
Mixing within particles is not possible, and growth can be limited by the diffusion of nutrients.	Vigorous mixing can be used, and diffusion of nutrients is usually not limiting.
Ability to remove metabolic heat is restricted, leading to overheating problems.	High water content and more dilute nature makes temperature control easier.
Process control can be difficult due to difficulties in making on-line measurements, and in measuring biomass. The addition of substances during the process is difficult.	Many on-line sensors are available and more are being developed. Additions of substances can be made to control the process.
Downstream processing may be simpler since products are more concentrated. However, extracts can be contaminated with substrate components.	Downstream processing requires removal of large volumes of water, and is more expensive. However, with defined media, product purification may be easier.
Liquid waste is not produced.	Usually large volumes of liquid wastes are produced.
Growth kinetics and transport phenomena have received little attention and are poorly characterized.	Much kinetic and transport information is available in literature, which can guide reactor design and operation.
Research results and information from the solid-state cultivation can be scaled-up, or even transferred and applied in liquid-state cultivation.	In scaling up fungal submerged cultivation processes, various technical problems need to be solved, such as an increased broth viscosity and oxygen supply.
Solid-state cultivation of fungal mycelia is less labor intensive.	Submerged cultivation is more demanding and labor intensive.

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V. Down Stream Processing

Disruption of medicinal mushrooms by mechanical, chemical or enzymatic methods is greatly required for the efficient extraction of active compounds from them. In addition, ultrafine powder of medicinal mushroom by mechanical method can be used for functional food or dietary supplement. Other products used for pharmaceuticals are produced at the stages of extraction, fractionation and purification by varying techniques including microwave assisted extraction, membrane separation, adsorptive separation and chromatograpy. Recrystallization, lyophilisation, drying and formulation are used as final product treatments [115].

Current disruption methods can be classified into mechanical, chemical and enzymatic methods in terms of their principles and characteristics. Mechanical methods are often preferred due to short residence time and lower operating costs [116]. The most common mechanical means for disruption are bead mill and homogenizer [117]. Another most frequently used sample disruption method is air jet milling which uses high velocity jets of gas to impart energy to particles for size reduction. The main features of air jet mills include [118].

Extraction is the first step to separate the desired products from the raw materials. Nearly 80–85 % of all medicinal mushroom products are extracted from their fruiting bodies while only 15 % are derived from mycelium culture [119].

Solvent is one of the most important parameters for a successful extraction. Selectivity, solubility, cost and safety should be considered in selection of solvents. Alcohols (ethanol and methanol) are universal solvents for the extraction of natural products although their low selectivity.

In recent years, advanced and greener extraction methods such as supercritical fuid extraction, pressurized liquid extraction, ultrasound assisted extraction, microwave assisted extraction, pulsed electric field extraction and enzyme assisted extraction have also been applied for extraction of natural products, and they offer some advantages such as lower organic solvent consumption, shorter extraction time and higher selectivity. In particular, supercritical fluid extraction gains increasing attention due to its higher efficiency and greener characteristics.

A brief summary of the various extraction methods used for medicinal mushroom products is shown in Table 4 [120].

Table 4: A brief summary of various extraction method used for medicinal mushroom product adapted from [120]

Applied Method	Solvent	Volume of consumed solvent	Temperature	Time	Pressure	Polarity of extracted products
Maceration	Various solvent	Large	Room temperature	Atmospheric	Long	Dependent on the solvent
Percolation	Various solvent	Large	Room temperature, sometimes under heat	Atmospheric	Long	Dependent on the solvent
Decoration	Water	None	Under heat	Atmospheric	Moderate	Polar compounds
Reflux extraction	Various solvent	Moderate	Under heat	Atmospheric	Moderate	Dependent on the solvent
Soxhlet extraction	Organic solvent	Moderate	Under heat	Atmospheric	Long	Dependent on the solvent
Pressurized liquid extraction	Organic solvent	Small	Under heat	High	Short	Dependent on the solvent
Supercritical fluid extraction	Supercritical fluid (usually CO_2)	None or small	Near room temperature	High	Short	None polar or moderate polar
Ultrasound assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Compounds dependent on the solvent
Microwave assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Dependent on the solvent
Pulsed electric field extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Dependent on the solvent

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Enzyme assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Moderate	Dependent on the solvent
Distillation	Water	Moderate	Under heat	Atmospheric	Long	Essential oil

The obtained fraction containing the desired products via fractionation steps described above may consist of several compounds with highly similar chemical and physical properties. Sometimes these compounds are analoges or even isomers as already stated, presenting a huge challenge for the purification. Column chromatography packed with separation media with small particle size is the most prevailing technique to implement the task, because it can offer very high number of theoretical plates and thus high resolution. However, in industrial scale, the particle size of the packed material is much larger because no pump can generate the pressure in manufacturing scale as that in a HPLC system [120].

VI. Conclusion

Various pharmaceutically active substances from medicinal mushrooms represent effective potential in human life. Great demand for medicinal fungi biomass production could be fulfilled using various cultivatiom techniques. Medicinal fungi biomass in a present time is mostly covered by farming. Farming cultivation represents cheap but long time consuming technology. Using cultivation on a wooden locks a few year coughing time is need. Cultivation on sawdust substrates in trays or beds and in sterilised plastic bags or in bottles represents an advance and much faster production of fungal fruit boddies than conventional farming.

Solid state a few week time cultivation is a comprehensive, well controlled technology that enables much faster medium scale technology for medicinal mushroom mycelia production. In this technology various secondary waste from wood and agriculture industry are successfully used. No fungal fruit boddies are produced. Final product delignolized, wooden material is overgrown by medicinal fungi biomass in two to four weeks, enreach with proteins and various pharmaceutically products need to be dryed and pulverized and in such form it could be directly used in a veterinary need. Solid state cultivation of medicinal mushroom biomass is cheap in non time consuming technology perfectlly suitable in veterinary use.

Submerged cultivation of medicinal fungi biomass represents fast and comprehensive technology method. Fungal biomass is in its final state from 10 to 28 days. The main benefit of cultivation of medicinal mushroom biomass in bioreactors is in using of higher sterility, comprehensive technology and bioprocess control, for large scale production of various pharmaceutically active compounds as are fungal polysaccharides, terpenoides or proteinoglucans in much shorter cultivation time. Reports on pharmacological activity of extracts, partly purified preparations and isolated compounds from biomass of *G.lucidum, G.frondosa, T.versicolor, H.erinaceus* and *C.militaris* and the other reported species in laboratory and pilot scale, are very convincing. Production of medicinal fungi polysaccharides enhanced by fed-batch or two-stage cultivation strategy was found very useful for improving the production. Some of present results of lab scale research of various medicinal fungi are already transferred to pilot and lower industrial scale and they represents suitable starting platform for development of medicinal fungi biomass in large scale pharmaceutical industrial production. Comparing the economy of the same product production solid state processing is 30 % less than those of the one produced in submerged cultivation.

Isolation of medicinal fungi pharmaceutically compounds in all three technologies is is based on precipitation with hot water and ethanol. Crude extracts often show equal or stronger pharmacological activity as purified compounds, which suggests potential synergistic effects of several naturally occurring compounds. In the future, more precise capture (or enrichment) and separation techniques such as affinity separation, and more integrated bioprocesses for medicinal mushroom products should be developed which would enable a higher product yield and better process performance.

As pointed out submerged cultivation of medicinal mushrooms has significant large scale industrial potential, but its success on a commercial scale depends on existing field-cultivation technology as well as pharmaceutical market economy. Production of various medicinal mushrooms products, terpenoids, polysaccharides and proteinoglucans represents great business in Asiatic space where it is traditional. Great demand of this active ingredients definitively need to include new and fast large scale industrial production technologies as are solid state and submerged cultivations. In opposite, Western Pharmaceutical Industry has no tradition in natural isolates from herbal and fungal sources. Unfortunately, it is too convinient and much basesed on classical pharmaco-chemical biosynthesis including its all side effects.

However, from the viewpoint of Western Science, pharmaceutical legislation and regulations might be one of the main obstacles hindering the introduction of medicinal mushrooms products as registered pharmaceuticals. In any case, further

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research is needed to fully understand all mechanisms of pharmaceutical effects of medicinal mushrooms products and to identify their potential side effects.

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Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for Writing a Good Quality Science Frontier Research Paper

Techniques for writing a good quality Science Frontier Research paper:

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of science frontier then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.



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6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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