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(TJAU)

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Araştırma Makalesi *Research Article* DOI: 10.62425/tjau.1693211

LANTANA CAMARA L.: A NATURAL TREASURE TROVE OF ANTIOXIDANTS AND ANTI-INFLAMMATORY AGENTS

LANTANA CAMARA L.: ANTİOKSİDANLAR VE ANTİ-İNFLAMATUAR AJANLARIN DOĞAL HAZİNESİ

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ABSTRACT

Lantana camara L. leaves were extracted using solvents of differing polarity—petroleum ether, acetone, and chloroform—to investigate their phytochemical composition, functional groups, and antioxidant potential. Preliminary phytochemical screening revealed a rich presence of bioactive metabolites such as flavonoids, phenolics, alkaloids, tannins, terpenoids, glycosides, and steroids predominantly in acetone and chloroform extracts, while petroleum ether showed limited polarity-associated compounds. Quantitative analysis demonstrated significantly higher total phenolic (54.86 mg GAE/g) and flavonoid content (39.44 mg QE/g) in acetone extracts compared to petroleum ether (8.72 mg GAE/g and 5.31 mg QE/g) and chloroform (28.17 mg GAE/g and 19.25 mg QE/g). Antioxidant assays corroborated these findings: acetone extract exhibited the strongest DPPH radical scavenging activity (IC_{50} = 41.25 µg/mL), highest ferric reducing antioxidant power (429.55 µmol Trolox equivalents/g), and ABTS inhibition (88.36%). Fourier Transform Infrared (FTIR) spectroscopy confirmed the presence of key functional groups, including hydroxyl, carbonyl, and aromatic moieties, varying with solvent polarity. These results highlight acetone as the most effective solvent for extracting antioxidant-rich phytochemicals from L. camara leaves, emphasizing the plant's potential as a natural source of therapeutic antioxidants for pharmaceutical and nutraceutical applications.

Keywords: *Lantana camara*, Bioactive compounds, Antioxidant activity, FTIR

ÖZ

Lantana camara L. yaprakları, fitokimyasal bileşimlerini, fonksiyonel gruplarını ve antioksidan potansiyellerini araştırmak için farklı polariteye sahip çözücüler (petrol eteri, aseton ve kloroform) kullanılarak ekstrakt edildi. Ön fitokimyasal tarama, aseton ve kloroform özütlerinde baskın olarak flavonoidler, fenolikler, alkaloidler, tanenler, terpenoidler, glikozitler ve steroidler gibi biyoaktif metabolitlerin zengin bir varlığını ortaya koyarken, petrol eteri sınırlı polariteyle ilişkili bileşikler gösterdi. Kantitatif analiz, aseton özütlerinde petrol eteri (8,72 mg GAE/g ve 5,31 mg QE/g) ve kloroform (28,17 mg GAE/g ve 19,25 mg QE/g) ile karşılaştırıldığında önemli ölçüde daha yüksek toplam fenolik (54,86 mg GAE/g) ve flavonoid içeriği (39,44 mg QE/g) gösterdi. Antioksidan analizleri bu bulguları doğruladı: aseton özütü en güçlü DPPH radikal temizleme aktivitesini (IC_{50} = 41,25 µg/mL), en yüksek ferrik indirgeyici antioksidan gücünü (429,55 µmol Trolox eşdeğeri/g) ve ABTS inhibisyonunu (%88,36) gösterdi. Fourier Dönüşümlü Kızılötesi (FTIR) spektroskopisi, çözücü polaritesine göre değişen hidroksil, karbonil ve aromatik gruplar dahil olmak üzere temel fonksiyonel grupların varlığını doğruladı. Bu sonuçlar, asetonu L. camara yapraklarından antioksidan açısından zengin fitokimyasalları çıkarmak için en etkili çözücü olarak vurgulayarak, bitkinin farmasötik ve nutrasötik uygulamalar için doğal bir terapötik antioksidan kaynağı olarak potansiyelini vurguladı.

Anahtar Kelimeler: *Lantana camara*, Biyoaktif bileşikler, Antioksidan aktivite, FTIR

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Introduction

Lantana camara, commonly known as lantana, is a perennial shrub native to tropical regions and recognized for its vibrant flowers and potential medicinal properties (Bhowmik et al., 2013). Historically, various parts of the plant have been employed in traditional medicine to treat ailments ranging from fevers and skin diseases to respiratory conditions (Chandra et al., 2016). Recent studies have highlighted the importance of phytochemicals, such as flavonoids and phenolic compounds, which contribute to the plant's therapeutic effects (Kumar et al., 2021). The rising interest in natural antioxidants and anti-inflammatory agents stems from their potential to combat oxidative stress and chronic inflammation, both of which are linked to numerous health issues, including cardiovascular diseases, diabetes, and cancer (Nita et al., 2020). This underscores the necessity to scientifically validate the traditional uses of Lantana camara by exploring its phytochemical profile and assessing its biological activities. Understanding these properties may provide insights into developing natural health products and pharmaceuticals derived from this plant.

Methods

Plant Collection and Authentication

Fresh leaves of Lantana camara L. were collected from areas in and around the vicinity of Hindusthan College of Arts & Science, Coimbatore, Tamil Nadu, India. The plant material was authenticated by a qualified Botanist at Tamil Nadu Agricultural University. After authentication, leaves were washed with distilled water to remove dust and debris, air-dried at ambient room temperature (25±2°C) for 7–10 days, and ground into a fine powder using a mechanical grinder. The powdered material was stored in airtight containers at room temperature until further analysis (Bhuvaneshwari et al., 2017).

Solvent Extraction Procedure

Bioactive compounds were extracted from the dried leaf powder using Soxhlet extraction with petroleum ether, acetone, and chloroform. Each solvent was used in triplicate extractions to ensure reproducibility. For each run, 20 g of powdered leaf material was placed in a thimble and extracted with 250 mL of the respective solvent for 6 hours. The extraction was conducted under the boiling point range specific to each solvent (petroleum ether: 40–60°C; acetone: ~56°C; chloroform: ~61°C). Post-extraction, the solvents were removed using a rotary evaporator under reduced pressure at 45°C. Residual moisture was eliminated by drying the concentrates in a hot air oven at 40°C to a constant weight. Dried extracts were stored in airtight containers at 4°C for further use (Hossain et al., 2016).

FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was performed to identify the functional groups present in the extracts. Solid extracts (1–2 mg) were mixed with 100 mg of spectroscopic-grade potassium bromide (KBr) and compressed into pellets. Liquid extracts were applied to an ATR (Attenuated Total Reflectance) crystal for direct measurement. Spectra were recorded in the range of 4000–400 cm^{-1} using an FTIR spectrometer calibrated with a background scan to correct for atmospheric interferences. The spectra were analyzed for characteristic absorption bands to infer the presence of functional groups such as O–H, C=O, C–H, and C–O (Mason et al., 2019).

Quantification of Phytochemicals

Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu method (Singleton et al., 1999). A calibration curve was established using gallic acid (10–100 µg/mL). To 1 mL of each standard or sample, 1 mL of Folin-Ciocalteu reagent and 7 mL of distilled water were added. After a 5-minute reaction period, 1 mL of 7.5% sodium carbonate was added. The mixture was incubated at room temperature for 30 minutes, and absorbance was recorded at 765 nm using a UV-Vis spectrophotometer. Results were expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g).

Total Flavonoid Content (TFC)

TFC was estimated using the aluminum chloride colorimetric method (Jiang et al., 2011). A standard curve was generated using quercetin (10–100 µg/mL). One mL of standard or sample was mixed with 0.5 mL of 5% sodium nitrite and incubated for 5 minutes. Subsequently, 0.5 mL of 2% aluminum chloride was added, followed by 1 mL of 1 M sodium hydroxide and 2.5 mL of distilled water. The absorbance was measured at 415 nm. Results were expressed as mg quercetin equivalents per gram of extract (mg QE/g).

Antioxidant Activity Assays

DPPH Radical Scavenging Activity

The DPPH assay was performed as described by Brand-Williams et al. (1995). A 0.1 mM DPPH solution in methanol was prepared and protected from light. Extract samples at various concentrations were mixed with DPPH solution and incubated in the dark for 30 minutes. The absorbance was recorded at 517 nm. Antioxidant activity was expressed as the percentage of DPPH radical inhibition.

FRAP (Ferric Reducing Antioxidant Power)

The FRAP assay was conducted as per Benzie & Strain (1996). FRAP reagent was freshly prepared from acetate buffer, TPTZ, and ferric chloride. Plant extract (100 µL) was mixed with 3 mL of FRAP reagent and incubated at 37°C for 30 minutes. Absorbance was measured at 593 nm. Results were expressed in µmol Trolox equivalents.

ABTS Radical Cation Decolorization Assay

The ABTS assay was performed according to Re et al. (1999). ABTS radical cations were generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating in the dark for 12–16 hours. The solution was diluted to an absorbance of 0.7 at 734 nm. Extracts were mixed with the ABTS solution and incubated for 6 minutes, followed by absorbance measurement at 734 nm. Antioxidant activity was calculated as the percentage inhibition.

Statistical Analysis

All experiments were performed in triplicate. Results were reported as mean ± standard deviation (SD). One-way Analysis of Variance (ANOVA) was used to determine statistical significance among extract groups, with p-values < .05 considered significant (Duncan, 1955; McHugh & Krieger, 2005).

Results and Discussion

This study systematically investigated the extraction efficiency, phytochemical composition, and antioxidant properties of Lantana camara leaf extracts using solvents of varying polarity: petroleum ether, acetone, and chloroform. The comparative evaluation provides critical insights into solvent-specific performance in isolating pharmacologically relevant secondary metabolites and establishes a basis for optimized extraction strategies in phytotherapeutic research.

Solvent extraction efficiency

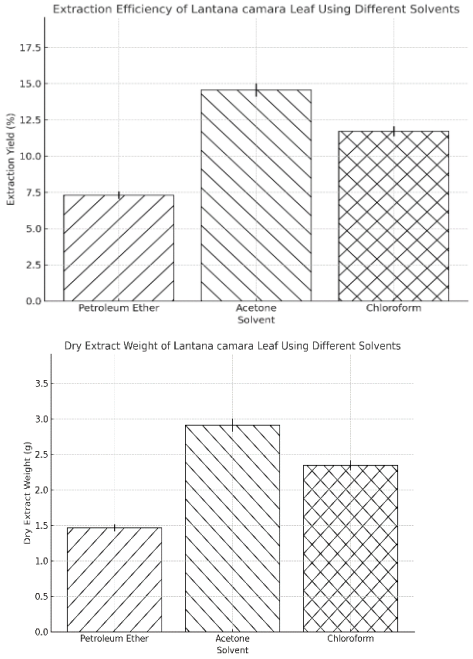

Figure 1. Extraction efficiency and dry extract weight of Lantana camara leaf using petroleum ether, acetone, and chloroform as solvents. Significant differences among groups were determined using one-way ANOVA ($p < 0.05$). Results are expressed as mean ± standard deviation ($n = 3$).

The solvent extraction efficiency (Figure 1) for Lantana camara leaves was evaluated by comparing the dry extract weight obtained from a fixed input of 20 g powdered leaf material for each solvent. The acetone extract provided the highest dry extract weight at 2.91 g, resulting in a proportional extraction yield of 14.55%. This was followed by chloroform with 2.34 g (yield 11.70%) and petroleum ether with 1.46 g (yield 7.30%). The proportional yield reflects the efficiency of each solvent in recovering extractable phytochemicals relative to the initial plant material. Acetone's superior extraction yield can be attributed to its intermediate polarity, allowing it to dissolve a broader spectrum of phytochemicals, including both polar and non-polar compounds (Do et al., 2014). Chloroform also demonstrated considerable efficacy, especially for moderately polar bioactives. Petroleum ether, a non-polar solvent, showed the lowest extraction yield, consistent with its selectivity for lipophilic constituents such as fats and waxes. This gradient in solvent extraction efficiency aligns well with the chemical nature of the bioactive compounds targeted and underscores the

importance of solvent choice in maximizing extraction output for phytochemical studies.

Preliminary Phytochemical Screening

Preliminary qualitative phytochemical analysis revealed distinct profiles across petroleum ether, acetone, and chloroform extracts of Lantana camara leaves, reflecting the differential solubility of phytochemicals in solvents of varying polarity (Figure 2). Alkaloids, which are known for their pharmacological activities including analgesic and antimalarial effects, were absent in the petroleum ether extract but detected in both acetone and chloroform extracts. The presence of alkaloids in these polar and mid-polar solvents indicates their higher solubility in such media, consistent with previous findings (Singh et al., 2013). Flavonoids and phenolics—classes of compounds widely reported for their antioxidant and anti-inflammatory activities—were notably more abundant in the acetone extract (++), with moderate presence in the chloroform extract (+) and absence in the petroleum ether extract (–). These results suggest that acetone is more efficient in extracting polar phenolic constituents from L. camara, in agreement with earlier reports that employed similar solvents for polyphenol extraction (Kaur & Arora, 2009).

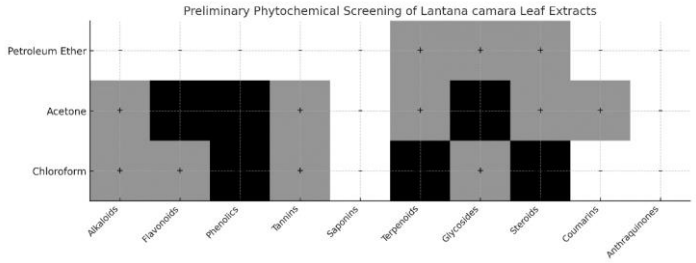

Figure 2: Preliminary phytochemical screening of Lantana camara leaf extracts using petroleum ether, acetone, and chloroform. (Symbolic representation: "–" = Absent, "+" = Present (low), "++" = Strongly Present (high). Data is encoded as a heatmap based on presence intensity)

Tannins, another group of phenolic compounds with astringent and antimicrobial properties, were moderately present in acetone and chloroform extracts, while absent in the petroleum ether extract. Their detection further supports the presence of phenolic-rich fractions in polar solvents. Saponins were not detected in any of the extracts, which might reflect either their absence in the leaf tissue or their insolubility in the selected solvents. Since saponins are typically extracted using aqueous or methanolic solvents, their absence here is not unexpected (Hostettmann & Marston, 1995). Terpenoids and steroids were observed in all three extracts, with strong presence (++ for chloroform) indicating that mid-polar solvents are particularly effective for triterpenoid extraction. These non-polar to mid-polar compounds are known to contribute to various bioactivities such as anti-inflammatory and cytotoxic effects (Jassim & Naji, 2003). Their abundance in the chloroform extract suggests it may contain significant lipophilic secondary metabolites. Glycosides, which are often associated with cardiotonic and antimicrobial functions, were most pronounced in the acetone extract (++), and also detected in petroleum ether and chloroform extracts (+). The broad solubility of glycosides across solvents highlights the diversity of glycosidic compounds within L. camara. Coumarins were detected only in the acetone extract, which may be due to their polar nature and affinity for medium-polar solvents. Coumarins possess anticoagulant and antimicrobial properties, and their presence supports the therapeutic potential of L. camara. Anthraquinones were not detected in any of the extracts. This suggests that such compounds may not be prevalent in the leaf tissues of L. camara or may require alternative solvents for extraction, such as ethanol or strong acid/base hydrolysis, commonly used for anthraquinone liberation. In summary, acetone emerged as the most efficient solvent for extracting a broad spectrum of phytochemicals from L. camara leaves, especially phenolics, flavonoids, glycosides, and coumarins. Chloroform was effective in extracting terpenoids, steroids, and alkaloids, whereas petroleum ether showed limited efficacy, extracting primarily non-polar compounds such as steroids and terpenoids. These findings are consistent with previous reports emphasizing solvent polarity as a key factor in phytochemical extraction efficiency (Harborne, 1998; Sasidharan et al., 2011).

This screening provides a foundational understanding of the phytochemical landscape of L. camara, informing solvent selection for targeted isolation of bioactive compounds in future studies. The predominance of antioxidant-associated groups such as flavonoids and phenolics in polar extracts correlates with their anticipated antioxidant potential, which is further explored through in vitro antioxidant assays in subsequent sections.

Total Phenolic and Flavonoid Content

The total phenolic content (TPC) and total flavonoid content (TFC) of the petroleum ether, acetone, and

chloroform extracts of Lantana camara leaves were quantified using the Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. The results are presented in Figure 3.

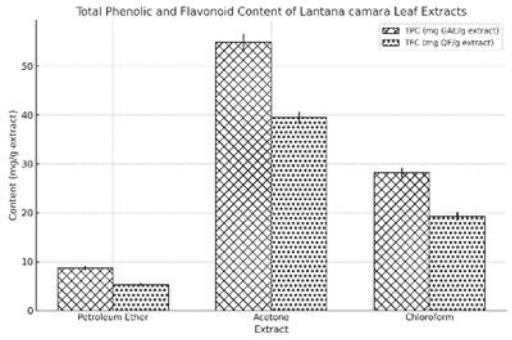

Figure 3: Grouped bar chart showing Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in Lantana camara leaf extracts using petroleum ether, acetone, and chloroform. (Values are represented as mean ± standard deviation (n=3). Significant differences among extract types were assessed using one-way ANOVA ($p < .05$) and Duncan's multiple range test)

The highest TPC and TFC values were recorded in the acetone extract, followed by chloroform, with petroleum ether showing the lowest levels. This trend reflects the influence of solvent polarity on the extraction efficiency of phenolic and flavonoid compounds. Acetone, being a polar aprotic solvent, is highly effective at dissolving phenolic hydroxyl groups and conjugated systems typically found in flavonoid structures (Do et al., 2014). Phenolic compounds are major contributors to the antioxidant potential of plant extracts due to their ability to donate hydrogen atoms or electrons to free radicals. The high phenolic content in the acetone extract (54.86 ± 1.72 mg GAE/g) suggests a significant antioxidant reservoir, correlating with the strong radical scavenging observed in subsequent DPPH and FRAP assays. The moderate TPC in chloroform extract (28.17 ± 1.03 mg GAE/g) indicates the presence of less polar phenolic constituents, while the low TPC in petroleum ether extract (8.72 ± 0.45 mg GAE/g) is attributed to its limited solubilizing ability for polar compounds. Similarly, flavonoids—recognized for their free radical scavenging and metal-chelating properties—were found in highest concentration in the acetone extract (39.44 ± 1.26 mg QE/g), moderate in the chloroform extract (19.25 ± 0.88 mg QE/g), and lowest in the petroleum ether extract (5.31 ± 0.39 mg QE/g). These findings align with studies suggesting that mid-to-high polarity solvents such as methanol, ethanol, and acetone are optimal for flavonoid extraction (Panche et al., 2016).

The elevated levels of phenolics and flavonoids in the acetone extract strongly support its use in further pharmacological investigations, particularly those exploring antioxidant, anti-inflammatory, and antimicrobial potentials. These secondary metabolites are known to interact synergistically, enhancing biological activity beyond the effects of individual compounds (Rice-Evans et al., 1997).

Antioxidant Activity

The antioxidant potential of Lantana camara leaf extracts were evaluated using three complementary in vitro assays: DPPH radical scavenging activity, FRAP (Ferric Reducing Antioxidant Power), and ABTS radical cation Decolorization as illustrate in Figure 4. These assays assess the extracts' capacity to neutralize free radicals or reduce oxidants, which collectively reflects their potential for mitigating oxidative stress.

DPPH Radical Scavenging Activity

The DPPH assay evaluates the ability of antioxidants to donate hydrogen atoms or electrons to the stable DPPH radical, resulting in a color change measurable at 517 nm. The acetone extract exhibited the highest DPPH radical scavenging activity with an IC_{50} value of 41.25 ± 1.03 µg/mL, followed by chloroform (76.43 ± 1.51 µg/mL) and petroleum ether (122.64 ± 2.06 µg/mL) extracts. These results align with the total phenolic and flavonoid content, reinforcing the role of phenolic compounds as primary radical scavengers. The lower IC_{50} value of the acetone extract suggests stronger antioxidant capacity, likely due to higher concentrations of hydroxylated phenolics capable of stabilizing free radicals through hydrogen donation (Brand-Williams et al., 1995).

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay measures the ability of an extract to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions in the presence of TPTZ, forming a blue-colored complex that absorbs at 593 nm. Among the tested extracts, the acetone extract displayed the strongest ferric reducing activity with a value of 429.55 ± 12.73 µmol Trolox equivalents/g, followed by chloroform (238.61 ± 9.64 µmol TE/g) and petroleum ether (105.47 ± 5.84 µmol TE/g). The trend observed in the FRAP assay is consistent with DPPH results, affirming the redox potential of phenolic-rich acetone extracts. These compounds can act as reducing agents, hydrogen donors,

and metal chelators (Benzie & Strain, 1996), which are critical mechanisms in combating oxidative stress.

ABTS Radical Cation Decolorization Assay

The ABTS assay evaluates both hydrophilic and lipophilic antioxidant capacities. $ABTS^{+}$ radicals are reduced upon interaction with antioxidants, with a decrease in absorbance at 734 nm. The acetone extract again demonstrated superior activity with 88.36 ± 1.92% inhibition, compared to 65.22 ± 1.68% for chloroform and 39.47 ± 1.41% for petroleum ether extracts at 100 µg/mL. The broad-spectrum sensitivity of ABTS to various antioxidant classes confirms the robust activity of the acetone extract and complements the observations from DPPH and FRAP assays. It also highlights the presence of both polar and non-polar antioxidant constituents (Re et al., 1999).

Comparative analysis and biological relevance: The consistent superiority of the acetone extract across all antioxidant assays underscores its efficiency in extracting a diverse range of antioxidant phytochemicals, notably flavonoids and phenolic acids. These molecules neutralize reactive oxygen species through multiple pathways, making them highly desirable for therapeutic development (Rice-Evans et al., 1997). In contrast, the petroleum ether extract, despite containing lipophilic constituents like terpenoids and steroids, exhibited comparatively lower antioxidant potential. This suggests that the bulk of antioxidative efficacy in L. camara leaves is attributable to polar compounds rather than non-polar ones. These findings align with earlier phytochemical and antioxidant studies on L. camara, reinforcing its potential as a source of natural antioxidants for pharmaceutical and nutraceutical applications (Mendoza-Castillo et al., 2017).

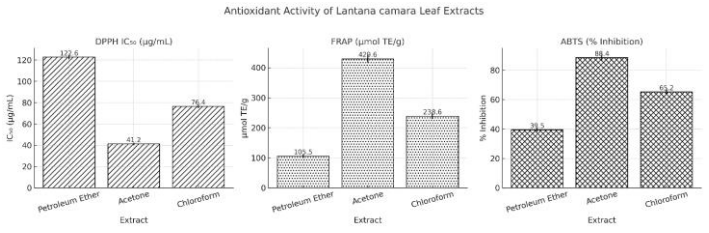

Figure 4: Comparative Antioxidant Activity of Lantana camara Leaf Extracts as Assessed by DPPH, FRAP, and ABTS Assays (Bar charts illustrate the antioxidant performance of petroleum ether, acetone, and chloroform extracts of Lantana camara leaves. (A) DPPH IC_{50} values reflect radical scavenging efficiency, where lower values denote stronger activity. (B) FRAP assay indicates reducing power, with higher values representing greater antioxidant potential. (C) ABTS assay shows percentage inhibition at 100 µg/mL, with higher values indicating stronger radical scavenging. Error bars represent standard deviation (n = 3). Statistical significance determined by one-way ANOVA ($p < .05$).

FTIR Spectroscopic characterization of Lantana camara leaf extracts

Fourier Transform Infrared (FTIR) spectroscopy was employed to identify major functional groups present in the petroleum ether, acetone, and chloroform extracts of Lantana camara leaves. The spectral profiles revealed the chemical diversity of the bioactive constituents extracted by solvents of differing polarities, thus offering insights into solvent–compound interactions and extract composition (Figures 5). All three extracts displayed a broad absorption band around 3400 cm^{-1}, indicating O–H stretching vibrations associated with hydroxyl groups found in alcohols and phenolics, or due to retained moisture. Prominent peaks at ~2920 cm^{-1} and ~2850 cm^{-1} were consistently observed across all samples, corresponding to asymmetric and symmetric C–H stretching vibrations, characteristic of long-chain aliphatic hydrocarbons and saturated compounds (Kumar et al., 2017). A strong peak near 1740–1745 cm^{-1} across the extracts was attributed to C=O stretching of carbonyl groups, indicating the presence of esters, aldehydes, or ketones—functional groups commonly found in lipids and flavonoid glycosides (Singh et al., 2018). The 1650–1400 cm^{-1} region showed signals corresponding to C=C stretching and CH_2/CH_3 bending, signifying both saturated and unsaturated hydrocarbons and suggesting the presence of aromatic systems or conjugated double bonds. In the 1250–1100 cm^{-1} region, C–O stretching vibrations indicated the presence of oxygenated compounds, such as esters, ethers, or phenolic glycosides. Notably, aromatic C–H out-of-plane bending vibrations were evident in the 950–750 cm^{-1} range, especially in the acetone and chloroform extracts, supporting the presence of polyphenolic or aromatic constituents (Ghosh et al., 2015). The petroleum ether extract, being non-polar, showed relatively intense bands related to lipids and long-chain hydrocarbons, with limited polar functional group signals. The acetone extract demonstrated strong O–H and C=O absorption, underscoring its efficiency in extracting phenolics and polar compounds. The chloroform extract, with intermediate

polarity, exhibited a wide array of functional groups and distinctive peaks below 700 cm^{-1} that could be attributed to C–Cl stretching vibrations, possibly from residual solvent, in addition to aromatic signals.

These results confirm that FTIR spectroscopy effectively highlights the chemical heterogeneity of Lantana camara leaf extracts and underscores the selective extraction capabilities of solvents with varying polarities. The spectral patterns reflect a complex matrix of phytochemicals including hydrocarbons, esters, phenolics, and possibly chlorinated residues, which may contribute to the observed bioactivity of the extracts.

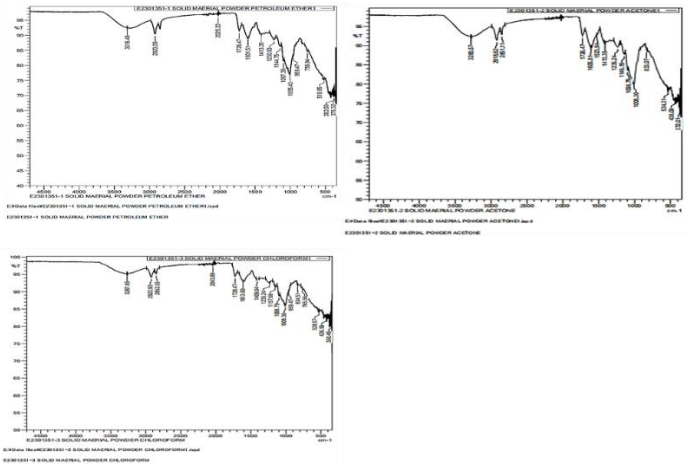

Figure 5. FTIR spectra of Lantana camara L. leaf extracts using petroleum ether, acetone, and chloroform as solvents. (The spectra exhibit characteristic absorption bands corresponding to functional groups such as O–H, C=O, C–H, and C=C, indicative of alcohols, phenolics, lipids, and hydrocarbons. Comparative analysis reveals solvent-specific variations in the chemical composition and functional group intensities of the extracts)

Conclusion

This study comprehensively evaluated the influence of solvent polarity on the extraction efficiency, phytochemical profile, and antioxidant activity of L. camara leaf extracts. Acetone, with its intermediate polarity, emerged as the superior solvent, yielding the highest total phenolic and flavonoid contents, which directly correlated with its potent antioxidant capacity demonstrated by DPPH, FRAP, and ABTS assays. Preliminary phytochemical screening and FTIR spectral analysis further supported the diverse and rich phytoconstituent profile in acetone extracts, including flavonoids, phenolics, and terpenoids, compounds known for their health-promoting bioactivities. Petroleum ether extracts, limited by its non-polar nature, extracted mainly lipophilic compounds with comparatively lower antioxidant potential. Chloroform extracts displayed moderate phytochemical richness and antioxidant capacity, consistent with its intermediate polarity. The study underscores the critical role of solvent selection in natural product extraction, providing a methodological framework for maximizing bioactive compound recovery. Given its robust antioxidant profile, L. camara leaf extract, especially when prepared with acetone, holds promising potential for development into natural antioxidant formulations. Future work should focus on isolation of individual bioactive compounds, mechanistic antioxidant studies, and in vivo validation to harness the therapeutic potential of this versatile plant.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this paper.

ETHICAL APPROVAL

This article does not contain any studies involving animals or human participants performed by any of the authors. Hence, ethical approval was not required.

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The author has read and approved the final version of the manuscript and consent to its publication

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In VIVO MODULATORY EFFECT of MALACHITE GREEN on CYTOCHROME P450 AND ANTIOXIDANT ENZYME ACTIVITIES in RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) LIVER

MALAHİT YEŞİLİNİN GÖKKUŞAĞI ALABALIĞI (*ONCORHYNCHUS MYKISS*) KARACİĞERİNDE SİTOKROM P450 VE ANTİOKSİDAN ENZİM AKTİVİTELERİ ÜZERİNE İN VİVO MODÜLATÖR ETKİSİ

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ABSTRACT

Malachite green is a mutagenic and carcinogenic chemical used as a dye in textile and paper industry and in fish farms against ectoparasites, bacterial and fungal infections. Aquatic organisms are at risk of malachite green exposure. In this study, our aim was to determine the effect of two different doses of malachite green on cytochrome P450 and antioxidant system in rainbow trout (*Oncorhynchus mykiss*). For this purpose, 24 fish were randomly divided into three groups: the control, the 0.1 mg/L malachite green administration group and the 0.5 mg/L malachite green administration group. 7-ethoxyresorufin-O-deethylase activity (EROD) in the 0.5 mg/L malachite green administration group was significantly higher than that in the control group. 7-pentoxyresorufin O-dependentylase (PROD) activities increased with increasing doses of malachite green. Catalase (CAT) and glutathione reductase (GR) activities in the 0.1 mg/L malachite green administration group were significantly higher than those in the control group. No statistically significant differences were found in erythromycin N-demethylase (ERND) and glutathione S-transferase (GST) activities among the groups. The results of this study clearly demonstrate that malachite green has a modulatory effect on EROD, PROD, CAT, and GR activities in rainbow trout.

Keywords: Antioxidant enzyme activities, Cytochrome P450, Malachite green, Rainbow trout (*Oncorhynchus mykiss*), Xenobiotic metabolizing enzymes.

ÖZ

Malahit yeşili, tekstil ve kâğıt endüstrisinde boya olarak ve balık çiftliklerinde ektoparazitlere, bakteriyel ve fungal enfeksiyonlara karşı kullanılan mutajenik ve karsinojenik bir kimyasaldır. Sucul organizmalar malahit yeşiline maruz kalma riski altındadır. Bu çalışmada amacımız gökkuşağı alabalığında (*Oncorhynchus mykiss*) iki farklı konsantrasyonda malahit yeşilinin sitokrom P450 ve antioksidan sistem üzerine etkisini belirlemektir. Bu amaçla, 24 balık üç gruba rastgele ayrılmıştır: kontrol, 0,1 mg/L malahit yeşili ile muamele edilen grup ve 0,5 mg/L malahit yeşili ile muamele edilen grup. 0,5 mg/L malahit yeşili ile muamele edilen grupta 7-etoksiresorufin O-deetilaz (EROD) aktivitesi, kontrol grubundan anlamlı düzeyde daha yüksektir. 7-pentoksiresorufin O-dependentilaz (PROD) aktivitesi artan malahit yeşili dozu ile artmıştır. 0,1 mg/L malahit yeşili ile muamele edilen grupta katalaz (CAT) ve glutatyon redüktaz (GR) aktiviteleri, kontrol grubundan anlamlı düzeyde daha yüksektir. Eritromisin N-demetilaz (ERND) ve glutatyon S-transferaz (GST) aktivitelerinde gruplar arasında istatistiksel olarak anlamlı fark bulunmamıştır. Bu çalışmanın sonuçları malahit yeşilinin gökkuşağı alabalığında EROD, PROD, CAT ve GR aktiviteleri üzerinde modülatör bir etkiye sahip olduğunu açıkça göstermektedir.

Anahtar Kelimeler: Antioksidan enzim aktiviteleri, Sitokrom P450, Malahit yeşili, Gökkuşağı alabalığı (*Oncorhynchus mykiss*), Ksenobiyotik metabolize eden enzimler

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Introduction

Malachite green (4-{[4-(Dimethylamino) phenyl] (phenyl)methylene}-N,N-dimethyl-2,5-cyclo hexadien-1-iminium) is an organic chemical, readily soluble in water, and used to color wool, silk and cotton in textile industry. It is also used in aquaculture as a therapeutic chemical due to its anti-parasitic and anti-fungal effects. It was previously used to give color to foods. However, its toxic and carcinogenic effects have been identified, leading to its prohibition in edible by the FDA in the US and EU countries (Dutta et al., 2024; Zu-yi et al., 2024). Nevertheless, recent studies have shown that fish are still exposed to malachite green (Hakami et al., 2021; Gharavi-nakhjavani et al., 2023; Lemos et al., 2023; Fakhri et al., 2025).

Malachite green is rapidly absorbed and metabolized to leucomalachite (Lemos et al., 2023). A study on the accumulation of malachite green in different organs revealed that the highest amount of malachite green is found in the liver (Gharavi-nakhjavani et al., 2023; Srivastav et al., 2016; Sinha et al., 2021). The toxic effect of malachite green has been shown in the liver tissue of rats, mice, and several fish species (Culp et al., 1999; Das et al., 2013; Hassan et al., 2014; Sinha et al., 2021). Oxidative biotransformation products, such as malachite green N-oxide and malachite green N-demethylated derivatives, have been detected in edible fish tissues (Doerge et al., 1998).

In vivo studies the biotransformation reactions of malachite green are limited, and contradictory results regarding its effects have been reported in the literature. Nebbia et al. (2017) reported the inhibitory effect of malachite green on GST and CYP1A enzyme activities with *in vitro* studies. In their study, they highlighted the necessity of *in vivo* studies to confirm their in *vitro* results. Phase I and Phase II enzymes play roles in the biotransformation reactions of xenobiotics. Most organic pollutants, including polycyclic aromatic hydrocarbons, solvents, pesticides, drugs, cosmetics, and food preservatives, are initially metabolized by phase I reactions. Most of the phase I reactions are catalyzed by cytochrome P450-dependent mixed function oxidases (Esteves et al., 2021). Cytochrome P450s (CYPs) have roles in detoxification reactions or the conversion of non-toxic chemicals to toxic forms (Veith & Moorthy, 2018; Esteves et al., 2021). CYP1A, one of the cytochrome P450 subfamilies metabolizes the initial metabolic reaction of many organic chemicals, including polycyclic aromatic hydrocarbons and polychlorinated biphenyls. It has also been demonstrated in many different organisms that the level of cytochrome P4501A increases by these organic substances (Goksoyr, 1995; Bozcaarmutlu et al., 2015; Rabuffetti et al., 2024; Sadauskas-Henrique et al., 2024). CYP3A and CYP2B are generally involved in detoxification reactions. CYP3A is responsible for the metabolism of the majority of drug molecules in the body (Xu et al., 2005). CYP2B also plays a role in the metabolism of endogenous substances such as androstenedione and testosterone (Nemoto & Sakurai, 1995).

Xenobiotics are generally further metabolized by phase II enzymes. These enzymes catalyze conjugation reactions of chemicals. Conjugation reactions convert molecules from their lipid-soluble forms to water-soluble forms which are then excreted from the body (Tew & Ronia, 1999; Rabuffetti et al., 2024). One of the phase II enzyme families is glutathione S-transferase (GST), which is involved in conjugation reaction between reduced glutathione and xenobiotics (Tew & Ronia, 1999). Additionally, free radicals are formed during metabolic and biotransformation reactions of xenobiotics (Tew & Ronia, 1999). Free radicals react with macromolecules in the cell, leading to protein or DNA damage. The effects of free radicals are eliminated by an antioxidant system (Vilchis-Landeros et al., 2024). Proteins in the antioxidant system include catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase enzymes.

In this study, our aim was to determine the *in vivo* effect of two different doses of malachite green on xenobiotic metabolizing enzyme activities, including CYP1A-associated 7-ethoxyresorufin-O-deethylase (EROD), CYP3A-associated erythromycin N-demethylase (ERND), CYP2B-associated 7-pentoxyresorufin O-depentylase (PROD), and glutathione S-transferase (GST) in the liver of rainbow trout (*Oncorhynchus mykiss*). In addition, the *in vivo* effect of malachite green on antioxidant system was determined by measuring catalase (CAT) and glutathione reductase (GR) activities in the liver of rainbow trout (*Oncorhynchus mykiss*). The involvement of CYP1A on the metabolism of malachite green was also supported through *in vitro* studies.

Methods

Fish Material and Treatment

The ethical approval for the animal-based studies was obtained from the Animal Experiments Local Ethics Committee of Bolu Abant Izzet Baysal Unıversıty University (Process number: 2018/21). Rainbow trout samples were purchased from a local trout farm in BoluTürkiye. The fish

were kept in a separate flow-through pond for 3 days before sampling. A total of 24 fish (26 ± 0.2 cm) were sampled from this pond and transferred to Fish Biology Laboratory in the Bolu Abant Izzet Baysal University. They were then randomly separated into three groups in 200 L aerated fiberglass tanks. The groups were named as control, 0.1 mg/L malachite green treatment group, and 0.5 mg/L malachite green treatment group. Malachite green oxalate was purchased from Merck KGaA (Darmstadt, Germany). The temperature of tanks was maintained constant at 11-12 °C. The tanks were drained and refilled every twelve hours. The fish were kept in 12 hours dark and 12 hours light cycle. Liver samples of fish treated with 0.5 mg/L malachite green were taken at the end of 24 hours by cervical dislocation and placed in liquid nitrogen for freezing. Liver sample of fish treated with 0.1 mg/L malachite green, and the control groups were taken at the end of 54 hours by cervical dislocation and placed in liquid nitrogen for freezing. They were then stored in a deep freezer at -80 °C until used.

Preparation of Microsomes and Cytosols

The liver tissue fractions of rainbow trout liver were prepared using the method described by Arınç & Şen (1993) with some modifications. Each tissue was prepared individually. The tissue pieces were homogenized with homogenization solution (1.15% KCl solution containing 1.0 mM EDTA pH 7.4, 0.1 mM PMSF and 0.25 mM ε-ACA) at a volume equal to 4 times the weight of the tissues. The homogenate was centrifuged at 10000×g for 20 minutes at 4°C. Microsomes and cytosols were then prepared as reported by Bozcaarmutlu et al. (2015). Finally, microsomes were resuspended in 25% glycerol containing 1.0 mM EDTA, pH 7.4, at a volume of 1.0 mL for each gram of rainbow trout liver tissues. Microsomes and cytosols were stored in a -80 °C deep freezer. Protein concentrations of microsomes and cytosols were determined using the Lowry Method (Lowry, 1951).

Enzyme Activity Measurements

The details of the procedures used for enzyme activity measurements were explained in previous reports (Bozcaarmutlu & Arinç, 2008; Bozcaarmutlu et al., 2020). Cytochrome P4501A associated 7-ethoxyresorufin-O-deethylase (EROD) and cytochrome P4502B associated 7-pethoxyresorufin-O-depenthylase (PROD) activities of fish liver microsomes were measured as described by Burke & Mayer (1974) and Arinc & Şen (1993). The optimum conditions for maximum activities were determined for rainbow trout in our laboratory through characterization studies. NADPH generating system was used in these activity measurements and prepared as reported by Bozcaarmutlu et al. (2015). A typical reaction medium contained 6.5 µM 7-ethoxyresorufin for EROD and 6.5 µM 7-penthoxyresorufin for PROD. The reaction was initiated with the addition of substrate of each reaction and followed at 25°C for 5 minutes for EROD and 10 minutes for PROD in a spectrofluorometer at 535 nm (excitation) and 585 nm (emission) wavelengths. Both EROD and PROD activities were determined by using standard calibration curve of resorufin.

Cytochrome P4503A associated erythromycin N-demethylase (ERND) activities in fish liver microsomes were measured as described by Cochin & Axelrod (1959) with some modifications. A typical assay mixture for rainbow trout contained 1 mM erythromycin. The reaction was carried out at 25°C for 15 minutes with shaking in a water bath. The amount of formaldehyde produced at the end of enzyme catalyzed reaction was measured by the method of Nash (1953). Standard calibration curve of formaldehyde was used to calculate the specific ERND activities.

Total glutathione S-transferase (GST) activities in rainbow trout liver cytosols were measured as described by Habig et al. (1974) with some modifications. A typical reaction mixture contained 3.0 mM GSH and 1.0 mM CDNB. The reaction was initiated by the addition of CDNB into reaction medium and measured at 340 nm for 2 minutes (ε=0.0096 $\mu M^{-1}cm^{-1}$).

Catalase activities in rainbow trout liver cytosol were determined as described by Aebi (1984) with some modifications. Rainbow trout liver cytosols were pretreated with 1% Triton X-100 for 10 minutes. A typical reaction mixture contained 50 mM H_2O_2. The reaction was initiated by the addition of H_2O_2 and measured at 240 nm for 1 minute (ε= 0.0364 mM^{-1} cm^{-1}).

Glutathione reductase (GR) activities in rainbow trout liver cytosol were determined as described by Carlberg & Mannervick (1985) with some modifications. A typical reaction medium contained 0.5 mM EDTA, pH 7.0, 100 mM potassium phosphate buffer, pH 7.0, 0.1 mM NADPH, 1 mM GSSG, 50 µL of enzyme source, and distilled water at a final volume of 2 mL. The reaction was initiated with the addition of GSSG. The decrease in NADPH amount was measured at 340 nm for 5 min spectrophotometrically (ε = 6.22 $mM^{-1}cm^{-1}$).

The *in vitro* effect of malachite was studied by adding different concentrations of malachite green (2.5-20.0 µM) before the addition of 7-ethoxyresorufin into the reaction mixture of EROD. The composition of reaction mixture was the same as in a typical EROD activity measurement. Microsomes from the control group were used in these activity measurements. The activities obtained at different concentrations of malachite green were compared with the control activity.

Statistical Analysis

In vivo enzyme activity results were expressed as average activity ± standard error of mean (SEM). All data were first tested for normality. The treatment groups were compared with the control group by using an independent samples t-test. The analyses were done by using SPSS statistical package (Version 21.0, Chicago, IL). Differences between means were considered significant when p≤0.05. *In vitro* enzyme activity results were given as percentage of enzyme activity ± standard deviation (SD).

Results

In this study, rainbow trout samples were divided into three groups and treated with two different doses of malachite green (0.1 and 0.5 mg/L). The treatment period for high dose (0.5 mg/L) was ended at 24th hour since five fish samples died. These fish were discarded, and the remaining fish were used for the activity measurements in this group. The treatment period for low dose (0.1 mg/L) was ended at 54th hour. Microsomes and cytosols were prepared from each liver samples. EROD, PROD, ERND, GST, CAT, and GR activities were measured, and the results of each treatment group were compared with those of the control group. All activities were measured in duplicates and, in some cases, in triplicates.

7-ethoxyresorufin O-deethylase (EROD) activities were 13.66 ± 1.24 pmole/min/mg protein in the control group, 12.99 ± 1.78 pmole/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 44.40 ± 7.00 pmole/min/mg protein in the 0.5 mg/L concentration malachite green treated group. EROD activities of fish treated with 0.5 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 1A).

7-Pentoxyresorufin O-depentylase activities (PROD) were 0.31 ± 0.02 pmole/min/mg protein in the control group, 0.40 ± 0.05 pmole/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 0.53 ± 0.01 pmole/min/mg protein in the 0.5 mg/L concentration malachite green treated group. PROD activities of fish in the 0.5 mg/L malachite green treated group were higher than those of fish in the 0.1 mg/L malachite green treated group and the control group. PROD activities of fish treated with 0.5 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 1B).

Erythromycin N-demethylase (ERND) activities were 91.63 ± 16.33 pmol/min/mg protein in the control group, 124.92 ± 11.34 pmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 115.69 ± 30.25 pmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. ERND activities of treatment groups were slightly higher than those of the control group. However, there were no statistically significant differences in ERND activities between the treatment groups and the control group (Fig. 1C).

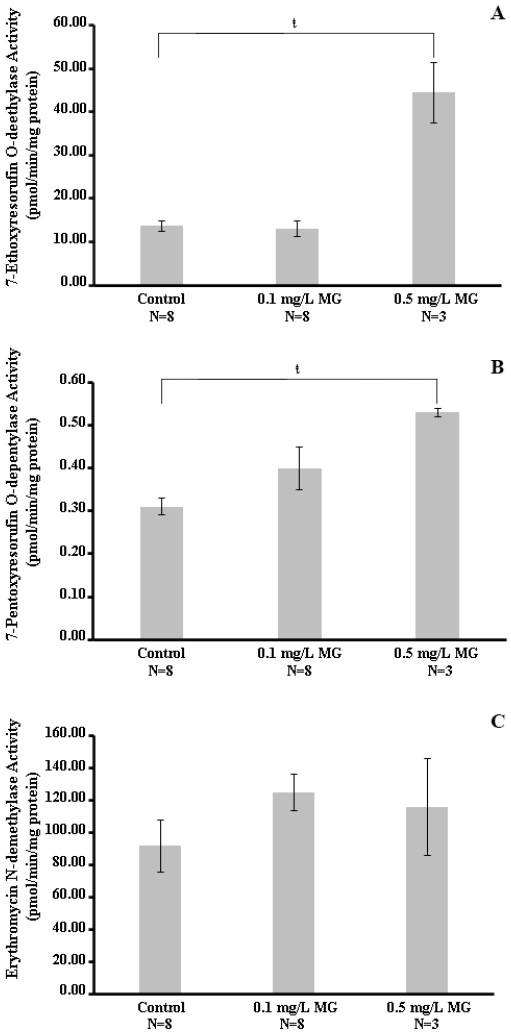

Figure 1 Effect of malachite green on cytochrome P450s. CYP1A-associated 7-ethoxyresorufin-O-deethylase activities (A), CYP2B-associated 7-pentoxyresorufin O-

dependylase (B) and CYP3A-associated erythromycin N-demethylase (C) The symbol (ŧ) indicates statistically significant difference between the 0.5 mg/L malachite green treated group and the control group (p≤0.05).

Glutathione S-transferase activities (GST) were 1162 ± 95 nmol/min/mg protein in the control group, 1109 ± 36 nmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 1165 ± 67 nmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. When the groups were compared, no statistically significant difference was found between the treatment groups and the control group (Fig. 2A).

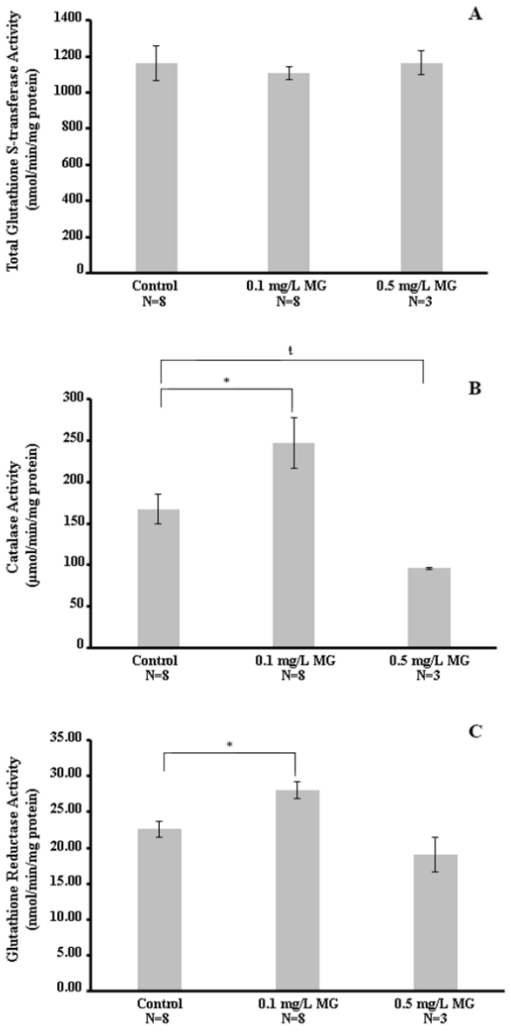

Figure 2 Effect of malachite green on glutathione S-transferase and antioxidant system. Glutathione S-transferase (A), catalase (B) and glutathione reductase (C) The symbol (*) indicates the significant difference between the 0.1 mg/L malachite green treated group and the control group (p≤0.05). The symbol (ŧ) indicates the significant difference between the 0.5 mg/L malachite green treated group and the control group (p≤0.05).

Catalase (CAT) activities were 167 ± 18 µmol/min/mg protein in the control group, 247 ± 31 µmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 96 ± 1 µmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. Catalase activities of rainbow trout samples treated with 0.1 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 2B). Catalase activities of fish treated with 0.5 mg/L malachite green were significantly lower than those of the control group (p≤0.05).

Glutathione reductase activities (GR) were 22.58 ± 1.09 nmol/min/mg protein in the control group, 28.05 ± 1.17 nmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 19.08 ± 2.40 nmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. GR activities of fish treated with 0.1 malachite green were significantly different from those of the control group (*p*≤.05) (Fig. 2C).

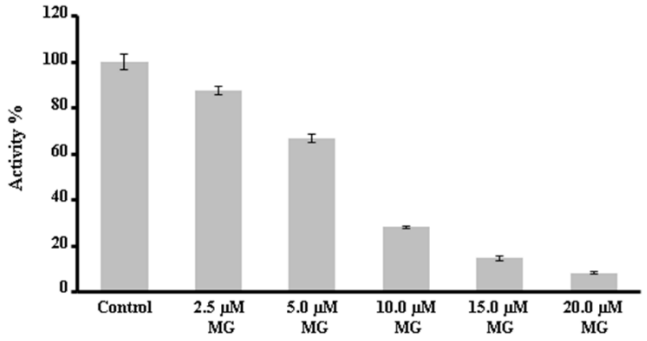

Figure 3 Inhibitory effect of malachite green on CYP1A-associated 7-ethoxyresorufin-O-deethylase activity.

In addition to *in vivo* studies, the effect of malachite green on EROD activities was determined by *in vitro* studies. As the concentration of malachite green increased in the reaction mixtures, EROD activities decreased (Fig. 3). At 20 µM concentration of malachite green, only 8 % of the initial EROD activity remained.Discussion

The use of malachite green has not been permitted in many countries. However, the monitoring studies clearly indicate its illegal usage (Hakami et al., 2021; Gharavi-nakhjavani et al., 2023; Lemos et al., 2023). High amounts of malachite green have also been reported in several effluents (Khan et al., 2019). The concentration of malachite green has been determined as 1.320 mg/L in

laundry effluent, 0.620 mg/L in paper effluent, 0.790 mg/L in printing effluent, and 1.680 mg/L in textile effluent (Khan et al., 2019). *In vivo* researches on its effect and metabolism in fish is limited. In the current study, 62% of fish samples died in the 0.5 mg/L malachite green treatment group within the 24 hours of malachite green administration. This result clearly shows that fish may die if exposed to the malachite green amounts present in these effluents. Considering the amount of accumulation and mortality at the dose of 0.5 mg/L, we ended the chemical administration period of the 0.1 mg/L malachite green treatment group at the end of 54 hours. Cytosols and microsomes were prepared from all samples. Cytochrome P450 associated enzyme activities and antioxidant enzyme activities were measured in microsomes and cytosols, respectively.

Cytochrome P450s metabolize both endogenous and exogenous molecules. Therefore, it is important to determine the effect of malachite green on cytochrome P450s. It has been shown that CYP1A-associated arylhydrocarbon hydroxylase activity increases with malachite green treatment in SHE primary cultures (Panandiker et al., 1992). The results of an *in vitro* study report that CYP1A-associated EROD activity decreases with the addition of increasing concentrations of malachite green into the reaction medium (Nebbia et al., 2017). However, there are contradictory reports about the effect of malachite green on CYP1A. For example, CYP1A has been measured in mRNA level in carp. Its level decreases with 0.146 mg/L chronic administration of malachite green (Sinha et al., 2021). Similarly, the mRNA expression level of CYP1A was inhibited significantly by the administration of 0.5 mg/L malachite green in Nile tilapia, grass carp, and Taiwan snakehead (Li et al., 2013). In the current study, elevated EROD activities were measured in the 0.5 mg/L malachite green treatment group. CYP1A activity is involved and stimulated by malachite green. Our *in vitro* studies also supported the involvement of CYP1A in the biotransformation of malachite green in rainbow trout (Fig. 3). Other cytochrome P450 associated activities measured in this study were ERND and PROD activities. CYP3A associated ERND activities were higher in the treatment groups. However, these results were not statistically different from the control group, indicating that this activity is not affected by the acute malachite green treatment. The PROD activity is generally associated with CYP2B enzyme activity in humans (Lubet et al., 1985). In the current study, this activity was higher in the treatment groups. Significantly high PROD activities were measured in the 0.5 mg/L malachite green treated group than in the control ($p \leq 0.05$). These findings suggest that cytochrome P450 enzymes associated with EROD and PROD activities are involved in the metabolism of malachite green.

Phase II enzymes also play important roles in the biotransformation of xenobiotics. Glutathione S-transferases are phase II enzymes catalyzing the conjugation reactions of many xenobiotics. In this study, GST activity in the 0.1 mg/L malachite green administration group was lower than that in the control group. However, this difference was not significant. A similar decrease in GST activity has been reported in carp (Sinha et al., 2021). In another study, administration of 0.1 mg malachite green for 30 days reduced GST activity in swiss albino mice (Das et al., 2013). Similarly, the mRNA expression level of GST was significantly inhibited by the administration of 0.5 mg/L malachite green in Nile tilapia, grass carp, and Taiwan snakehead (Li et al., 2013). The involvement of GST in the biotransformation of malachite green has been shown with *in vitro* inhibition studies (Nebbia et al., 2017). However, the results of the current *in vivo* study clearly show that this activity was not modulated by acute treatment of malachite green. The insignificant decrease in GST activity in this study may have potentially resulted from the depletion of GSH. It has been shown that GSH level decreases with malachite green treatment in common carp and rainbow trout (Yonar & Yonar, 2010; Sinha et al., 2021).

In addition, studies indicate that malachite green causes free radical formation (ROS) and oxidative stress (Kovacic & Somanathan, 2014). In this study, catalase and glutathione reductase activities were measured to determine the effect of malachite green on antioxidant enzyme activities in the cytosols of rainbow trout. Catalase activity was significantly higher in the 0.1 mg/L malachite green treatment group than the control group, whereas this activity was significantly lower in the 0.5 mg/L malachite green administration group than the control group ($p \leq 0.05$). Similarly, it has been reported that 0.146 mg/L malachite green administration increases catalase activity when carp samples are treated with malachite green for 15 days (Sinha et al., 2021). In the same study, catalase activity decreased in the 30- and 60-day administration groups. In the current study, the reason for low activities measured in 0.5 mg/L malachite green treated group might result from high malachite green administration in a short period of time. A similar decrease in catalase activity has been reported in the liver of rainbow trout treated with 6.67 mg/L malachite green (Yonar & Yonar, 2010). Significantly higher glutathione reductase activities were measured in the 0.1 mg/L malachite green administration group compared with the control group ($p \leq 0.05$). These results indicate that glutathione reductase

activity was also modulated by the treatment of malachite green. The modulation patterns of catalase and glutathione reductase activities were similar, higher in the low dose malachite green administration group than in the control and lower in the high dose malachite green administration group. Both activities are related to the elimination of reactive oxygen species from the body. The results of this study clearly show that malachite green affects oxidative stress defense system in rainbow trout.

Conclusion and Recommendations

As a result of this study, CYP1A-associated activity increased in the high malachite green administration group. Antioxidant enzyme activities were also modified by malachite green treatment. CYP1A is generally found in the conversion of toxic chemicals to more toxic forms and generation of reactive oxygen species. The high mortality observed in the current study at 0.5 mg/L may be related to generation of excess oxygenated cytochrome P450 related biotransformation products and reactive oxygen species. Further studies are needed to determine whether the high mortality rate in the high malachite green administration group is directly caused by malachite green or by other factors related to modified enzyme activities.

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MARINE DERIVED CHITIN AS A PROMISING BIO- STIMULANT FOR SUSTAINABLE AGRICULTURE

SÜRDÜRÜLEBİLİR TARIM İÇİN UMUT VERİCİ BİR BİYOSTİMULAN OLARAK DENİZ KAYNAKLI KİTİN

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ABSTRACT

Chitin and chitosan are natural compounds that are biodegradable and nontoxic, have garnered significant attention for their positive impact on crop yield and agro-environmental sustainability. As sustainable agriculture becomes more imperative, biocontrol using natural compounds such as chitin, a carbohydrate chain polymer, and its derivatives, is a promising strategy. Chitin and its derivatives induce or enhance natural defensive mechanisms in plants. They are recognized as plant growth regulators, growth stimulants, and elicitors for the production of secondary metabolites. They have beneficial effects as fertilizers, soil conditioning agents, plant disease control agents, antitranspirants, ripening retardants, and seed and fruit coatings. Chitin and its derivatives are obtained from renewable sources, mainly shellfish waste, having a great potential for the development of bioproducts as alternatives to synthetic agrochemicals. Recent studies have provided evidence that the use of these biopolymers can help control postharvest diseases, increase the content of nutrients available to plants, and elicit positive metabolic changes that lead to higher plant resistance against pathogens. Hence, the present study was aimed to collect crustaceans from coastal region and chitin extraction. The extracted chitin is fortified with soil mixture as manure and subjected to detect the growth of plants such as okra and tomato. The formulation and application of Chitin manure to soil and Analysis of Plant Growth Parameters at different time intervals is recorded and the data interpretation carried out.

Keywords: Chitin, marine source, crustaceans, okra, tomato, sustainable agriculture

ÖZ

Kitin ve kitosan, biyolojik olarak parçalanabilir ve toksik olmayan doğal bileşikler olup, mahsul verimi ile tarımsal-çevresel sürdürülebilirliği üzerindeki olumlu etkileri nedeniyle önemli ölçüde dikkat çekmiştir. Sürdürülebilir tarım daha zorunlu hale geldikçe, karbonhidrat zincirli bir polimer olan kitin ve türevleri gibi doğal bileşiklerin kullanıldığı biyokontrol, umut verici bir stratejidir. Kitin ve türevleri bitkilerde doğal savunma mekanizmalarını tetikler veya güçlendirir. Bunlar; bitki büyüme düzenleyicileri, büyüme stimülanları ve ikincil metabolit üretimi için elisitörler olarak kabul edilir ve gübre, toprak düzenleyici ajan, bitki hastalık kontrol ajanı, antitranspiran, olgunlaşma geciktiricileri ve tohum ile meyve kaplaması olarak faydalı etkilere sahiptirler. Kitin ve türevleri, başlıca kabuklu deniz ürünü atıkları olmak üzere yenilenebilir kaynaklardan elde edilmekte olup, sentetik tarım kimyasallarına alternatif biyo-ürünlerin geliştirilmesi için büyük bir potansiyele sahiptir. Son çalışmalar, bu biyo-polimerlerin kullanımının hasat sonrası hastalıkların kontrolüne yardımcı olabileceğine, bitkilere mevcut besin madde içeriğini artırabileceğine ve patojenlere karşı daha yüksek bitki direncine yol açan olumlu metabolik değişiklikleri ortaya çıkarabileceğine dair kanıtlar sağlamıştır. Bu nedenle, mevcut çalışma kıyı bölgesinden kabukluları toplamayı ve kitin ekstraksiyonunu amaçlamıştır. Elde edilen kitin, gübre olarak toprak karışımı ile zenginleştirilmiş ve bamya ve domates gibi bitkilerin büyümesini tespit etmek için uygulanmıştır. Kitin gübresinin formülasyonu ve toprağa uygulanması ile farklı zaman aralıklarında Bitki Büyüme Parametrelerinin Analizi kaydedilmiş ve veri yorumlaması gerçekleştirilmiştir.

Anahtar Kelimeler: Kitin, denizel kaynak, kabuklu, bamya, domates, sürdürülebilir tarım

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Introduction

Chitin, a naturally occurring polysaccharide abundant in the exoskeletons of crustaceans, insects, and fungi, has emerged as a versatile biomaterial with diverse applications spanning agriculture, biomedicine, and materials science. Derived predominantly from discarded crustacean shells, chitin serves as a renewable resource that can be enzymatically converted into chitosan a derivative with enhanced functional characteristics and eco-friendly attributes (Aklog et al., 2016). This comprehensive introduction aims to provide an in-depth overview of chitin and chitosan, exploring their extraction methods, physicochemical properties, applications in sustainable agriculture, advanced materials development, and their significant contributions to environmental sustainability. This introduction aims to provide an overview of the extraction methods, physicochemical properties, and diverse applications of chitin and chitosan, particularly focusing on their roles in sustainable agriculture and advanced material development (Bhatnagra & Sillanppa, 2009). Chitin extraction is a multi-step process involving demineralization and deproteinization techniques, as outlined by various researchers (Casadidio et al., 2019; Devi & Dhamodharan, 2017). These methods focus on isolating chitin from crustacean shells while eliminating impurities like minerals and proteins. The resulting chitin exhibits unique physicochemical properties, including a Nano fibrous structure, which plays a crucial role in its bioactivity and surface area, as evidenced by studies conducted by (Duan et al., 2017; Entsar et al., 2008).

Chitin and chitosan have gained significant traction in agriculture, functioning as potent bio stimulants and biofertilizers (Gadgey & Bahekar, 2017; Gallert & Winter, 2008). These natural biopolymers offer multifaceted benefits, including improved soil properties, enhanced nutrient uptake, and increased crop resilience. Research studies (Huang et al., 2021; Jie et al., 2017) highlight their efficacy in controlling phytopathogens, thus reducing reliance on synthetic agrochemicals and promoting sustainable agricultural practices. Innovations in material science have capitalized on chitin's unique properties to develop advanced biomaterials (Kaya et al., 2015). Chitin nanofibers and nanoparticles exhibit promise in bioplastics, wound healing, and drug delivery systems due to their biocompatibility and biodegradability. Furthermore, the synthesis of chitosan from chitin enables the production of nanostructured films, scaffolds, and membranes with applications in tissue engineering and regenerative medicine (Mohan et al., 2021; Pandharipande & Prakash, 2019). The valorization of agricultural waste, specifically crustacean shells, into chitin and chitosan aligns with circular economy principles and sustainable development goals (Amiri et al., 2022).

The application of chitin and chitosan in agriculture has gained prominence, with researchers (Surinder & Gurpreet Singh, 2015; Wang & Chio, 1998) exploring their potential as bio stimulants and biofertilizers. These natural biopolymers exhibit multifaceted roles in enhancing soil properties, stimulating plant growth, and mitigating environmental impact by reducing the need for synthetic agrochemicals. Studies (Huang et al., 2021; Pichyangkura & Chadchawan, 2015) have highlighted their effectiveness in improving nutrient uptake, enhancing crop resilience, and controlling phytopathogens, thus contributing to sustainable agricultural practices. Chitin and its derivatives serve as renewable and versatile resources with wide-ranging applications across various industries. From enhancing agricultural productivity and developing eco-friendly biomaterials to promoting environmental sustainability, chitin-based materials offer innovative solutions towards a more sustainable future (Puglia et al., 2021). Chitin is a versatile polysaccharide with potential applications ranging from pharmaceuticals to environmental remediation. Recent advancements, such as the base-free preparation method demonstrated by (Rkhaila et al., 2021), have shown promise in efficiently obtaining low molecular weight chitin (LMW-chitin) with high purity and desirable molecular characteristics.

Attention due to their potential to address environmental challenges and promote circular economy principles. (Bhatnagra & Sillanppa, 2009) highlighted the importance of bioconversion compost, such as larvae bioconversion compost and vermicomposting, in containing bio-stimulant components like betaines, chitin, hemic substances, protein hydrolysates, and beneficial microorganisms. These bio-stimulants have demonstrated agricultural benefits, contributing to improved soil health, plant growth, and nutrient cycling. Moreover, the study underscored the successful large-scale application of Musca domestic in reducing antibiotic residues in pig manure, emphasizing the potential of bioconversion technologies in waste management and sustainable agriculture (Rouphael & Colla, 2020a). Bio stimulants encompass a wide array of biological and inorganic materials, such as microbial fermentations from animal or plant feedstock, live microbial cultures, macro and micro-algae, protein hydrolysates, as well as humic and fulvic

substances (Rouphael & Colla, 2020b). Through their analysis, Baltazar et al., (2021) discovered that carbohydrates found in bio stimulants may positively influence plant signaling cascades, ultimately benefiting plant growth and development. This research underscores the importance of understanding bio stimulants and their potential applications in modern agriculture (Sawssen et al., 2015).

Shellfish processing, particularly involving crustaceans like lobster, shrimp, and crab, results in a substantial amount of waste, with around 40-50% of the total mass ending up as discarded shells. This equates to about 6-8 million tons globally each year. The challenge lies in effectively managing this waste, as only a small portion is repurposed for animal feed or fertilizer (Shahrajabian, et al., 2021). The majority often finds its way to landfills or coastal waters, posing environmental pollution risks. These shells are comprised of approximately 50-60% chitin, along with other components such as calcium, proteins, and pigments. Chitin is not only abundant but also versatile (Shinsuke et al., 2009). It exists in the shells as ordered crystalline micro fibrils, and it's found in various living organisms. Harnessing this chitin could offer solutions beyond waste management, potentially leading to the development of biodegradable plastics, wound dressings, drug delivery systems, and more (Sirajudheen et al., 2021). In this present study, the Chitin is extracted from crab shells is used as bio-stimulant for sustainable agriculture.

Methods

Sample Collection and Preparation

Crab shells were collected from the Kanathur fish market, Chennai, India and Identified as *Portunus sanguinolentus* by taxonomical classification (Figure 1). And transported using ice packs to maintain freshness. The collected crab shells were washed with tap water to remove unwanted contaminants and then dried for 24 hours in a hot air oven at 100°C. The dried samples were subsequently crushed using a motor and pestle and sieved using 0.5 nm sieve to obtain a uniform powder. Then the chitin is extracted using chemical extraction method.

Kingdom	Animalia
Class	Malacostraca
Order	Decapoda
Family	Portunidae
Genus	*Portunus*
Species	*Portunus sanguinolentus*

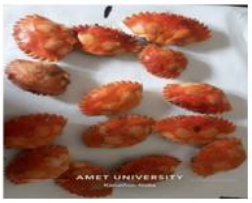

Figure 1. Taxonomical Classification of collected crab shells

Extraction of Chitin

The powdered crab shells were depolymerized using chemical methods to obtain chitin. 20g of Powdered crab shells was treated with 400 mL of sodium hydroxide NaOH (2N) solution for deproteinization. Then the powdered crab shells were washed with distilled water and dried for 1 hour in hot air oven. The powdered crab shells were again treated with 800ml of hydrochloric acid HCl (2N) solution for demineralization. Then the powdered crab shells were washed twice with distilled water and dried for 12 hrs. Then the powdered shells were soaked in acetone for 3 hours to remove pigmentation and washed with ethanol to remove impurities. Finally, the powdered crab shells are dried at 100°C in hot air oven for 12 hours to obtain chitin powder. The obtained chitin powder is used for further experiments and analysis.

Characterization of Chitin

The Chitin yield was calculated by deducting weight from dried crab shells powder. The physicochemical properties of chitin were analyzed using different instrumental techniques such as Fourier transforms Infrared Spectroscopy (FTIR) analysis (FT/IR-6600typeA), Field Emission Scanning Electron Microscopy (Carl Zeiss NTS GMBH, Germany, SUPRA 55) and Nuclear Magnetic Resonance (CPMAS; MAS 10 kHz, 5mm Quadrupole Inverse Probe with gradient).

Seed Germination Technology

The seeds of tomato (*Solanum lycopersicum*) and okra (*Abelmoschus esculentus*) were used in this experiment. The seeds were inoculated in boiling tubes and kept for 24hrs incubation. After the germination, the seeds are transferred into the germination tray. The soil is mixed with chitin powder in different concentration (10%, 25% and 50%) were used for both tomato and okra seeds and named as (T1, T2 and T3) (O1, O2 and O3) respectively. The soil is separately added to both tomato and okra seeds as control (C). Distilled water is added daily to the germination tray to ensure the seeds are adequately moist.

$$\text{Germination Rate (\%)} = \frac{\text{Total Number of Seeds} \times 100}{\text{Number of Germinated Seeds}}$$

Monitoring Plant Growth

Monitoring Plant Growth in chitin involves evaluating how plants interact with and respond to chitin, a natural polymer derived from the extraction of crab shells. Chitin can influence plant growth and health in several ways, including enhancing resistance to pathogens, promoting beneficial microbial populations, and improving soil structure. The plant growth is monitored daily and compared with different concentration of chitin powder mixed with soil. After 45 days, the height and length of the plant and root measured using measuring scale respectively. Along with that leaf also counted and noted as a parameter. The experiment is triplicated in the germination tray and the values were observed and noted. To test the significant differences among the treatment One- way ANOVA was performed.

Phytochemical Analysis (Shaikh & Patil, 2020)

Test for Alkaloids

Mayers test

- To a few ml of sample extract, two drops of Mayer's reagent are added along the sides of test tube.
- Appearance of white creamy precipitate indicates the presence of alkaloids.

Test for carbohydrates

Benedict's test

- To a 2 ml of sample extract, 2ml of benedicts reagent was added and the mixture is heated on boiling water bath for 2mins.
- Appearance of red orange precipitate indicates the presence of Carbohydrates.

Test for phenol compounds

Ferric chloride test

- Extracts were treated with 3-4 drops of ferric chloride solution.
- Formation of bluish black color indicates the presence of Phenols.

TEST FOR FLAVONOIDS
Alkaline reagent test

- Extracts were treated with few drops of sodium hydroxide solution.
- Formation of intense yellow color which becomes colorless on addition of dilute acid indicates the presence of Flavonoids.

TEST FOR PROTEINS

Biuret test

- It is used to determine the presence of peptide bonds in protein.
- To 3ml of test sample 3% of NaOH is added and few drops of 1% CuSO4 was added.
- The solution turns Blue to Violet or Pink indicates the Presence of Protein.

TEST FOR STEROIDS

- To 1 ml of the test solution, equal volume of Chloroform and 3 drops of concentrated sulphuric acid was added.
- Formation of Brown color ring indicates the presence of steroids.

TEST FOR TANNIS

- To 0.5 ml of extract solution, 1 ml of water and 1-2 drops of ferric chloride solution was added.
- Blue color was observed for Gallic tannins and Black color for catecholic tannins.

TEST FOR TERPENOIDS

- 2ml of extract was mixed with 2ml of chloroform in a test tube.
- To this 3ml of concentrated sulphuric acid was added along the walls of the tube to form a layer and interface with reddish brown colorization confirmed the presence of terpenoids.

TEST FOR GLYCOSIDES

- To the solution of extract glacial acetic acid was added, few drops of 5% ferric chloride and concentrated was added and reddish-brown colorization at the junction of two layers and bluish

green color in upper which indicates presence of glycosides.

TEST FOR AMINO ACIDS

Ninhydrin test

- To a few ml of extract, two drops of ninhydrin reagent was added in the test tube.
- Appearance of purple black color indicates the presence of amino acids.

Fourier Transform Infrared Spectroscopy (FTIR)

The characterization was done by Fourier transformed infrared (FTIR) spectroscopy (FT/IR-6600typeA, FTIR spectrometer) was used in the range of 400 to 4000 cm-1. FTIR is commonly employed to characterize its molecular structure and to confirm its presence in chitin sample. Previous tests have pointed out (Van de Velde et al., 2003) that chitin rapidly absorbs rather large amounts of water which would interfere with IR measurements. Combining literature data with own experience led to following procedure designed to avoid presence of moisture. A small amount of sample was dried at 105°C for at least 1 h and consequently grinded. For the deproteinization assessment, proteins contained in 20 mg of chitin samples were leached out in 0.85 % v/v phosphoric acid at 60°C under stirring for 3 h. The peaks confirm the chitin group in the sample.

Nuclear Magnetic Resonance (NMR)

Solid state cross-polarization CP/MAS 13C NMR is a direct way of chitin determination. The 13C-NMR were obtained at CPMAS; MAS 10 kHz, 5mm Quadrupole Inverse Probe with gradient. The purified samples were dissolved at a concentration of 70 mg/mL in deuterated water (99.9%) for 13C NMR. A drop of concentrated HCl or NaOH, to dissolve the samples, was added. The expected 13C signals are: C1 (d104.5–104.6 ppm), C2 (d55.6–55.7 ppm), C3 (d74.0 ppm), C4 (d83.6–83.8 ppm), C5 (d76.1 ppm), C6 (d60.9–61.5 ppm), and CH3 (d23.2–23.3 ppm) (Van de Velde, Kiekens, 2004). In all cases 3072 scans were accumulated. The contact time was 1 mS, the repetition time 5 s, and the acquisition time 50 mS. DSS was used as an internal reference (0 ppm).

Field Emission Scanning Electron Microscope (FESEM with Edax)

The morphology of the crab shells, the chitins extracted by chemical extraction method and the residue of CaCO3 isolated from crab shells were examined by Field-Emission Scanning Electron Microscopy (Carl Zeiss NTS GMBH, Germany, SUPRA 55). Field-Emission Transmission Electron Microscopy coupled with Energy Dispersive X-ray spectroscopy (EDX) was used to determine the elemental content. From the EDX result of elemental analysis, the percentage of proteins remaining in chitin was calculated from the following equation:

$$P\% = (N\% - 6.9) \times 6.25$$

where P% represents the percentage of proteins remaining in the chitin and N% represents the percentage of nitrogen measured by elemental analysis with 6.9 corresponding to the theoretical percentage of nitrogen in fully acetylated chitin and 6.25 corresponding to the theoretical percentage of nitrogen in proteins.

Results

Extraction of Chitin using Chemical Method

In this present study, the yield of chitin is extracted from 20g of raw crab shell powder is 5.230± 0.81. This experiment is repeated to produce more yield of chitin for sustainable agriculture as bio-stimulant (Figure 2).

Figure 2. Chitin extracted from crab shells

Germination Seed Technology

All the seeds germinated very quickly; on the first day, the germination rates of C, T1, T2, T3, O1, O2 and O3 were 80, 82, 84, 90, 86, 88 and 95% respectively (Table 1). The germination percentage of T3 and O3 was the highest. Therefore, the highly effective germination of T3 and O3 was a result of the homogeneous microporous structure of the chitin, which maintained structure and a lot of water. The results also supported that chitin as a plant growth regulator could promote seed germination. The higher T3

and O3 indicated better seedling growth, which could be attributable to the microporous structure. In our findings, T3 and O3 had preferable mechanical properties but also a high ESR, which is important for plant growth. Moreover, chitin is biodegradable and its degradation products contain a plant growth regulator. Therefore, chitin is suitable for use as soilless for seed germination and growth (Figure 3).

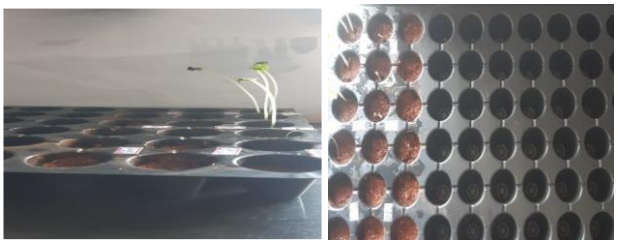

Figure 3. Seed germinated and transferred to germination tray

Table 1. Chitin Concentration on Growth Parameters

Treatment	Mean Plant Height (cm) ± SD	Leaf Count	Mean Root Length (cm) ± SD
T_0	18.1± 0.5	6.3 ± 0.5	12.4 ± 0.3
T_1	20.4± 0.3	7.3 ± 0.5	14.8 ± 0.4
T_2	22.8± 0.2	8.3 ± 0.5	17.2 ± 0.3
T_3	25.6 ± 0.5	9.3 ± 0.5	20.1 ± 0.4
O_0	17.9 ±1.4	6.1 ±0.5	12.5±0.4
O_1	20.2 ±1.1	7.0 ±0.6	14.9 ±0.5
O_2	22.7 ±1.6	8.2 ±0.7	17.4 ±0.6
O_3	25.3 ±1.2	9.4 ±0.6	20.0 ±0.5

Monitoring Plant Growth

In order to determine the effect of chitin on plant growth, tomato and Okra seeds were grown under similar in vitro conditions for 45–70 days, following the growth parameters. An increase in root length (up to 5%) and shoot length (up to 28%) was observed and relative to the control. The plant grown in 50% Chitin concentration showed more effective results compared to control and other concentrations (Figure 4).

Figure 4. Plant growth monitored after 14 days

Phytochemical Analysis

The shoot and leaves of the plant grown in the T3 and O3 is crushed using motor and pestle adding distilled water. The extract is used for phytochemical test and results are observed. The presence of Flavonoids, Steroids and Glycosides are observed in both tomato (Figure 5) and okra (Figure 6) plant. At the same time, there is absence of Alkaloids, Carbohydrates, Ferric chloride, Protein, Tannins, Terpenoids and Ninhydrin in both tomato and okra plant.

Figure 5. Phytochemical analysis for tomato plant extract

Figure 6. Phytochemical analysis for okra plant extract

Fourier Transform Infrared Spectroscopy (FTIR)

The Chitin is characterized by FTIR and the peaks shows absorption bands for chitin extracted from crab shells. A broad peak around 3200-3550 cm^{-1} indicates the presence of hydroxyl groups. A peak around 1310-1320 cm^{-1} shows the C-N stretching and N-H deformation vibrations. A peak around 1420 cm^{-1} represents the bending vibrations of the CH2 groups. Peak around 895-950 cm^{-1} shows C-O-C Stretching and indicates the presence of glycosidic linkages, which are crucial for the polysaccharide backbone of chitin (Figure 7). The presence of these characteristic absorption bands confirms the extraction of chitin, as these are consistent with the known structure and functional groups of chitins. Any deviations or additional peaks might suggest impurities or the presence of other compounds.

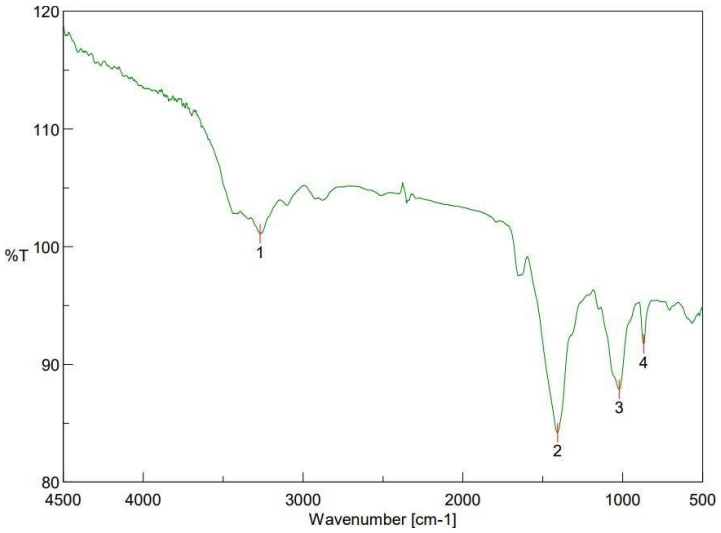

Figure 7. FTIR peaks shows the absorption bands for chitin

Nuclear Magnetic Resonance (NMR)

The 13C NMR spectrum provides detailed information about the carbon skeleton of the polymer (Figure 8). Characteristic peaks for chitin include Carbonyl Carbon (C=O) of the acetamido group resonates downfield, typically around 170-175 ppm. The carbons in the pyranose ring exhibit resonances in the range of 55-110 ppm. C-1 around 100-105 ppm, C-2, C-3, C-4, C- 5 around 70-80 ppm and C-6 around 55-65 ppm. Methyl Carbon of Acetamido attached to the nitrogen resonates up field, typically around 20-25 ppm. The clear resonance for the carbonyl carbon, the pyranose ring carbons, and the methyl carbon provide detailed information about the carbon framework of chitin.

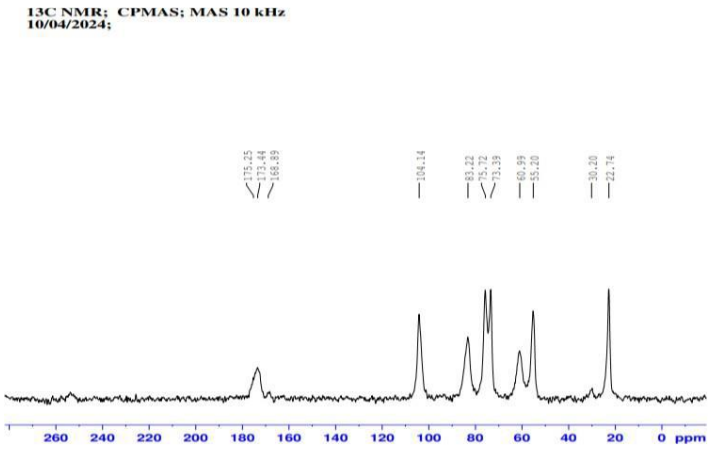

Figure 8. 13C NMR confirms chitin extracted from crab shells

Field Emission Scanning Electron Microscope (FESEM WITH EDAX)

FESEM provides high-resolution images of the surface morphology. Chitin usually exhibits a fibrous or layered structure. FESEM images may show interconnected fibers or a sheet-like morphology, indicating the presence of chitin microfibrils. EDAX (or EDS) provides information on the elemental composition of the chitin sample. For chitin extracted from crab shells, typical elements detected and their approximate compositions might include Carbon, Oxygen, Nitrogen and Calcium (Figure 9). The EDAX spectrum will show peaks corresponding to the energies of X-rays emitted by different elements in the sample. Major peaks for carbon, oxygen, and nitrogen will be prominent, while smaller peaks for trace elements might also appear (Figure 10). FESEM images provide detailed insight into the surface morphology and microstructure of chitin, while EDAX analysis gives precise elemental composition data.

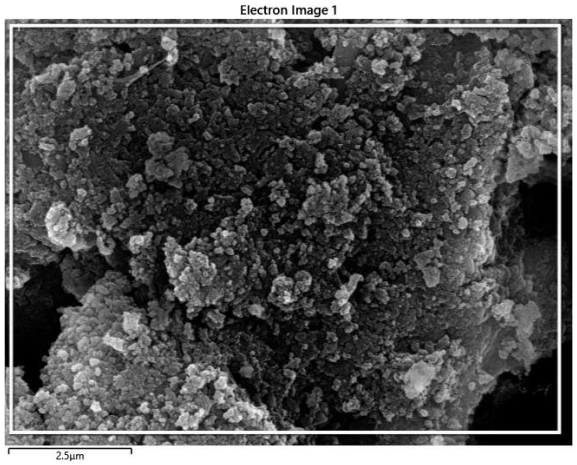

Figure 9. SEM Analysis Image

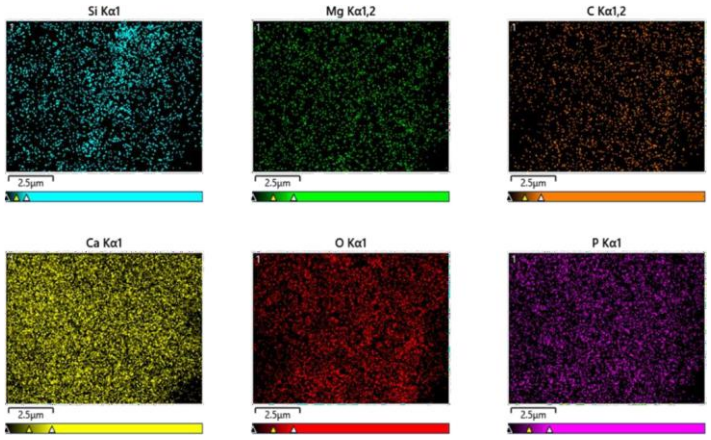

Figure 10. Edax images for Chitin Extracted from Crab shells

Discussion

This study demonstrates the extraction and characterization of chitin from crab shells which can be used as a bio- stimulant in the field of agriculture for its sustainable use. 20g of dry crab shell powder yields 5.230±0.81 g of chitin as early studies had reported chitin extraction using chemical method (Aranaz et al., 2009; Younes & Rinaudo, 2015). During Field Emission Scanning Electron Microscope observation, the chitin possesses a microporous and fibrous nature that helps chitin water retention capacity and enhances the plant nutrient absorption as well as plant hydration (Li et al., 2021). The structural integrity and purity of extracted chitin from crab shell were confirmed using FTIR and MNR analyses. Functional groups such as glycosidic, hydroxyl, amide linkages are identified as reported on structural fingerprint of crustacean derived chitin (Kasaai, 2010). The Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy revealed the elemental 10 composition, mainly the presence of nitrogen and calcium that plays an essential role in plant physiological functions like root and shoot growth. Chitin not only improves the plant growth but also nourishes the soil nutrient need for plant development (Karthik et al., 2017). The Chitin stimulates and enhances the ability of seeds to uptake water that made the seed to be properly hydrated for its development. Chitin interacts with plant hormones like auxins and gibberellins that are responsible for plant growth (Sharp, LeNoble, & Else, 2000; Saharan & Nehra, 2011). The tomato and okra plants treated with 50% chitin exhibited the highest plant height (25.6 ± 0.5 and 25.3 ± 1.2), leaf count (9.3 ± 0.5 and 9.4 ± 0.6) and root length (20.1 ± 0.4 and 20.0 ± 0.5) respectively. This study showed efficient improvement in seed germination rates especially in T3 (90%) and O3 (95%) treatments. Seeds treated with 50% of the chitin concentration showed effective growth by blanching the nutrient availability and biocompatibility as previously reported by (Sreekumar et al., 2018). As the concentration increases the effectiveness also increases. This helps seeds to have a consistent aeration and moisture for seed germination this makes the chitin as suitable bio stimulant for sustainable agriculture. Chitin extracted from crab shell effectively stimulates the auxin pathways that improves cell wall formation. Up to 5% root and 28% shoot development was observed in chitin treated plant (El Hadrami et al., 2010). In orderly to it the role of phytochemicals plays a major role in plant development and growth. The presence of secondary metabolites like glycosides, steroids, flavonoids were observed in O3 and T3 treatments when compared to control group. According to (Hadwiger, 2013; Muzzarelli, 2009) application of chitin in farming increased antioxidant levels and other defense related compounds in plants. The results of this study showed the dual role of chitin as plant growth promotor and soil enrichment. The significance of chitin is, it is eco-friendly to nature and biodegradable. Due to all such uses chitin can be used as a surrogate to synthetic agrochemicals and manures (Barka et al., 2004; Bautista-Banos et al., 2006).

Conclusion

This study demonstrates the successful extraction and characterization of chitin from crab shells using a chemical method. The extracted chitin yield was found to be 5.230±0.81 grams from 20 grams of raw crab shell powder. The high- quality chitin, characterized by its homogeneous microporous structure, shows promising applications in sustainable agriculture, particularly as a bio-stimulant for seed germination and plant growth. Germination Seed Technology for chitin significantly improved seed germination rates, with T3 and O3 treatments showing the highest rates of 90% and 95% respectively. The enhanced germination and seedling growth can be attributed to the chitin's ability to retain water and its beneficial structural properties. Over a period of 25-30 days, plants treated with chitin showed notable increases in root length (up to 5%) and shoot length (up to 28%) compared to the control. A 50% chitin concentration was found to be the most effective in promoting plant growth. Phytochemical tests on plants grown with chitin (T3 and O3) revealed the presence of beneficial compounds such as flavonoids, steroids, and glycosides, indicating enhanced nutritional and medicinal properties. FTIR Analysis confirms the characteristic of functional groups of chitins, including

hydroxyl groups, C-N stretching, N-H deformation, CH2 bending, and glycosidic linkages. NMR Analysis provides detailed the carbon framework of chitin, with characteristic peaks for the carbonyl carbon of the acetamido group, pyranose ring carbons, and methyl carbon. FESEM with EDAX revealed the fibrous or layered structure of chitin and provided precise elemental composition data, confirming the presence of carbon, oxygen, nitrogen, and calcium. The comprehensive analysis confirms the successful extraction and purity of chitin, highlighting its potential as a valuable bio-stimulant in agriculture. The study supports the use of chitin not only for enhancing seed germination and plant growth but also as a sustainable and biodegradable material with significant benefits for crop production and soil health.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this paper.

ETHICAL APPROVAL

This article does not contain any studies involving animals or human participants performed by any of the authors. Hence, ethical approval was not required.

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CONSENT FOR PUBLICATION

The author has read and approved the final version of the manuscript and consent to its publication

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